Culture potential and population structure of the indigenous oysters *Striostrea margaritacea* and *Saccostrea cucullata* in South Africa

By

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Declaration

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Abstract

Oyster culture has a long standing history with mankind and movement of indigenous oysters, in South Africa, dates back to the 1600s. *Crassostrea gigas* (the Pacific oyster) accounts for 80% of global oyster culture and is the sole oyster species cultured in South Africa. Marine environments are vulnerable to invasion, not only by the introduced species but also their associated epifaunal organisms and parasites, as well as the potential for intraspecific disease transmission between introduced and indigenous species. The culture of indigenous oysters, within their range, could reduce these risks as well as relieve commercial harvesting pressure on the current wild stocks. Of South Africa's five indigenous oysters the two most palatable ones were chosen for this study, *Striostrea margaritacea* (Cape rock oyster) and *Saccostrea cucullata* (Natal rock oyster). To develop a new culture species requires knowledge of population densities, distribution, biology, growth rates, and settlement success. To minimize the potential impact of such mariculture, genetic structure and diversity should also be examined. This study focused on genetic structure and diversity, as well as spat settlement and the impact of a Harmful Algal Bloom on spat settlement.

Samples of *S. margaritacea* were taken from five equidistant locations throughout the species' range in South Africa, from the Breede River to Westbrook in KwaZulu-Natal; the same was done for *S. cucullata* from three equidistant locations from Mtakatye in the Eastern Cape up to Umdloti in KwaZulu-Natal. Genetic analyses assessed the CO1 and 16S mtDNA gene regions and both showed that *S. margaritacea* populations have relatively high genetic diversity and high levels of gene flow along South Africa's coastline ($Fst>0.02$, $p>0.05$ in both cases). The populations that were thought to be *S. cucullata* have a high level of fixation between populations for CO1 and 16S ($Fst>0.86$ in both cases, $p<0.01$); the fact that this Fst value is so high suggested that these populations are not related.

What was thought to be *Saccostrea cucullata* in South Africa is now show to be two distinct species (*S. cucullata* and, what appears to be, *S. mordax*) that appear to be separated at some point between Port Edward (south KwaZulu-Natal) and Mtakatye. However, there appears to be an overlap in range as one sample from Mtakatye groups with those from the northern populations. Although the northern population samples do not form a single clade with *S. mordax* in the phylogenetic tree, there is a shell morphological characteristic (radial groves) that seems to be shared between the two that is not shared by other *Saccostrea* species. This is the first record of more than a single *Saccostrea* species on South Africa's coastline. If

Saccostrea were to be used for mariculture the species identity would have to be confirmed before breeding in hatcheries could occur. Both *S. cucullata* and *S. mordax* are exceptionally similar morphologically and the most accurate method of identification would be using CO1 barcoding. A small tissue sample could be extracted by anesthetising the oyster with magnesium chloride and extracting a small section of mantle tissue. Further study should be conducted to confirm the identity of the two *Saccostrea* species; this study should include a taxonomic study and genetics using nuclear and mitochondrial markers. The true range of the *Saccostrea* species should also be determined as they may have overlapping geographic ranges.

Spat settlement trails were performed in Port Alfred in the Eastern Cape over a six month period (November 2013-April 2014). Two temporally separate trials were undertaken throughout the oyster spawning season from 6 November 2013 to 11 February 2014 and 21 February to 23 April 2014 respectively. Few true oysters settled throughout both trials (total of 74 true oysters) and only 19 of the targeted *S. margaritacea* settled. The majority of the oyster settlement occurred during the second trial period. As oysters, along with other marine bivalves, settled on all culch types and the fact that these culch types are used globally for oyster settlement, it can be assumed that the key to the lack of settlement lay elsewhere. During the settlement trials an extensive Harmful Algal Bloom (HAB), comprising of predominantly the toxic dinoflagellate *Lingulodinium polyedrum*, occurred; this bloom ranged from Mossel Bay to East London, its epicentre was at Port Elizabeth (approximately 125 km west of the settlement trail site in Port Alfred), and it lasted from November 2013 to February 2014. Harmful Algal Blooms have an impact on several environmental conditions (pH, temperature, dissolved O_2 and CO_2 levels, and ammonium levels), which may have affected the settlement of oyster spat.

To assess the impact of the HAB (if any) on spat settlement at the study site in summer 2013-2014, three locations within Marine Protected Areas (MPAs) were chosen to perform a size class analysis. One of these, the southern-most, was within the range of the algal bloom (Gulu), another fell on the border of the HABs extent (Gonubie), and the third, northern-most location fell outside of the bloom range (Kei Mouth). I expected a gradient, with Gulu having the lowest number of recruits and Kei Mouth (outside of the HAB range) the highest. However, pre-existing population structure (most likely due to anthropogenic factors) seemed to influence the number of recruits more than the HAB. The location with the most recruits was Gonubie, within a densely human-populated area, whereas the other two sites (north and

south of Gonubie) had far fewer recruits and have a smaller human population. The low settlement experienced during the settlement trials show the unreliable nature of wild oyster settlement as it is subject to unpredictable natural phenomena. A more reliable method for oyster cultivation would be to use hatcheries where most, if not all, conditions could be regulated and monitored.

This study is the first step in the investigation into indigenous oyster culture, and growth trials and other studies still need to be performed. The finding that there are two *Saccostrea* species in South Africa, indicating a need for further resolution of oyster taxonomy in this country, is important for both biodiversity conservation and mariculture.

Key words: Oyster culture, CO1 and 16S, spat settlement, HAB, size class analysis.

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Chapter 1: General Introduction

1.1 Mariculture

Mariculture (marine aquaculture) has a long standing history with mankind, and oysters in particular have frequently been moved around the world in the last century as very successful mariculture species (Andrews, 1980). Some introductions of non-native species are deliberate, for mariculture purposes or to replace depleted native stocks (Cognie, et al., 1996; Haupt, et al., 2010). Initial movements of mariculture species resulted in introduced species becoming naturalised or invasive before the impacts of moving non-native species were entirely understood (Robinson, et al., 2005). Marine ecosystems are highly vulnerable to invasion (Escapa, et al., 2004); and the introduction of potentially invasive species has resulted in, among others, native species being outcompeted and a reduction in biodiversity due to non-native species overwhelming native species' niches (Grabowski, et al., 2004). The Pacific oyster, *Crassostrea gigas*, is used extensively around the world as a mariculture species and has been successfully introduced in many countries for mariculture purposes (Grabowski, et al., 2004) and accounts for over 80% of the world's oyster production (Ayers, 1991). *Crassostrea gigas* has, however, become invasive in 17 of the 66 countries to which it has been introduced (Ruesink, et al., 2005). Pacific oysters have also become invasive in Australia (Ayers, 1991), South America (Escapa, et al., 2004), North America (Hedgecock & Sly, 1990), and northern Patagonia (Escapa, et al., 2004).

1.1.1 Mariculture in South Africa

In the 1950s, the Knysna Oyster Company was established and from the 1970s the Fisheries Development Corporation, a parastatal organisation, was funding it to develop mariculture on a commercial scale (Sauer, et al., 2003). Culture of *C. gigas* has been present in South Africa since 1973 when the Knysna Oyster Company discovered that it had higher growth rates and lower mortalities than indigenous *S. margaritacea* and that importing spat from overseas hatcheries was more cost effective and reliable than wild-spat fall (Cowley, et al., 1998; Coastal & Environmental Services, 2007). Pacific oysters are the sole basis of the South African oyster culture industry, which has resulted in feral populations in a few Southern Cape estuaries (Keightley, et al., 2015; Robinson, et al., 2005). These feral populations are not self-sustaining and have either disappeared or decreased dramatically in size since the culture of *C. gigas* has stopped in these estuaries however, a new population of *C. gigas* has arisen in the Swartkops estuary and is of substantial size (Keightley, et al., 2015). It is likely

that the population of *C. gigas* in the Swartkops is due to several mariculture attempts in that estuary over the years. Another option is that the oyster farms in Algoa Bay are seeding the population in the nearby estuary (de Keyser, 1987). Cultivating indigenous oysters could reduce the risk of *C. gigas* populations becoming established and self-sustaining. More importantly utilizing indigenous oysters would reduce the possibility of importing alien pathogens and epifaunal organisms from countries of origin, as well as reducing the risk of disease transmission between *C. gigas* and indigenous oysters.

Indigenous oysters are well adapted to the environmental conditions within their range which is why their culture potential may be superior to that of *C. gigas* within these ranges. *Crassostrea gigas* is farmed in four locations in South Africa; in Hamburg in the Keiskamma River oysters are farmed on racks, in Algoa Bay and Saldanha Bay oysters are farmed on long-lines, and in Kleinsea oysters are farmed in onshore ponds (Pieterse, et al., 2012). However, not all of these locations are ideal for mariculture of this species, specifically Algoa Bay where *C. gigas* has a higher shell to meat ratio than those in Saldanha Bay (Pieterse, et al., 2012). Pieterse et al. (2012) compared the condition and growth of *C. gigas* grown in Saldanha and Algoa bays; Saldanha Bay falls outside of the range of South Africa's indigenous oysters, but Algoa Bay is well within the range making it a well suited site for indigenous oyster mariculture (indigenous oysters and their ranges are discussed below in section 1.3), as well as already having the required infrastructure. It is not possible to completely phase out the culture of *C. gigas*, but there is a market for indigenous oysters and filling this niche should be explored. The current market for indigenous oysters is being supplied entirely by wild harvesting.

1.2 Wild harvest of indigenous oysters

Wild harvesting of oysters is practiced in many countries around the world on a commercial scale (de Bruyn, 2006). In South Africa wild populations of indigenous oysters are currently harvested recreationally, for subsistence, and on a commercial scale (de Bruyn, 2006; Haupt, et al., 2010). Commercial wild harvesting is allowed in KwaZulu-Natal and in the Eastern Cape; the fishery in the Eastern Cape was previously poorly managed as there was no license requirement and catch returns were not monitored (Dye, et al., 1994); the current status of the *S. margaritacea* fishery in KwaZulu-Natal is listed as Green but that in the Eastern Cape is Orange according to the latest SASSI assessment (2015, http://wwfsassi.co.za/fish-detail/73/) , indicating that it is not necessarily sustainable. To try and correct the poor management of the Eastern Cape fishery, as of 2002, it was placed under the same national fishery as the

KwaZulu-Natal fishery and commercial permits have since been issued (de Bruyn, 2006). Usually, to manage the harvesting pressure a daily bag limit would be issued: however in the case of commercial oyster harvesting, the number of pickers per license is limited and a closed season is also in place (DEAT, 2006). As with any fishery, the harvesting rate must be limited to remain sustainable. To relieve wild harvesting pressure, expand the niche market for local oysters, and reduce the extent of *C. gigas* mariculture the cultivation of indigenous oysters should be investigated in South Africa.

1.3 Candidate indigenous species for culture in South Africa

There are five species of oyster which are native to South Africa (Haupt, et al., 2010). Of these five, the two most palatable species were selected for this study. *Striostrea margaritacea* (formerly *Crassostrea margaritacea* and known commonly as the Cape rock oyster) is indigenous to South Africa and occurs sub-tidally, which makes it well suited to long-line suspended culture, the predominant culture method in South Africa (Kilburn & Rippey, 1982). *Saccostrea cucullata* (formerly *Crassostrea cucullata* and known by the common name the Natal rock oyster or the hooded oyster) is more widely distributed globally and can be found throughout the Indo-Pacific (Lam & Morton, 2006); it is cultivated and harvested on a commercial scale in several countries including Thailand, Guam, Australia, and other tropical countries indicating that it has culture potential (Braley, 1982; Nell, 2001; Klinbunga, et al., 2003).

Both *S. margaritacea* and *S. cucullata* are cupped oysters, hermaphroditic, and change sex from male to female as they age, but are capable of changing sex throughout their life time (Kilburn & Rippey, 1982). These two species both reproduce via broadcast spawning which can be triggered by the presence of sperm or eggs in the water column; this presence can induce an entire colony to spawn simultaneously (Branch, et al., 2007; Kilburn & Rippey, 1982). *Striostrea margaritacea* ranges from False Bay to Mozambique (Figure 1.1) and reaches a maximum size of 180 mm (Robinson et al., 2005; Kilburn & Rippey, 1982; Haupt et al., 2010; Branch et al., 2007). *Striostrea margaritacea* is the dominant species south of the Transkei and forms beds at and below the extreme low tide mark (Kilburn & Rippey, 1982). et al. *Saccostrea cucullata* is cosmopolitan and found from Algoa Bay northwards along the African coast to Somalia, east through the Seychelles and Madagascar to Asia and the Pacific islands, and south to Australia and New Zealand (Braley, 1982; Branch et al., 2007; Haupt et al., 2010; Kilburn & Rippey, 1982); South African range shown in Figure 1.1. An intertidal

species, found in the mid- to upper-intertidal zone, *S. cucullata* can reach sizes of 70mm (Branch, et al., 2007).

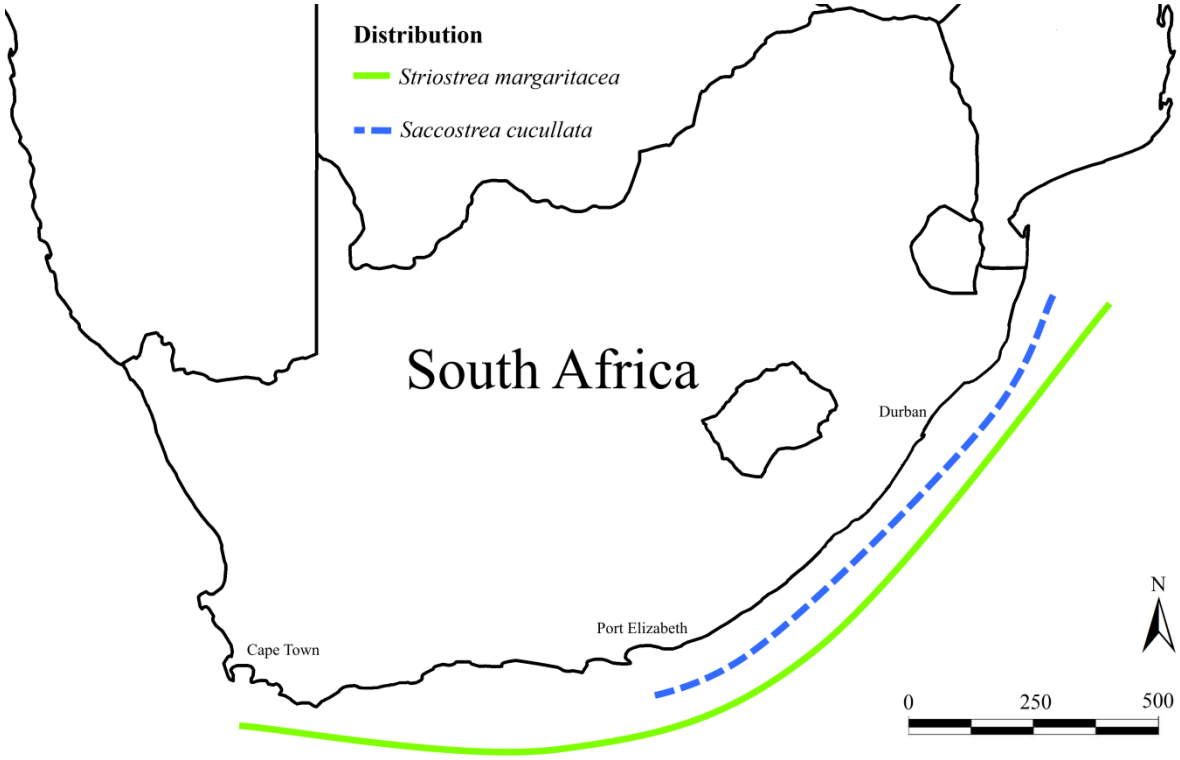

Figure 1.1 Distribution of *S. cucullata* and *S. margaritacea* along South Africa's coastline. *Saccostrea cucullata* ranges from South Africa through Mozambique and throughout the Indo-Pacific (Kilburn & Rippey, 1982; Lam & Morton, 2006). *Striostrea margaritacea* ranges from False Bay to Mozambique (Kilburn & Rippey, 1982).

1.4 Structure of this Thesis

Development of new species for culture requires knowledge of their population size and structure, distribution, genetic structure and diversity, biology and growth rates; much of this knowledge is currently lacking in South Africa. Before translocations of indigenous oyster spat should be attempted, current genetic structure and diversity should be determined; which is the topic of Chapter 2. Spat settlement is an integral part of oyster culture as hatcheries would require a large financial input and may not yield results. Spat settlement is affected by several environmental factors, such as changes in dissolved CO_2 and O_2 concentrations, pH, temperature, and ammonium concentrations. Chapter 3 reports a settlement trial, during which an extensive Harmful Algal Bloom (HAB) occurred. The dominant species of this bloom was *Lingulodinium polyedrum*, which may have altered many of the above conditions. To determine the impact of the HAB on oyster settlement, population size/age structure needed to be analyzed, both of which are addressed in Chapter 3.

Chapter 2: Genetic diversity and biogeographic distribution of indigenous oysters in South Africa

2.1 Introduction

As previously mentioned oyster-culture has a long standing history with mankind. To successfully culture indigenous oysters in South Africa it is important to understand their genetic diversity and natural distribution. There is an expectation of great potential for marine species with pelagic larvae to have a high degree of gene flow between local populations and therefore low genetic structure (Hellberg, 2009). However this is not always the case, for example Kelly and Palumbi (2010) show that 13 rocky intertidal community species, with pelagic larvae show significant genetic structure. Oysters have high fecundity and long pelagic larval duration (PLD of *S. cucullata* and *S. margaritacea* is 20 to 50 days), but low survival rates due to selective pressures (Kilburn & Rippey, 1982; Sukumar & Mohan Joseph, 1988; Taris, et al., 2006). Genetic structure of populations is influenced by adult ecology or post-settlement survival which can temper the influence of dispersal, or pre-settlement processes (Hellberg, 2009). Pre-settlement processes (larval input) are of great importance in forming the genetic structure of populations (Todd, 1998). Many species that span multiple biogeographic regions have different evolutionary units that can only be distinguished at the genetic level (Teske, et al., 2011). To hypothesise about the genetic structure of indigenous oysters various aspects need to be considered, such as patterns of marine biogeography, population genetics approaches and factors affecting oyster population structure.

2.1.1 Patterns of marine biogeography in South Africa

Southern Africa's coastline is home to very complex oceanographic features and is unique in that it has two contrasting currents on the opposing east and west coasts (Griffiths, et al., 2010; Lombard, et al., 2004). The west coast is influenced by the cold Benguela Current, while the east and south coasts are influenced by the warm Agulhas Current (Griffiths, et al., 2010) (Figure 2.1). The west coast experiences periodic, wind-driven upwelling and the intense upwelling results in this coast having high biological productivity; high nutrient supply to upper layers and dense plankton blooms are characteristic of this water (Griffiths, et

al., 2010; Lombard, et al., 2004). The warm Agulhas Current brings nutrient poor waters, flowing from Mozambique and East Madagascar, and has sporadic upwelling resulting in the east and south coast's being less productive than the west (Griffiths, et al., 2010; Lombard, et al., 2004). The Agulhas east coast is characterised by low nutrient levels but highly diverse biota from the Indo-Pacific region (Lombard, et al., 2005). The continental shelf off the east coast narrows to only three kilometres in the northern region and slopes steeply; further south the continental shelf then widens, pushing the current away from the coastline and causing it to retroflect (Heydorn, et al., 1978). Some eddies, however, continue to move westwards and mix with the cold Benguela Current (Figure 2.1) (Heydorn, et al., 1978; Lutjeharms & de Ruijter, 1996). Natal Pulses occur on an irregular basis (mean frequency of 4-6 per year) and these can be found anywhere between the Natal Bight (between Cape St Lucia and Durban) and Port Elizabeth on the east coast (Figure 2.1) (Rouault & Penven, 2011; de Ruijer, et al., 1999; Bryden, et al., 2005). The pulse (a meander in the current associated with cold upwellings and circular rotations in the inshore current known as the Agulhas Rings) pushes the Agulhas Current further away from the coastline and from this point a domino effect is observed where, throughout its length, the current will be pushed off course; this variability in the Agulhas Current results in movement of the exact point where the two currents meet (Lutjeharms & de Ruijter, 1996).

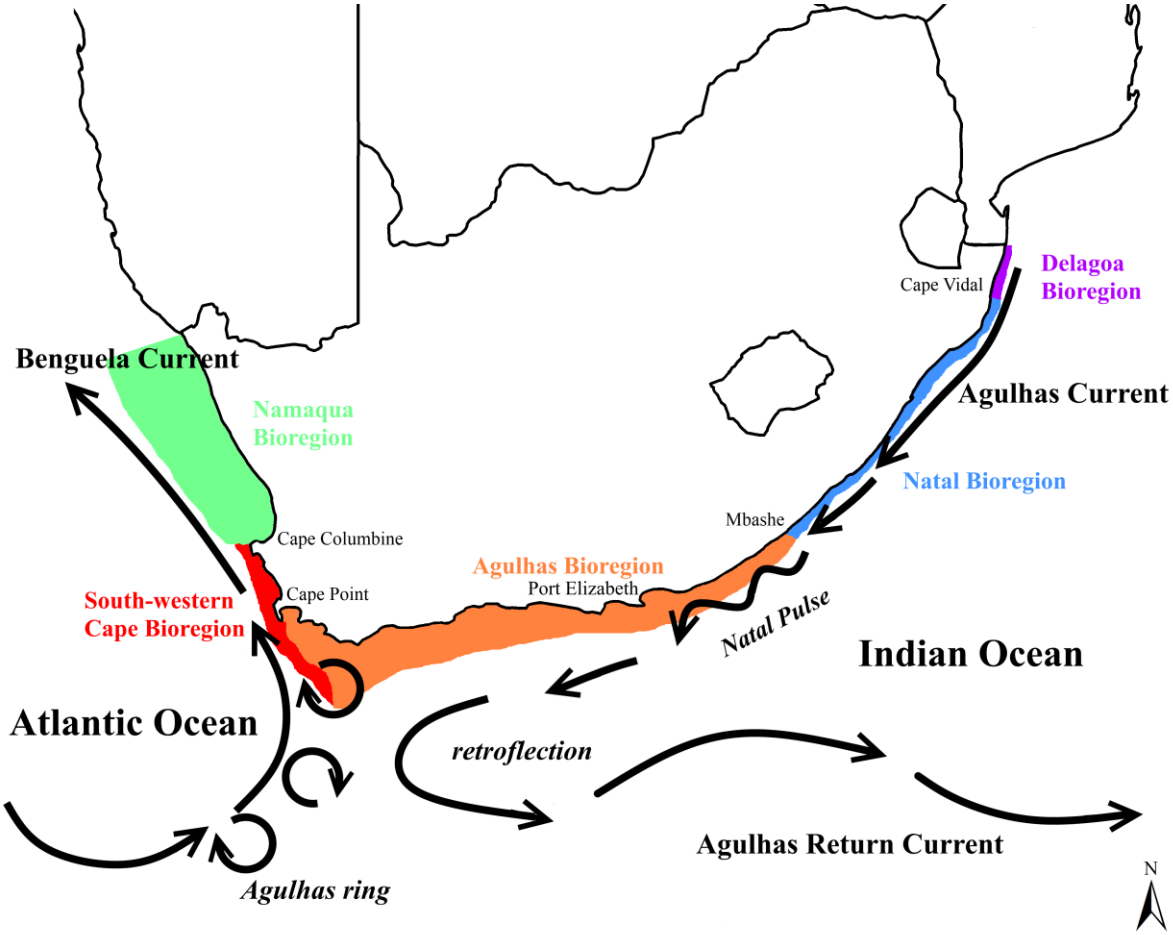

Figure 2.1 South Africa: major ocean currents and coastal biogeographic regions. Ocean currents and current features, such as the Natal Pulse and Agulhas rings are shown (Lutjeharms & de Ruijter, 1996; Rouault & Penven, 2011). The names and locations of inshore biogeographic regions along with biogeographic breaks are also shown (Sink, et al., 2005).

2.1.2 South African biogeography and phylogeography

Currents, temperatures and other oceanography influence species diversity and distribution which results in different biogeographic regions (Spalding, et al., 2007). Although several the bioregions and biogeographic zones have been proposed, South Africa's coastline can be divided into five main coastal biogeographic regions. These are: the Namaqua, South-western Cape, Agulhas, Natal, and Delagoa bioregions (Figure 2.1) (Lombard, et al., 2004; Griffiths, et al., 2010). There is a region of overlap between the South-western Cape and the Agulhas bioregions, this may be due to the fact that the meeting point of the Agulhas and Benguela Currents is constantly in a state of flux; this area is a mixing point for various mollusc and fish communities and is thus considered an area of contact (Griffiths, et al., 2010; Sink, et al., 2005; Lutjeharms & de Ruijter, 1996; von der Heyden, et al., 2011). The Agulhas and Natal bioregions meet on South Africa's east coast. The location that separates the Agulhas and Natal Bioregions has been heavily debated (Sink, et al., 2005). Mbashe was ultimately chosen

as the exact site of contact (Figure 2.1) but other localities put forward for this boundary were (in order of northern- to southern-most) Waterfall Bluff, Mbotyi, Port St Johns[1], and Woody Cape (Sink, et al., 2005); the first three sites fall within a distance of approximately 100 km North of the ultimately chosen Mbashe site whereas Waterfall Bluff is approximately 290 km South of said site.

Phylogeography falls within the broader construct of biogeography; a phylogeographic region refers to the geographic limits of a genetic population or species, whereas a biogeographic region refers to the geographic limits of biologically-recognised species (Arbogast & Kenagy, 2001; Avise, 2009). Phylogeographic barriers restrict genetic exchange which could result in separate reproductive stocks (Avise, 2009); biogeographic barriers are barriers that mark the separation of morphologically- or biologically-recognised species, community of species, and higher taxonomic levels. The Cape Agulhas phylogeographic barrier (Figure 2.1), for example, is apparent in several species, including *Haliotis midae* (South African abalone), and has resulted in separate reproductive stocks either side of this barrier (Evans, et al., 2004). On the east coast, there appear to be multiple phylogeographic barriers and regions of overlap (Sink, et al., 2005). The Mbashe phylogeographic barrier is present in several species such as *H. midae*, *Panulirus homarus* (east coast rock lobster), and various estuarine fish communities (Sink, et al., 2005; Harrison, 2002). The phylogeographic barriers on the east coast are not as distinct as the Cape Agulhas barrier as there have been far fewer studies on this coast (Sink, et al., 2005).

The biogeographic regions around South Africa's coastline are important if one were to cultivate indigenous oysters, as it should be known whether there are natural, pre-existing distinct phylogenetic populations. If such populations were to exist, the onus would be on mariculturists and government to maintain them, and not move oysters from one genetically distinct population to the other. Mixing of historically distinct populations through agriculture and mariculture has occurred frequently in human history but maintaining natural genetic structure would be preferable (Grant, 2007). Cross breeding may result in hybridization that reduces the fitness and genetic integrity of the original, breeding population (Rhymer, 2006).

2.1.3 Population genetic approaches

An important aspect to understanding and successfully culturing indigenous species is knowledge of their genetic structure and diversity. Genetic diversity, genetic structure and

[1] Port St Johns is located between two of the chosen sample sites for *Saccostrea cucullata*

gene flow are vitally important to the evolutionary potential of a species (Laikre, et al., 2009). Oyster farmers frequently move oysters around the globe to get the best results or for the best growth conditions. It may be found empirically that the best location for oyster spat collection is in one biogeographic region whereas the best location for growth is in another. It is of vital importance that we determine the genetic structure of indigenous oysters before such movements occur so as to not tamper with the natural genetic diversity and structure (Bester-van der Merwe, et al., 2011). Genetic diversity is important to maintain fitness which is important for the perseverance of a population or species and aquaculture production (Rhymer, 2006).

To determine population structure two gene regions were chosen for this study, Cytochrome oxidase subunit I (CO1, mtDNA) and 16S (rRNA); both have been used extensively in the analysis of population genetics in marine bivalves and, in particular, oysters (Klinbunga, et al., 2005; Boudry, et al., 1998; Banks, et al., 1993; Zardi, et al., 2007; Boudry, et al., 2003; Salvi, et al., 2014; Liu, et al., 2011). The 16S gene region is more slowly evolving than the CO1 but to determine genetic phylogenies and oyster relationships most studies have relied on fragments of ribosomal genes of either mitochondrial or nuclear subunits (16S or 28S respectively (Salvi, et al., 2014). As analysis of nuclear DNA is more complex analytically (due to its double-stranded nature), the mitochondrial 16S rRNA gene region was chosen to support the CO1 data for this study.

The CO1 gene region was used as it is one of few fast-evolving gene regions for which there are available data for the *Saccostrea* and *Striostrea* genera (Liu, et al., 2011; Salvi, et al., 2014). DNA barcoding and identification of a species are done using the CO1 gene region (Dawnay, et al., 2007; Liu, et al., 2011), which makes its usefulness in this study two-fold, not only can it be used to calculate genetic analysis indices and examine patterns of genetic structure, but the CO1 sequence can also be used to ensure that a sample has been correctly identified when collected. Unfortunately sequence identity can only be confirmed if there is an accurate reference library and there are no CO1 sequences for *S. margaritacea* in GenBank which means it cannot be identified as such. There are, however, sequences of *Saccostrea* (including *S. cucullata* and *S. mordax*) from other countries; therefore these could be identified. There are many genetic markers suitable for population genetic approaches including the use of fragments of mitochondrial DNA, tandem repeated microsatellite markers; and the use of nuclear DNA markers, to name a few (Salvi, et al., 2014). Mitochondrial markers (at equilibrium) are generally more sensitive indicators of population

structure than nuclear markers (Zink & Clough, 2008). Mitochondrial markers (mtDNA) have been chosen over microsatellites or nuclear markers, as the use of microsatellites is contingent on a pre-constructed library; if one does not exist (as is the case) the construction of one is a time consuming and expensive endeavor (Zane, et al., 2002; Squirrell, et al., 2003; Thiel, et al., 2003). Microsatellites for a congener, such as *Saccostrea glommerata*, could be tested on the target species however they may not work successfully.

2.1.4 Factors affecting oyster population structure (in South Africa)

Many factors could affect population structure some of which are abiotic factors such as ocean currents, temperature, salinity, and oxygen availability; all of which would impact larval dispersal and juvenile survival (Bricelj & Lonsdale, 1997; Calbrese & Davis, 1966; Cockcroft, 2001; Branch, et al., 2013). The two oyster species chosen for this study both have a pelagic larval duration (PLD) of 20 to 50 days (Kilburn & Rippey, 1982; Sukumar & Mohan Joseph, 1988). Most species which disperse via planktonic larvae show little genetic structure however, this is not always the case, for example *Perna perna* and *Mytilus galloprovincialis* (Teske, et al., 2011; Zardi, et al., 2007). A multispecies study by Kelly and Palumbi (2010) of intertidal species with pelagic larvae showed 13 species with significant genetic structure, this finding corroborates those of Teske et al. (2011) and Zardi et al. (2007). Another factor affecting oyster population structure is the availability of hard substratum for attachment of settled juvenile and adult oysters. Some bivalves fasten themselves to the substratum: for example, mussels use byssus threads to attach to a substrate, while oysters cement their left shell valve to hard sediment such as rock or, in sandy conditions, shells, mangrove roots and even jetty piers (Kilburn & Rippey, 1982; Joseph, 1998).. In South Africa it is illegal to collect oysters on SCUBA, making subtidal hard ground a good location for oyster breeding stock of natural populations. Since oysters require hard ground for survival, data from the National Biodiversity Act (NBA) (Sink, et al., 2012) were used to determine available hard ground along the coast line that is between 500 meters above sea level and 30 meters below (Figure 2.2); these data were used to aid site selection and to allow hypothesising of genetic structure.

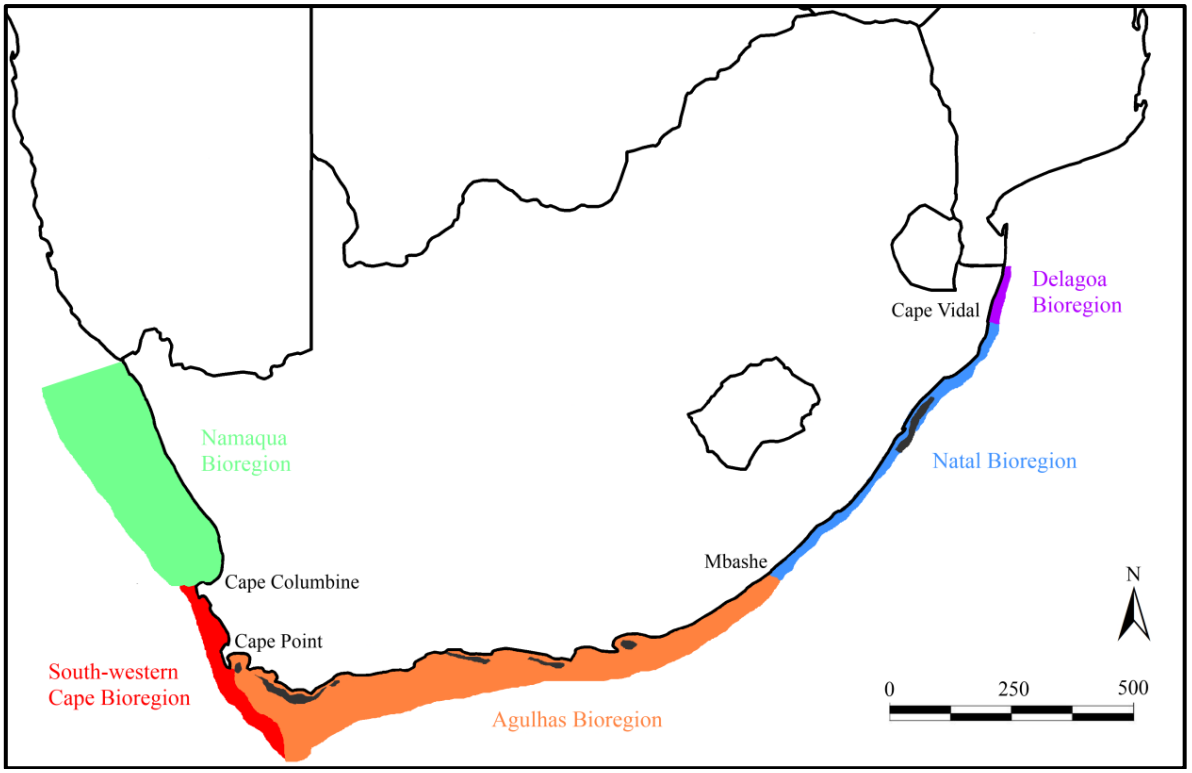

Figure 2.2 Hard-ground sites between +500m and -30m including biogeographic regions around South Africa's coastline; a very small portion of the coastline is shallower than 30m but the hard-ground has been kept to scale. (Sink, et al., 2012)

2.1.5 Candidate indigenous species for culture in South Africa

Most oysters can be found in the intertidal zone and below the low water mark down to approximately 5 meters (Kilburn & Rippey, 1982). There are five species of oyster which are native to South Africa (Haupt, et al., 2010). Of these five, two are palatable species and are listed below, along with reasons for their choice. *Striostrea margaritacea* (formerly *Crassostrea margaritacea*, also known as the Cape rock oyster) is endemic to South Africa, and occurs sub-tidally which makes it well suited to long-line suspended culture, the predominant culture method in South Africa (Kilburn & Rippey, 1982). *Saccostrea cucullata* (formerly *Crassostrea cucullata*, commonly known as the Natal rock oyster or the hooded oyster) is more widely distributed and can be found throughout the Indo-Pacific from Algoa Bay northwards along the African coast to Somalia, east through Seychelles and Madagascar to Asia and the Pacific islands, and south to Australia and New Zealand (South African range: Figure 2.3) (Braley, 1982; Branch, et al., 2007; Haupt, et al., 2010; Kilburn & Rippey, 1982; Klinbunga, et al., 2003).

Saccostrea cucullata forms conspicuous belts and can be found in the mid- to upper-intertidal zone in South Africa (Kilburn & Rippey, 1982; Branch, et al., 2013) while in the Mediterranean it can be found sub-tidally down to 15 meters (CIESM, 2005). *Striostrea*

margaritacea ranges from False Bay (South Africa) to Mozambique and reaches a maximum size of 180 mm, Figure 2.3 (Robinson, et al., 2005; Kilburn & Rippey, 1982; Haupt, et al., 2010; Branch, et al., 2007). *Striostrea margaritacea* is the dominant species south of the Transkei and forms beds at and below the extreme low tide mark, down to 5 meters below sea level (Kilburn & Rippey, 1982). Both *S. margaritacea* and *S. cucullata* are cupped oysters, and transition from male to female as they age but can change from female to male or vice versa throughout their life time (Kilburn & Rippey, 1982). They both reproduce via broadcast spawning which can be triggered by the presence of either eggs or sperm in the water column; this can induce an entire colony to spawn simultaneously (Branch, et al., 2007; Kilburn & Rippey, 1982).

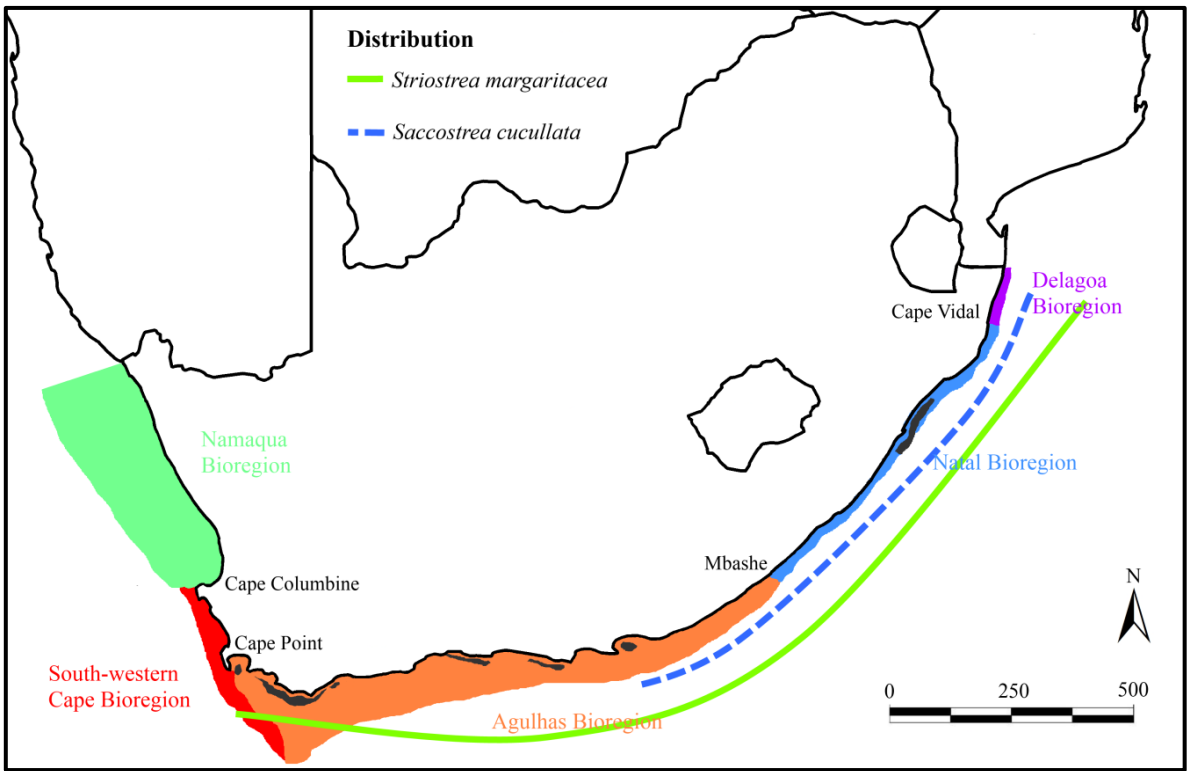

Figure 2.3 Distribution range of *S. margaritacea* and *S. cucullata* around South Africa's coastline bioregions and hard ground (Griffiths, et al., 2010) are also shown alongside distribution (Haupt, et al., 2010).

2.1.6 Aims

This study aimed to determine the genetic structure of *S. margaritacea* and *S. cucullata* along South Africa's coastline using mitochondrial DNA markers CO1 and 16S. Due to the sampling sites spanning the Agulhas and Natal Bioregions, there is an expectation of a biogeographic break between the samples from locations south-west of and north-east of the Mbashe River. Patchy hard-ground suggests that there is limited habitat for oyster settlement.

The hypothesis was that there would be genetic structure between locations given the different biogeographic regions and patchy hard-ground along the coastline.

2.2 Methods

2.2.1 Collection of samples

All oysters were collected under a research permit from the Department of Agriculture Forestry and Fisheries (DAFF). Thirty *Striostrea margaritacea* were collected from each of the following sites: Breede, GouKou, Mossel Bay, Knysna, Plettenberg, Kromriver, Algoa Bay, Port Alfred, Hamburg, Kwelera, Mtakatye and Westbrook (Figure 2.4). The range of a species changes over time due to changes in abiotic environmental factors and changes in communities, biotic factors (Sexton, et al., 2009). Communities can change due to introductions of other species or influences of anthropogenic nature or natural environmental changes. Since the most recent distribution of *S. margaritacea* was determined in the 1980s by Kilburn and Rippey (1982) and, as mentioned, ranges can change over time, samples had to be taken from multiple locations within the previously given range. After samples had been collected the new, approximate range could be determined and sites, that were equidistant within this range, could be chosen for genetic analyses (Figure 2.4). As the sampling was not intensive between Breede (most western site where *S. margaritacea* was found) and Cape Agulhas (western site where *S. margaritacea* could not be found) it cannot be stated that the new range of *S. margaritacea* has its western limit at the Breede River. However its limit is somewhere between the Breede River Mouth and Cape Agulhas and the new range is further east than the previous western limit, False Bay (Kilburn & Rippey, 1982).

Striostrea margaritacea are subtidal oysters but some samples can be found above the low-water mark during spring tides. The samples which were sub-tidal were collected using a mask and snorkel, and harvesting was accomplished using either a concrete chisel and hammer or a screwdriver and hammer to prise the oysters from the substrate. Those found above the low-water mark were collected using a screwdriver and hammer. *Striostrea margaritacea* samples were collected by Jenna Keightley and Sue Jackson, Doug and Ann Davis (in Knysna), Ken and Les Keightley, and those collected in Westbrook were collected by Erica Steyn (courtesy of Mike Schleyer) (Table 2.1).

Table 2.1 List of sampling locations and collectors

Species	Location	Collecting team	Collection method	Date
Striostrea margaritacea	Breede	Jenna & Sue	Hammer and Chisel	2012
	GouKou	Sue	Hammer and Chisel	2012
	Mossel Bay	Jenna & Sue	Hammer and Chisel	2013
	Knysna	Doug & Anne	Hammer and Chisel	2015
	Bitou	Jenna & Sue	Hammer and Chisel	2013
	Krom	Jenna & Sue	Hammer and Chisel	2013
	Swartkops	Jenna & Sue	Hammer and Chisel	2013
	Port Alfred	Jenna & Sue	Hammer and Chisel	2013
	Hamburg	Jenna & Sue	Hammer and Chisel	2013
	Kwelera	Jenna & Sue	Hammer and Chisel	2013
	Mdumbi	Dave Krebser	Hammer and Chisel	2013
	Mtakatye	Jenna	Hammer and Chisel	2013
	Westbrook	Erica Steyn	Hammer	2013
Saccostrea cucullata	Mtakatye	Ken & Les	Hammer and Chisel	2015
	Port Edward	Erica Steyn	Hammer	2013
	Umdloti	Erica Steyn	Hammer	2013

Thirty *Saccostrea cucullata* were collected from Mdumbi, Mtakatye, Port Edward, and Umdloti; the samples from Mdumbi were not used for genetic analyses as the location was too close to the Mtakatye sample site and the sites used were approximately equidistant (Figure 2.4). Samples collected from Mtakatye were collected by Jenna Keightley, those from Mdumbi were collected by Dave Krebser, and the samples from Port Edward and Umdloti were collected by Mike Schleyer and Erica Steyn. *Saccostrea cucullata* is an intertidal species in South Africa which is visible above the low-water mark during neap tide (Kilburn & Rippey, 1982). *Saccostrea cucullata* tissue samples were collected by breaking open oysters on the rocks with a hammer and collecting the tissue in the field and preserving it as mentioned below.

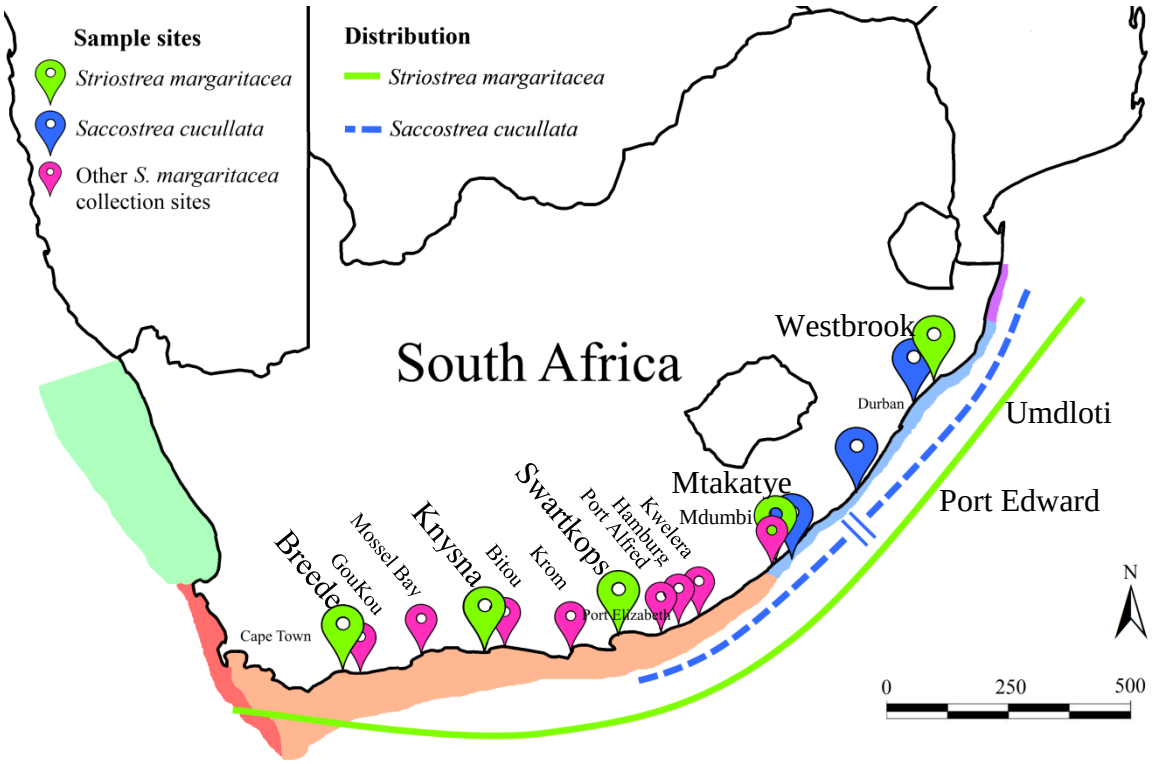

Figure 2.4 Locations of collection sites for *S. margaritacea* and *S. cucullata* along South Africa's coastline. Samples collected from the locations indicated in pink were not sequenced, for reasons detailed below.

2.2.2 Tissue extraction

Striostrea margaritacea samples, once collected, were shucked and a section of mantle and/or gill tissue, weighing approximately 0.6 grams was taken from each oyster sample. All tissue samples were put into 1.5 mL Eppendorf tubes containing 99% ethanol and stored at -4°C to -20°C. A slightly different method was used to extract tissue from *S. cucullata*; the left valve (bottom valve) of *S. cucullata* attaches them very securely to rocky substrate which makes removing an intact oyster problematic. If the oyster was removed and remained intact the sample would be treated in the same fashion as samples of *S. margaritacea*, otherwise gill and mantle tissue were removed from the damaged oysters (in the field) and immediately placed in Eppendorf tubes with 99% ethanol. Storage of *S. cucullata* tissue was the same as that of *S. margaritacea*.

2.2.3 Sequence data

DNA was extracted and sequenced from samples of *Striostrea margaritacea* from Breede, Knysna, Algoa Bay, Mtakatye and Westbrook. The locations were chosen due to their proximity to hard ground according to Sink et al. (2012), as well as the fact that these locations were relatively evenly dispersed along the known range of *S. margaritacea*. Locations from which DNA extraction and sequencing were performed for *S. cucullata* were

Mtakatye, Port Edward, and Umdloti; these locations are evenly distributed along the coastline within the known range of *S. cucullata*. The same methods were used for both *S. margaritacea* and *S. cucullata*. DNA extractions were performed using either GeneJET Genomic DNA Purification Kit or Macherey-Nagel's NucleoSpin® Tissue Kit (difference in kit choice due to stock availability). All extractions were performed as per the user manual of each kit, respectively.

Polymerase Chain Reaction (PCR) was performed using KAPA Biosystems PCR reagents (Taq and PCR buffer with Magnesium chloride) or Super-Therm Polymerase PCR reagents (Taq, PCR buffer, and Magnesium). Primers utilized in the PCR reactions for the Cytochrome Oxidase subunit I (CO1) gene region were LCO1490 (forward primer) 5'-ggtcaacaaatcataaagatattgg-3' and HC02198 (reverse primer) 5'-taaacttcagggtgaccaaaaaatca-3' (Folmer, et al., 1994). The PCR amplification reaction was carried out in a 25 µl reaction volume however the combination of the different PCR reagents differed between the brands of PCR reagents. KAPA Biosystems PCR reagents required the following reaction volumes and concentrations : 11.9µl distilled water, 200µM Deoxynucleotide Triphosphates (dNTPs), 2.5µl 1X PCR buffer, 5µM forward and reverse primers, and 0.5 units of Taq; the amount of DNA used in each reaction was approximately 300 ng. When using Super-Therm Polymerase PCR reagents, the volumes for each reagent was as follows: 12.4µl distilled water, 2.5µl PCR buffer, 100µM dNTPs, 2mM Magnesium Chloride , 0.5µM Forward (LCO1490) and reverse primer (HCO2198), 0.5 units Taq polymerase (Hoffman-La Roche) and 3µl of extracted DNA. The PCR protocol above, for *S. margaritacea* was adapted from Klinbunga et al. (2005) for *Striostrea mytiloides*. A gradient PCR was used to determine the annealing temperature which resulted in the following successful protocol: initial denaturation at 94° C for 3 minutes, 45 cycles consisting of a denaturing step at 94° C for ten seconds, followed by annealing at 42° C for 30 seconds and then elongation at 72° C for 90 seconds, a final elongation at 72° C for five minutes was carried out at the end.

For the 16S gene region PCR was performed using KAPA Biosystems PCR reagents (Taq, PCR buffer, and Magnesium Chloride). 16S primers designed by Palumbi (1996) were used: 16Sar (5'-cgcctgtttatcaaaaacat-3', forward) and 16Sbr (5'-ccggtctgaactcagatcagatcacgt-3', reverse). PCR was carried out in a 25 µl reaction volume comprising: 13.9 µl water, 2.5 µl 1X PCR buffer, 100µM dNTPs, 1.5mM Magnesium Chloride, 5 µM of each primer, 0.5 units of Taq, and approximately 200ng DNA. A similar PCR protocol was used as that for CO1 the initial denaturing at 94° C for 3 minutes; 35 cycles of a denaturing step at 94° C for 10

seconds, followed by annealing at 42° C for 30 seconds, and then elongation at 72° C for one minute 30 seconds. The final elongation step was at 72° C for five minutes.

Five microliters of each PCR product were visualised on a 1% agrose gel stained with ethydium bromide or with Pronosafe to determine if the amplification was successful[2]. If the bands were distinct the remainder of the PCR product was sent to the Sequencing Unit at Stellenbosch University. BigDye terminator chemistry (Applied Biosystems) was used to generate sequences on an ABI-3100 automated sequencer. Samples that appeared as distinct bands when visualized on a 1% agrose gel were sequenced in a single direction (using the forward primer) and for those whose bands appeared dull but still visible, bi-directional sequencing was used (both forward and reverse primers).

2.2.4 Molecular analyses

Sequence identity was confirmed by using the BLAST function in GenBank (Benson, et al., 2012; Altschul, et al., 1990). To determine whether pseudogenes had been amplified in CO1 sequences, an Open Reading Frame (ORF) Finder program (Gene Infinity LLC, 2014) was used to convert nucleotide sequences into protein sequences; this program finds all open reading frames and prepares protein sequences for each one. The longest protein sequence would be the one with the correct ORF. Most, if not all, of the protein sequences that do not have the correct ORF will have a stop codon within the sequence All amplified CO1 sequences were checked for pseudogenes and none were found.

BioEdit (Hall, 1999) and Geneious v 7.1 (Kearse, et al., 2012) were used to edit and align sequences; edited CO1 sequences had a length of 491 basepairs (bp) and 16S sequences, a length of 315bp. Network v 5 was used to create Median Joining haplotype networks (Bandelt, et al., 1999; Fluxus Technology Ltd, 1999-2015). Arlequin v 3.5.1.2 (Excoffier & Lischer, 2010) was used to calculate haplotype (h) and nucleotide (π) diversity, AMOVA (Excoffier, et al., 1992), and pair-wise Fst values for all species, locations and markers. Haplotype and nucleotide diversity are used to infer inter- and intra-population variation, this variation is important as it alludes to the evolutionary potential of the populations. Fst values are used to infer genetic structure and inter-population gene-flow. Fst values were calculated using an AMOVA for each species and marker (data set), to determine overall gene flow; pairwise Fst values were also calculated, these were used to determine more specific inter-

[2] Ethidium bromide is used in the EGG lab in Stellenbosch whereas Pronosafe is used in the genetics lab at the Oceanographic Research Institute.

population fixation. MEGA 6 (Tamura, et al., 2013) was used to create Maximum Likelihood phylogenetic trees and Fig Tree (Rambaut, 2014) was used to visualise these outputs.

Maximum likelihood (ML) analyses and best fit model tests were performed using Mega 6 (Tamura, et al., 2013). As the minimum number of discrete gamma categories allowed in a ML analysis in MEGA 6 is two, this was the gamma value used for both CO1 and 16S along with 1000 replicates to determine bootstrap support (Kennedy & Schumacher, 1993). Maximum likelihood analysis on the CO1 gene region included sequences of *S. cucullata*, *S. margaritacea*, and *O. atherstonei* (outgroup) from South Africa. Sequences of species from other countries were also included in these analyses: *Saccostrea mordax* (China), *S. cucullata* (Thailand and Japan), and *Saccostrea echinata* from GenBank. Maximum likelihood analysis on the 16S gene region included *S. cucullata* but excluded *S. margaritacea* sequences from South Africa as sequences with large gaps could not be aligned with confidence and were therefore omitted. Other species included in the 16S analysis were *Saccostrea kegaki* (China and Japan), *S. mordax* (China), *Saccostrea malabonensis* (Japan), *S. echinata* (China), and *S. cucullata* (China and Australia), with *Ostrea edulis* being the out group. *Ostrea atherstonei* and *O. edulis* are used as out groups as the *Ostrea* genus is shown to be a separate, monophyletic clade to *Saccostrea* for combined CO1 and 16S sequences by Salvi et al. (2014). The ML trees were edited in TreeGraph2 (Stöver & Müller, 2010). Trees were rooted with their respective outgroup species.

Fu's Fs, Tajima's D and Fu and Li's D* and F* statistics were calculated using DNAsp (Librado & Rozas, 2009). Fu's Fs and Tajima's D statistical values describe the difference between observed and expected diversity in polymorphic sites and are used to detect selection, deduce population changes (increase, decrease, or stable) and determine adaptive selection (Fu, 1997; Peck & Congdon, 2004). A significantly negative Tajima's D value suggests that there are fewer haplotypes and rare alleles are present in a higher than expected frequencies, and that there has been a population expansion after a recent bottle neck (Stephens, et al., 2001). A significantly positive Tajima's D suggests that rare alleles are present at lower than expected frequencies, there are more haplotypes, and the population has undergone a sudden population contraction (Stephens, et al., 2001). A Tajima's D of 0 suggests that the observed variation is similar to the expected variation and the population is evolving as per mutation-drift equilibrium (Stephens, et al., 2001). Fu's Fs and Fu and Li's D* and F* statistics are the same as Tajima's D but are more powerful tests (Fu, 1997).

Inter- and intra-population distances were calculated using Arlequin. These analyses where performed for both CO1 and 16S for *Saccostrea* samples collected from South Africa as well as *S. cucullata* and *S. mordax* from other countries (sourced from GenBank). Inter- and intra-specific distances were also calculated for known species of *Crassostrea* and *Ostrea* from GenBank to compare with the distances shown by *Saccostrea* to substantiate whether or not the *Saccostrea* samples represent different species. *Crassostrea* and *Ostrea* genuses were chosen as they are closely related to *Saccostrea* and *Striostrea* all forming part of the Ostreidae family and are distinct species as shown by Salvi et al. (2014).

2.3 Results

All of the below results show that what was previously recognised as a single species of *S. cucullata* along South Africa's coastline is in fact two distinct species; one being *S. cucullata* while the other may be *S. mordax* or another *Saccostrea* species. Samples collected from Mtakatye are more closely related to *S. cucullata* (except one sample, sample 13) and all samples collected from both Umdloti and Port Edward are more closely related to *S. mordax* (10% inter-population distance between them) or another *Saccostrea* species other than *S. cucullata*. Due to this discovery when these samples are being discussed as a collective they will be referred to as *Saccostrea* (as they all fall within the genus), when referred to separately: Mtakatye samples will be referred to as such and the Umdloti and Port Edward samples will be grouped and referred to as the northern population.

2.3.1 Haplotype and nucleotide diversity indices

Haplotype and nucleotide diversity of *Saccostrea* for both CO1 and 16S gene regions show that samples from Mtakatye have a higher diversity than the northern population (Table 2.2). Haplotype and nucleotide diversity for *S. margaritacea* for both CO1 and 16S gene regions show that no population has higher or lower diversity than another (Table 2.3). Both CO1 and 16S show similar patterns in each species where *Saccostrea* has one site (Mtakatye) with much higher diversity than the other two and *S. margaritacea* shows little difference in diversity across all populations. For both *Saccostrea* and *S. margaritacea* the 16S gene region shows lower levels of diversity than the CO1 gene region.

Table 2.2 Haplotype (h) and nucleotide (π) diversity of *Saccostrea* CO1 and 16S gene regions

location	haplotype (h)	nucleotide (π)
CO1		
Umdloti	0.54 +/- 0.14	0.0036 +/- 0.0024
Port Edward	0.42 +/- 0.12	0.0012 +/- 0.0011
Mtakatye	1.00 +/- 0.02	0.0644 +/- 0.0334
16S		
Umdloti	0.00 +/- 0.00	0.0000 +/- 0.0000
Port Edward	0.22 +/- 0.17	0.0005 +/- 0.0007
Mtakatye	0.87 +/- 0.11	0.0470 +/- 0.0260

Table 2.3 Haplotype (h) and nucleotide (π) diversity of *S. margaritacea* CO1 and 16S gene regions

location	haplotype (h)	nucleotide (π)
CO1		
Breede	0.88 +/- 0.06	0.0060 +/- 0.0036
Knysna	0.84 +/- 0.09	0.0050 +/- 0.0032
Swartkops	0.82 +/- 0.07	0.0058 +/- 0.0035
Mtakatye	0.87 +/- 0.07	0.0041 +/- 0.0027
Westbrook	0.88 +/- 0.03	0.0065 +/- 0.0038
16S		
Breede	0.00 +/- 0.00	0.0000 +/- 0.0000
Knysna	0.18 +/- 0.14	0.0004 +/- 0.0006
Swartkops	0.00 +/- 0.00	0.0000 +/- 0.0000
Mtakatye	0.20 +/- 0.15	0.0004 +/- 0.0007
Westbrook	0.38 +/- 0.18	0.0009 +/- 0.0010

2.3.2 Fixation indices (Fst values)

The AMOVA Fst values for *Saccostrea* are high for both CO1 and 16S whereas AMOVA Fst values for *S. margaritacea* for CO1 and 16S are strikingly lower (Table 2.4). This suggests that there is restriction in the flow of alleles between the populations of *Saccostrea*, whereas *S. margaritacea* has little or no fixation between its populations, indicating higher levels of gene flow. Assessing pairwise Fst values for *Saccostrea* shows the high AMOVA Fst values are indicative of potnetial species level divergence, as corroborated by the ML trees below (Figure 2.9 and Figure 2.10). Pair-wise Fst values for *Saccostrea* show that the Mtakatye samples have a high level of fixation when compared to the two northern populations (Umdloti and Port Edward) (Table 2.5). Pairwise Fst values for *S. margaritacea* show that all populations have similarly low levels of fixation for alternative alleles for both CO1 and 16S gene regions (Table 2.6).

Table 2.4 AMOVA Fst values for all species and gene regions

		AMOVA	
		Fst Values	p-values
Northern population & Mtakatye 13	CO1	0.04	>0.05
	16S	<0.01	>0.05
Saccostrea	CO1	0.87	<0.01
	16S	0.89	<0.01
S. margaritacea	CO1	0.02	>0.05
	16S	0.01	>0.05

Table 2.5 Pairwise Fst values for *Saccostrea* for the CO1 and 16S gene region. Significant values are marked with *.

	Umdloti	Port Edward	Mtakatye
CO1			
Umdloti			
Port Edward	0.016		
Mtakatye	0.812*	0.846*	
16S			
Umdloti			
Port Edward	<0.001		
Mtakatye	0.782*	0.785*	

Table 2.6 Pairwise Fst values of *S. margaritacea* CO1 and 16S gene regions.

	Breede	Knysna	Swartkops	Mtakatye	Westbrook
CO1					
Breede					
Knysna	0.000				
Swartkops	0.000	0.000			
Mtakatye	0.000	0.045	0.003		
Westbrook	0.018	0.000	0.016	0.085	
16S					
Breede					
Knysna	0.000				
Swartkops	0.000	0.000			
Mtakatye	0.000	0.001	0.000		
Westbrook	0.000	0.004	0.000	0.000	

2.3.3 Fu's, Fu and Li's, and Tajima's statistics

For these analyses, Mtakatye sample 13 was removed to separate the lineages. Only the CO1 gene region for the northern population shows significant negative Fu's Fs, Fu and Li's D* and F* statistic, and Tajima's D. These negative statistics suggest that this population has undergone a recent population expansion and has fewer than expected haplotypes; the lower number of haplotypes is shown in the haplotype networks (Figure 2.5).

Table 2.7 Population expansion and selection statistics for *Saccostrea* samples

		Fu & Li's statistics			
		Fu's Fs	F*	D*	Tajima's D
CO1	Northern population	-9.67*	-3.83*	-3.67*	-2.39*
	Mtakatye	-2.90	-1.93	-1.65	-1.30
16S	Northern population	-0.79	-1.61	-1.50	-1.16
	Mtakatye	1.39	-1.06	-0.86	-0.80

2.3.4 Haplotype networks

Median Joining haplotype networks for *Saccostrea* show a high level of allelic differentiation between samples from the southern, Mtakatye, and northern, Port Edward and Umdloti, locations demonstrating that the gene flow is limited. Both the CO1 and 16S haplotype networks show the same pattern (Figure 2.5 and Figure 2.6). The CO1 haplotype network shows that the Mtakatye haplotypes are separated from the northern population by 102 mutational steps (between the two closest haplotypes) while the 16S haplotype network shows 36 mutational steps between these haplotypes; the inter-populationdivergence between Mtakatye and the northern populations is 20.35% and 9.75% for CO1 and 16S, respectively. Median joining haplotype networks for *S. margaritacea* CO1 and 16S show that there is little fixation of alternative alleles and the populations are panmictic (Figure 2.7 and Figure 2.8). The star-shaped network suggests levels of unique mutations which suggest rapid population expansion.

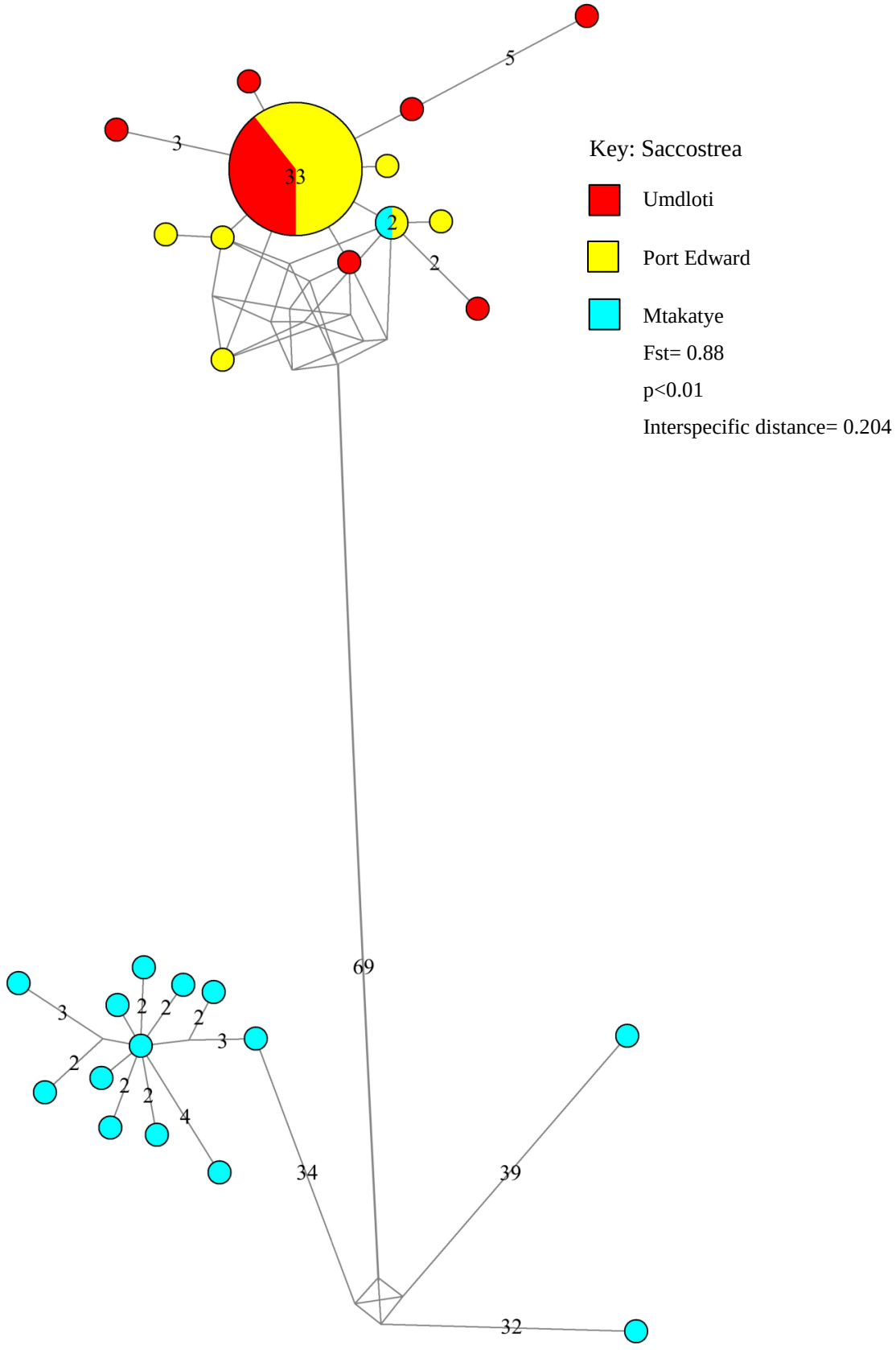

Figure 2.5 Haplotype network of *Saccostrea cucullata* CO1 gene region. Haplotype frequency and branch lengths are shown on nodes and branches respectively; any node of branch with no given value has a value of 1. The AMOVA Fst value, as well as the inter-population distance, are both shown above.

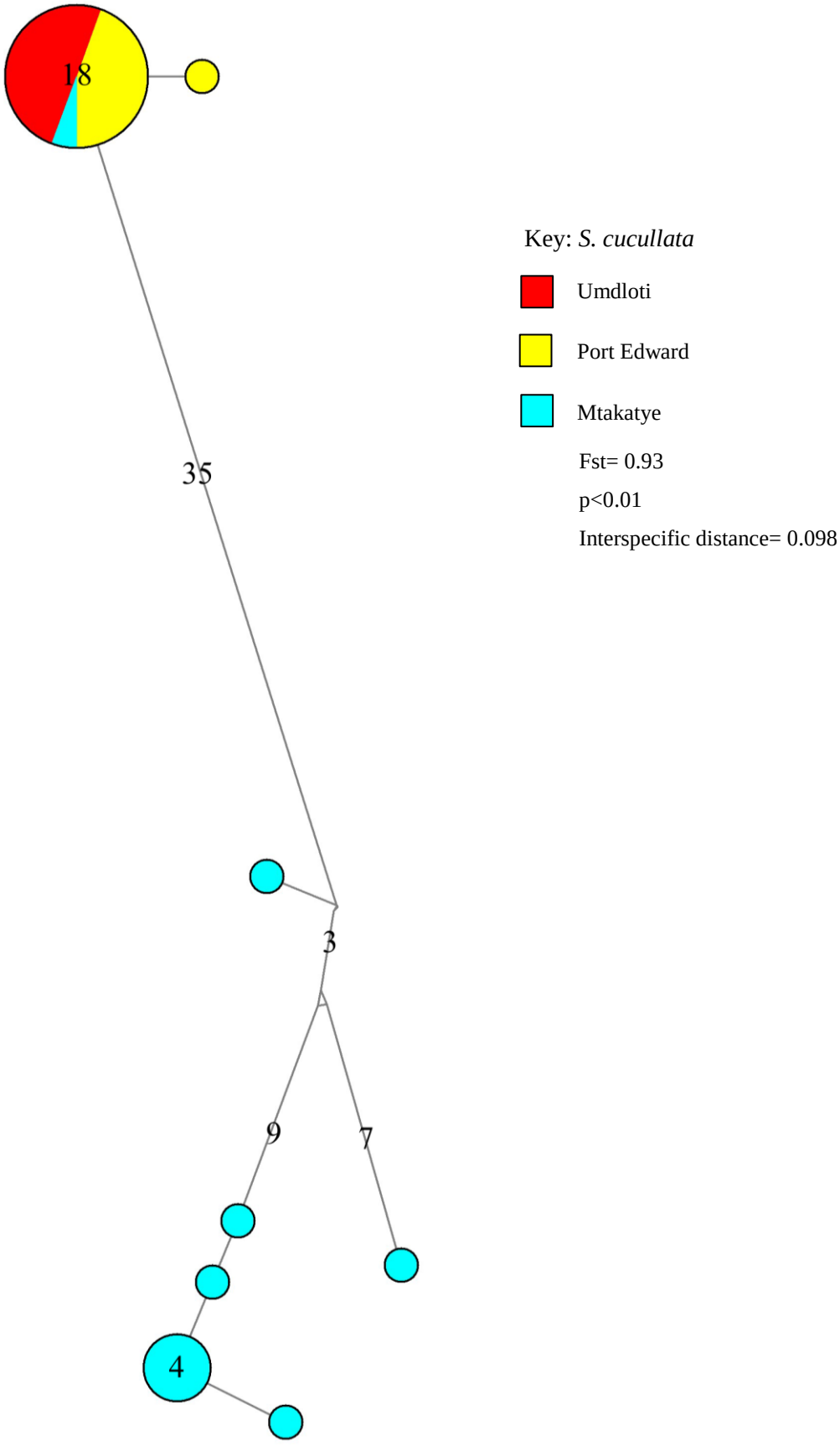

Figure 2.6 Haplotype network of *Saccostrea cucullata* 16S gene region. Haplotype frequency and branch lengths are shown on nodes and branches respectively; any node of branch with no given value has a value of 1. The AMOVA Fst value and inter-population distance are both shown above.

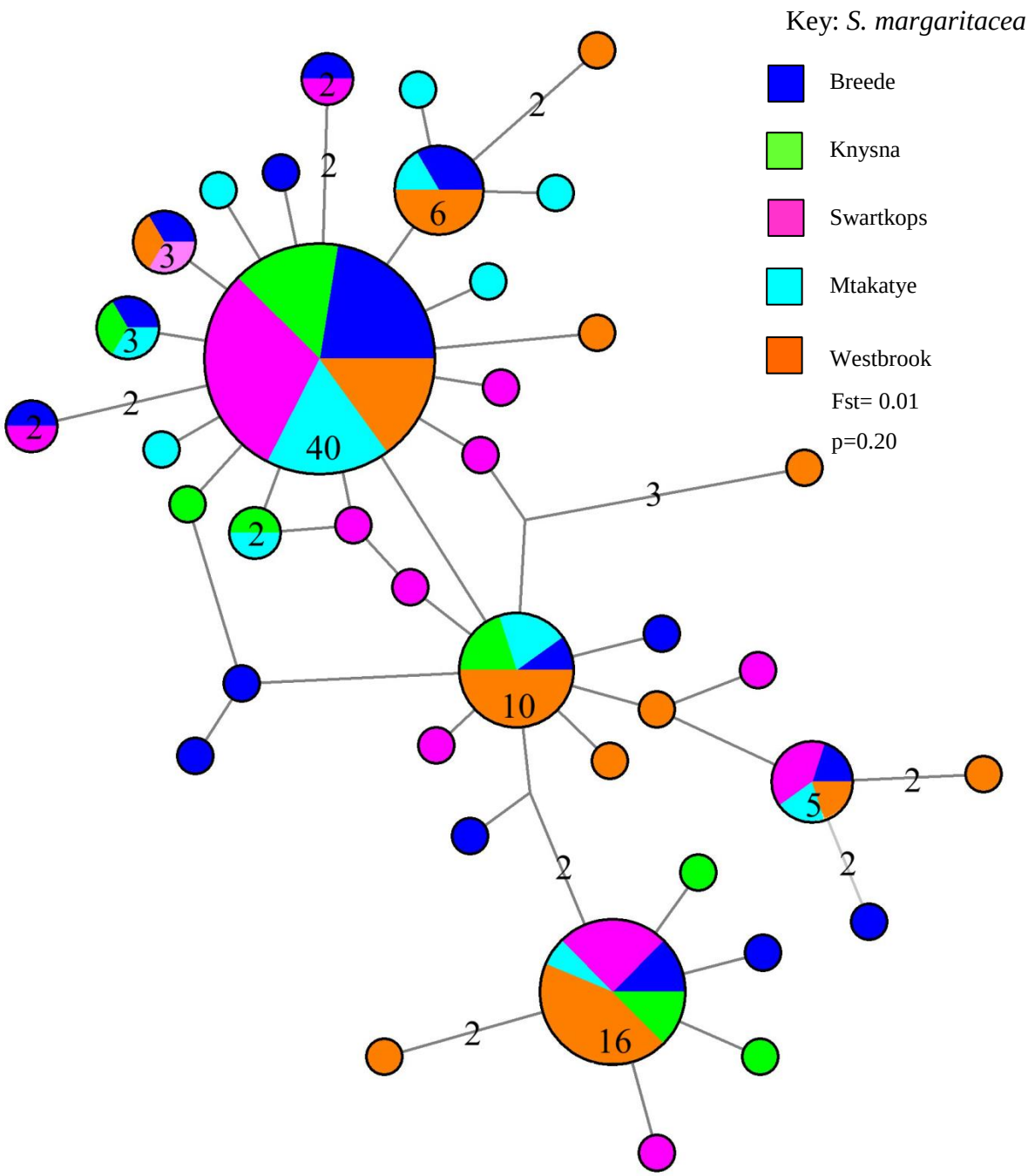

Figure 2.7 Haplotype network of *Striostrea margaritacea* CO1 gene region. Median Joining haplotype networks; Haplotype frequency and branch lengths are shown on nodes and branches respectively; any node of branch with no given value has a value of 1. The AMOVA Fst value is shown above.

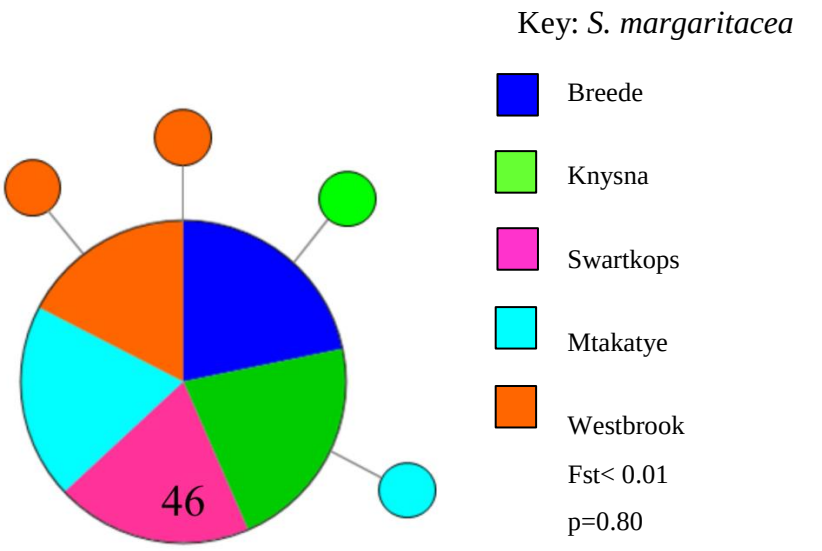

Figure 2.8 Haplotype network for *Striostrea margaritacea* 16S: Median joining haplotype networks; haplotype frequency and branch lengths are shown on nodes and branches respectively; any node of branch with no given value has a value of 1. The AMOVA Fst value is shown above.

2.3.5 Phylogenetic trees

The best model for CO1 was Hasegawa-Kishino-Yano (Hasegawa, et al., 1985) plus gamma of 0.74 (many sites evolve slowly but some evolve rapidly) and invariant sites (sites that have no mutations or do not vary); the best model for 16S was Tamura 3-parameter model (Tamura, 1992) plus gamma of 0.1 (many sites evolve slowly but some evolve rapidly). The maximum likelihood phylogenetic tree for *Saccostrea* and *S. margaritacea* CO1 gene region shows that the Mtakatye samples are more closely related to *S. cucullata* from other countries, such as Japan and China, than to the Northern population in South Africa (Figure 2.9). *Saccostrea mordax* shows to be paraphyletic as a recognised species (Salvi, et al., 2014), and the northern population may be more closely related to *S. mordax* (Figure 2.9). An individual Mtakatye sample (sample number 13) groups with the Northern populations and *S. mordax* for both the CO1 and 16S gene regions (Figure 2.7 and Figure 2.8, respectively).The phylogenetic tree for CO1 (Figure 2.9) shows the same grouping as that shown in Salvi et al. (2014) where the clades of *Striostrea* and *Saccostrea* are paraphyletic.

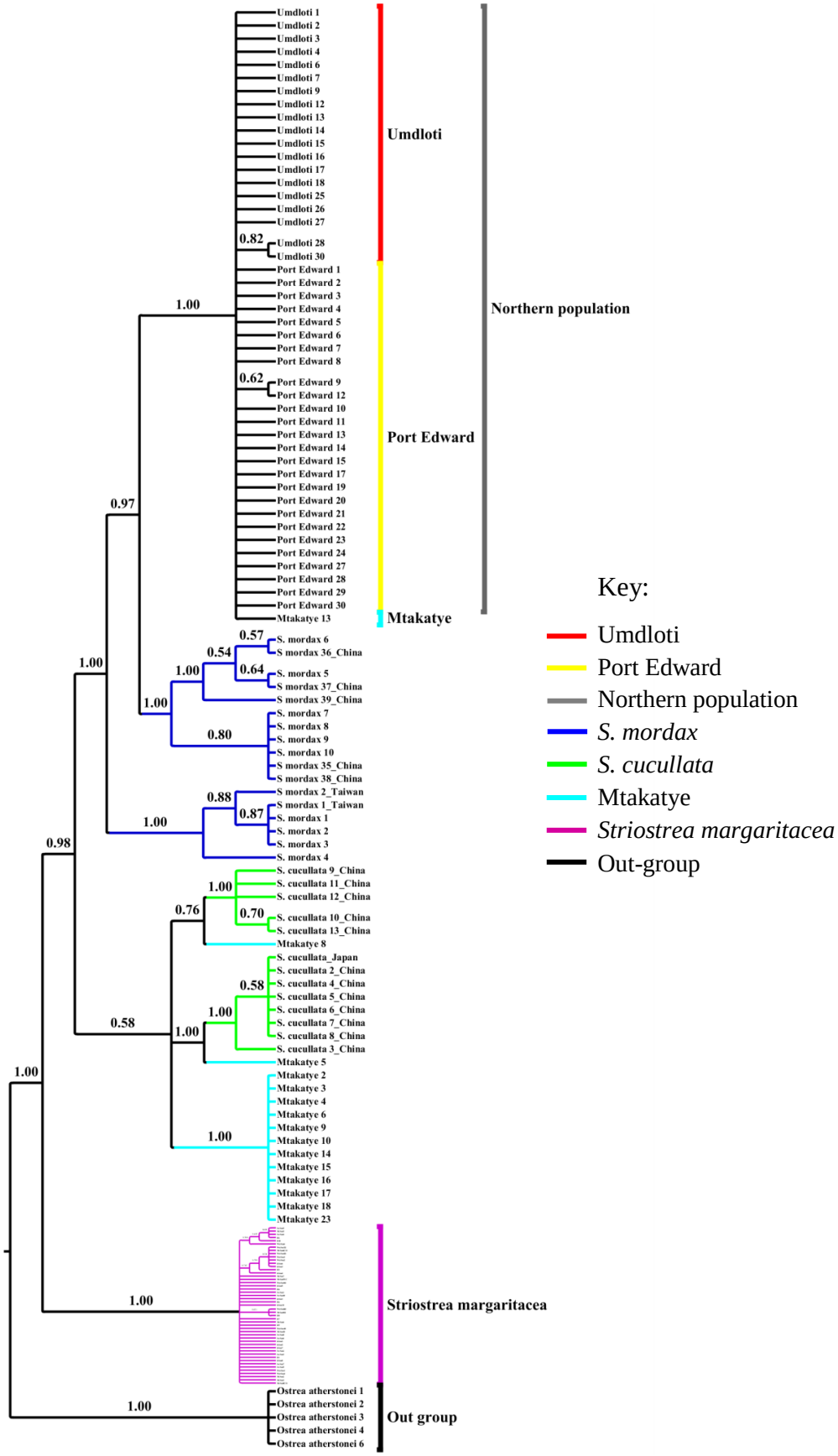

Figure 2.9 Maximum Likelihood Phylogenetic tree of *Saccostrea* and *S. margaritacea* for the CO1 gene region Nodes show bootstrap probabilities and 1 000 bootstrap replications were used for this tree. All sequences had a length of 491 base pairs.

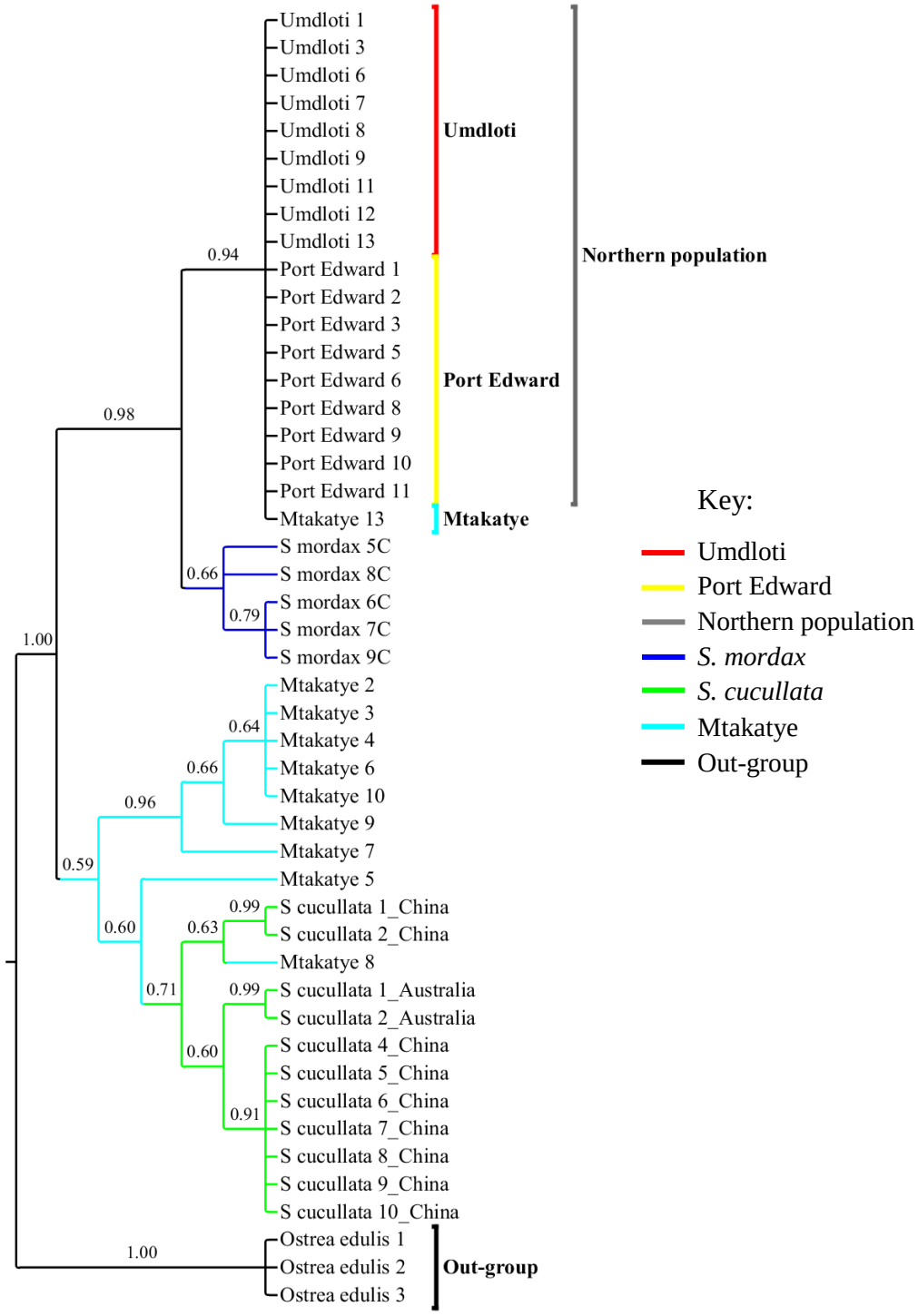

Figure 2.10 Phylogenetic tree of *S. cucullata* for the 16S gene region. Bootstrap probabilities are shown on nodes and 1 000 bootstrap replicates were used; all sequences had a length of 402 base pairs.

2.3.6 Inter-population and within site distances

Inter- and within site distances should be analysed in conjunction with the corresponding phylogenetic tree. Mtakatye *S. cucullata* sample number 13 was removed from the distance analyses as it grouped with the northern population (Figure 2.9). Due to the grouping of the Mtakatye sample it would either have to be considered a group on its own and, with a sample size of 1, this would give no informative results; alternatively the Mtakatye sample could be grouped with the other Mtakatye samples but this would blur the distinction between the Mtakatye and northern population samples by mixing the groupings. Cytochrome Oxidase 1 within site distances for Umdlolti and Port Edward are 0.003 and 0.001, respectively (Table 2.8); the inter-population distance between Umdolti and Port Edward is lower than the within site distance of Umdloti (0.002), indicating that these two populations are the same species. There is very high inter-population distance between Mtakatye samples and the Northern population, which is much higher than the within site distance for either indicating that these are two distinct species (Table 2.8). The 16S inter-population and within site distances mirror trends shown in the CO1 inter-population and within site distances where Umdloti and Port Edward are the same species, or population, which is distinct from Mtakatye (Table 2.9). *Striostrea margaritacea* shows low inter- and intra-population distances for both CO1 and 16S, suggesting that these are the same species, or population (Table 2.10 and Table 2.11, respectively).

Table 2.8 *Saccostrea* CO1 intra- and inter-population distances: Umdloti, Port Edward, Mtakatye, *S. cucullata*, and *S. mordax*. Numbers, 1 to 5, running horizontally correspond with the locations next to the same number running vertically in the first column.

***Saccostrea* CO1**

		Intra-population distance	Inter-population distances				
			1	2	3	4	5
1	Umdloti	0.003					
2	Port Edward	0.001	0.002				
3	Mtakatye	0.053	0.204	0.203			
4	*S. cucullata*	0.095	0.209	0.208	0.168		
5	*S. mordax*	0.058	0.086	0.086	0.186	0.197	

Table 2.9 *Saccostrea* 16S intra- and inter-population distances: Umdloti, Port Edward, Mtakatye, *S. cucullata*, and *S. mordax*.

***Saccostrea* 16S**

		Intra-population distance	Inter-population distances				
			1	2	3	4	5
1	Umdloti	0.000					
2	Port Edward	0.001	0.000				
3	Mtakatye	0.024	0.097	0.098			
4	*S. cucullata*	0.024	0.104	0.105	0.056		
5	*S. mordax*	0.007	0.017	0.017	0.094	0.105	

Table 2.10 *Striostrea margaritacea* CO1 inter- and intra-population distances

Striostrea margaritacea		Average between group distance= 0.005				
Intra-population distance		**Inter-population distance**				
		1	2	3	4	5
1	Breede	0.006				
2	Knysna	0.004	0.005			
3	Swartkops	0.006	0.006	0.005		
4	Mtakatye	0.004	0.005	0.004	0.005	
5	Westbrook	0.007	0.006	0.005	0.006	0.006

Table 2.11 *Striostrea margaritacea* 16S inter- and intra-population distances

***Striostrea margaritacea* 16S**		Average between group distance= 0.005				
Within group distance		**Inter-population distance**				
		1	2	3	4	5
1	Breede	0.000				
2	Knysna	0.001	0.000			
3	Swartkops	0.000	0.000	0.000		
4	Mtakatye	0.001	0.000	0.001	0.000	
5	Westbrook	0.001	0.001	0.001	0.001	0.001

Table 2.12 Crassostrea CO1 intra- and inter-population distance (data sourced from GenBank).

Crassostrea		Average interspecific distance= 0.197										
Intraspecific distance		**Interspecific divergence**										
		1	2	3	4	5	6	7	8	9	10	11
1	*C. angulata* 0.004											
2	*C. ariakensis* 0.001	0.164										
3	*C. belcheri* 0.004	0.173	0.182									
4	*C. gigas* 0.003	0.028	0.162	0.182								
5	*C. gryphoides* 0.002	0.167	0.201	0.176	0.168							
6	*C. hongkongensis* 0.004	0.140	0.146	0.191	0.138	0.183						
7	*C. iredalei* 0.007	0.180	0.182	0.178	0.181	0.142	0.175					
8	*C. madrasensis* 0.013	0.188	0.204	0.180	0.188	0.154	0.192	0.037				
9	*C. rhizophorae* 0.002	0.256	0.276	0.257	0.267	0.283	0.260	0.291	0.303			
10	*C. sikamea* 0.011	0.101	0.174	0.168	0.117	0.171	0.144	0.192	0.207	0.255		
11	*C. virginica* 0.015	0.251	0.283	0.262	0.259	0.283	0.262	0.262	0.256	0.178	0.248	

Table 2.13 Ostrea CO1 intra- and interspecific distance (data sourced from GenBank).

Ostrea		Average distance= 0.166					
Intraspecific distance		**Interspecific Divergence**					
		1	2	3	4	5	6
1	*O. angasi* 0.001						
2	*O. chilensis* 0.027	0.155					
3	*O. edulis* 0.009	0.019	0.147				
4	*O. permollis* 0.011	0.208	0.204	0.218			
5	*O. puelchana* 0.017	0.195	0.209	0.203	0.033		
6	*O. stentina* 0.003	0.222	0.225	0.214	0.117	0.117	

2.4 Discussion

Genetic analyses were performed on the CO1 and 16S gene regions of indigenous oyster samples of *S. margaritacea* and *S. cucullata* collected from several locations around South Africa's coastline. The aim of this study was to determine genetic diversity and structure to inform the possibility of aquaculture of indigenous oysters. *Striostrea margaritacea* shows high levels of diversity within the species with panmictic populations along the coastline and no fixation or structure across biogeographic regions. The CO1 phylogenetic tree shows better resolution than 16S, as was also shown by Liu et al. (2011). The Natal rock oyster was previously recognised as a single species, *S. cucullata*, along South Africa's coastline but is now shown to be two genetically distinct *Saccostrea* species, one of which is *S. cucullata* and the other may be *S. mordax*. The northern population, consisting of samples from Umdloti and Port Edward, is more closely related to *S. mordax* than *S. cucullata* while the samples from Mtakatye on the Wild Coast group more closely with *S. cucullata*. The northern population samples of *Saccostrea* have much lower levels of diversity than the Mtakatye samples and there is significant fixation between the northern population and Mtakatye; this is due to the fact that these group as two separate species. More investigation is required to determine the reason for the low diversity but it may be due to high levels of gene flow among the northern populations due to these samples being in more exposed sites allowing for better propagation of larvae.

2.4.1 *Saccostrea*

Oysters are frequently misidentified and are known to have nomenclature confusion due to the plasticity of shell morphology (Lam & Morton, 2004). For example *S. cucullata* and *S. echinata* have been genetically identified as a single species (Lam & Morton, 2006). Due to the similarities in shell morphology, *S. cucullata* and *S. mordax* are frequently misidentified as the other (Lam & Morton, 2009). What was previously accepted as a single species of *S. cucullata* along South Africa's coastline is now shown to be two distinct *Saccostrea* species, *S. cucullata* and, perhaps, *S. mordax*. The two species appear to be separated at some geographic point between Mtakatye and Port Edward but there may be a region of overlap as one sample from Mtakatye grouped with the northern population for both CO1 and 16S gene regions. Fixation values, Maximum likelihood trees, haplotype networks, and inter- and intra-population distances all support the separation, potentially, at the species level between Mtakatye and the northern population of *Saccostrea* on South Africa's coastline (Figure 2.5, Figure 2.6, Figure 2.9, and Figure 2.10). The CO1 inter-population distance, between

Mtakatye and Northern population samples are similar to those of recognised species of *Saccostrea* (*S. cucullata* and *S. mordax*, Table 2.8), Crassostrea (Table 2.12) and Ostrea (Table 2.13); *Crassostrea* species have an average inter-population distance of 0.197 and *Ostrea* species of 0.166. The inter-population distance between *S. mordax* and the northern populations are close to the within site distance of *S. mordax*, and the inter-population distances between them are also lower than the average distance between established species within *Crassostrea* and *Ostrea*, indicating that these two groups could be the same species. The phylogenetic trees of CO1 and 16S, however, indicate that the northern population is distinct from *S. mordax* and may be another *Saccostrea* species even though the inter-population distance is comparatively low (0.086). Low interspecific distance in the CO1 gene region is shown between recognised species: *Crassostrea madrasensis* and *Crassostrea iredalei* (0.037), *Crassostrea gigas* and *Crassostrea angulate* (0.028), *Ostrea edulis* and *Ostrea angasi* (0.019), and *Ostrea puelchana* and *Ostera permollis* (0.033). The inter-population distance between Mtakatye and *S. cucullata* is very high and may indicate that the Mtakatye samples are not the same species as *S. cucullata*. There is very high within site distance for Mtakatye, *S. mordax*, and (especially) *S. cucullata*; this could be attributed to a species complex. This is the first record of more than a single *Saccostrea* species on this coastline. The Haplotype networks show 102 and 36 mutational steps between the northern population and Mtakatye for CO1 and 16S, respectively. The Maximum likelihood phylogenetic trees show *Saccostrea* from Mtakatye groups more closely with *S. cucullata* from China and Taiwan (CO1), and China and Australia (16S), whereas the northern population groups with *S. mordax* from China. These South African samples group more closely with *Saccostrea* species from other countries than they do with samples found within 130 km on the same coastline.

Fst value between the northern population and Mtakatye sample 13 could not be calculated as there is only a single Mtakatye sample. There are two possibilities for the occurrence of sample 13 from Mtakatye grouping with the northern population: the first being human error where samples were incorrectly labelled; this is, however, unlikely as the Mtakatye samples were not handled in the laboratory at the same time as the northern samples. The second possibility is that the northern population and sample 13 of Mtakatye create a sympatric lineage and there is range overlap and/or habitat preference. Further study would be needed to determine the true cause of the occurrence of this individual. The possibilities of habitat preference, and range overlap are explored below.

2.4.1.1 Distinguishing between *Saccostrea* species

Saccostrea cucullata and *S. mordax* can be found throughout the Indo-West Pacific (Lam & Morton, 2006). *Saccostrea cucullata* is found on sheltered rocky shores or in estuaries attached to mangrove roots or rocky substrate; *S. mordax* is found on exposed rocky shores that are exclusively marine (Lam & Morton, 2004). As mentioned previously, it is difficult to differentiate between *S. cucullata* and *S. mordax*. Size and shape of the shell are plastic characteristics that are dependent on the substrate to which the oysters attach (Lam & Morton, 2004); colour is also a plastic feature and external features are often obscured by weathering resulting in difficulty deciphering morphological distinctions (Lam & Morton, 2004). All *Saccostrea* species are deeply-cupped, oval and the right valve is smaller than the left and usually flat (Lam & Morton, 2006). There are, however, some morphological features that can be used to distinguish *S. cucullata* from *S. mordax*. Lam and Morton (2009) examined *S. cucullata* and *S. mordax* from Malaysia and Singapore, and found many similarities in the shell morphology, but there are approximately five characteristics that separate *S. cucullata* from *S. mordax* which are summarised in Table 2.14 (Figure 2.11 below defines the morphological features mentioned in Table 2.14). There is a single feature shared only by *S. mordax* and is not found in *S. cucullata*, *S. glomerata* or *S. kegaki*; this feature is radial grooves on the exterior of the right valve (Lam & Morton, 2006). Samples of *Saccostrea* species were collected from Ballito, north of Umdloti, and the morphology examined; all ten samples displayed radial groves and, although this is a small sample size, personal observation suggests that many of the *Saccostrea* oysters in Ballito and Umdloti share this feature. As shell morphology is not a definitive identification method it cannot be stated outright that these oysters are *S. mordax* but they do share many physical characteristics as *S. mordax*.

Table 2.14 Morphological characteristics differentiating *S. cucullata* from *S. mordax*

Characteristic	*S. cucullata*	*S. mordax*
Shell outline / shape	Oval to subtriangular	Either triangular or D-shaped
Adductor muscle scar	D- shaped	Circular to elongated-oval
Chomata	May be present in juveniles	Present throughout life-time
Features only present in *S. mordax*		Radial grooves from half way along the right valve (dorso-ventral axis) to the ventral shell margin
		Dorsal margin thin and easily broken when collected

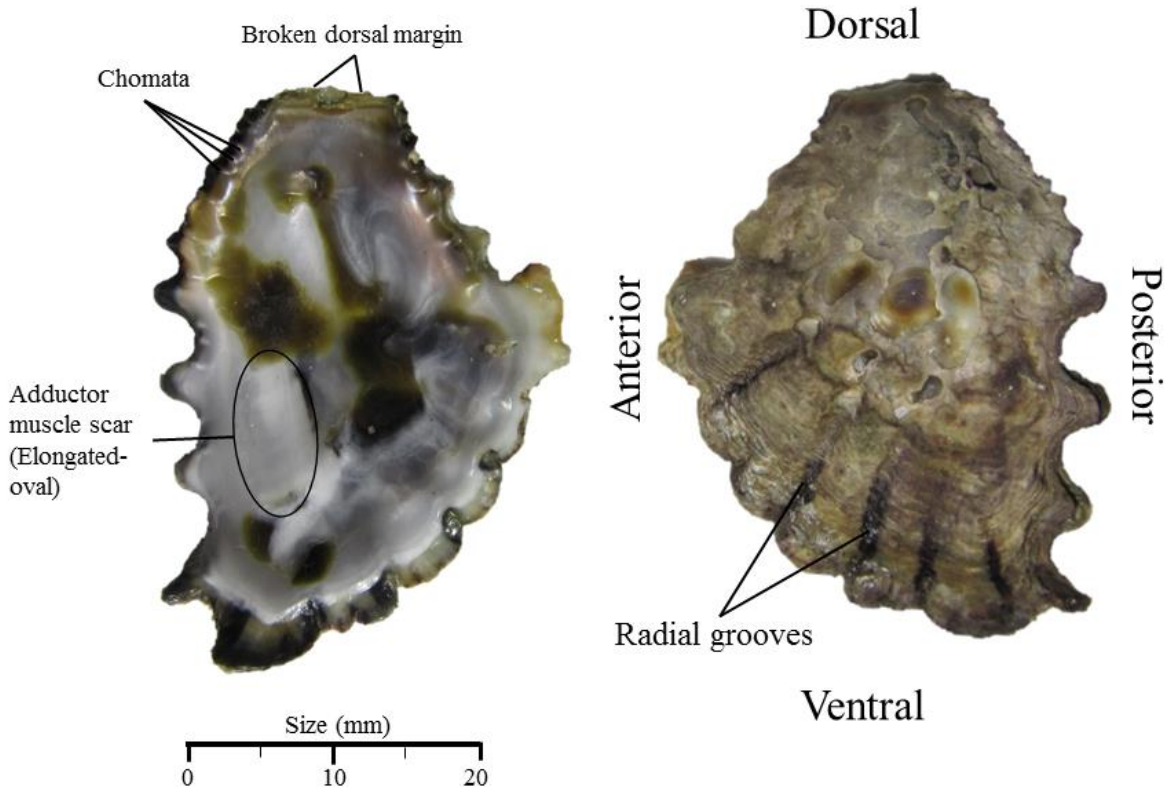

Figure 2.11 Oyster shell morphology on the Right Valve (*S. mordax* shell): The adductor muscle scar is cryptic in this image as the scar is the same colour as the shell, however there are faint vertical striations and when handling the shell the texture of the scar is apparent.

2.4.1.2 Population expansion or adaptive selection

The northern population has very low levels of diversity compared to *Saccostrea* from Mtakatye and *S. margaritacea* (Table 2.2). Low diversity may be due to the fact that samples at each northern population site were taken from a localized area and they may all be related. However, this is unlikely as the samples from the northern population come from two distinct locations, 150 kilometres apart, further analyses could determine relatedness. Another possibility is population expansion or adaptive selection. Tajima's D and Fu's Fs statistic are statistical tests used on sequence data to determine population expansion, contraction or stability by determining the difference between expected and observed sequence diversity in polymorphic sites (Ramos-Onsins & Rozas, 2002; Peck & Congdon, 2004); expansion would be represented by a negative value, contraction a positive value, and stability by zero (Ramos-Onsins & Rozas, 2002; Peck & Congdon, 2004). Fu's Fs is also used to detect background selection, and Fu and Li's D* and F* statistics are used to distinguish if Fs is indicative of adaptive selection or population changes (Fu, 1997). Tijima's D and Fu's Fs for the northern population are both significantly negative indicating either that the gene region

is under selection or the population has undergone an expansion (Table 2.7); the fact that Fu and Li's F* and D* statistic are both significant suggests that CO1 is under selection (Fu, 1997). Fu's Fs is more accurate when used with larger sample sizes and as this data set is relatively small it is likely that the significant values are misleading (Ramos-Onsins & Rozas, 2002).

2.4.2 *Striostrea margaritacea* population genetics

Striostrea margaritacea have pelagic larvae which are in the water column for up to 50 days before settlement occurs (Sukumar & Mohan Joseph, 1988). However, several studies show that larval duration is not always correlated with distribution distance (Kelly & Palumbi, 2010; Palumbi, 1994; Todd, 1998). Shanks et al. (2003) noted that 6 species in their multispecies study showed lower than expected dispersal for their given pelagic larval duration; the larvae of these species all displayed similar behaviours such as swimming against the current, sinking and remaining near the bottom substrate, and grouping together in sheltered places near the bottom (such as beneath boulders). It has been shown that the larvae of the oyster, *Crassostrea virginica*, shows similar behaviour of remaining near the bottom (within 1 centimetre) and of "dive-bombing", accelerating towards the bottom substrate, and remaining at that depth (Finelli & Wethey, 2003). The behaviour results in limited dispersal and higher genetic diversity which is shown in *S. margaritacea*. However this species does not show distinct genetic populations. Populations of *S. margaritacea* show higher diversity than the *Saccostrea* samples for the CO1 and 16S gene regions across all populations and show low levels of fixation; indicating gene flow between the populations. The 16S gene region of *S. margaritacea* shows lower levels of diversity than CO1, but the populations are still shown to be panmictic with a low level of fixation. Both *S. cucullata* and *S. margaritacea* show lower levels of diversity for the 16S than the CO1 gene region. It has been shown in a previous study by Nicolas et al. (2012) that 16S is less variable than the CO1 gene region as it evolves more slowly. However, in this study, 16S was used as an additional gene region to be compared with the phylogeny of CO1 to corroborate the findings as using nuclear markers was beyond the scope.

Saccostrea and *S. margaritacea* have very similar life histories (Kilburn & Rippey, 1982). However they can be found in different tidal zones. *Saccostrea* can be found in the mid-intertidal zone, whereas *S. margaritacea* is only found in the very low-intertidal to subtidal region (Kilburn & Rippey, 1982). Species in the mid- to upper-intertidal zone usually show more genetic structure than do those in the lower-intertidal (Kelly & Palumbi, 2010; von der

Heyden, et al., 2013). Other than life history traits, human mediated movement can play a role in altering genetic diversity and structure. *Striostrea margaritacea*, as a historically economically important species, has been moved around the country on multiple occasions and at times in large numbers (Korringa, 1956). Movements of *S. margaritacea* have been documented from as early as the 1670s by European settlers and, more recently, in the early to mid-1900s, where large numbers of the species were moved around by the Knysna Oyster Company as they experimented with cultivating indigenous oysters (Korringa, 1956; DAFF, 1970). This artificial movement likely resulted in inter-population crossbreeding which would blur or erase any pre-existing genetic structuring of the population. Hybridised populations can be susceptible to reduced fitness or persistence when exposed to changing environmental conditions (Rhymer, 2006). However, *S. margaritacea* shows no fixation between populations and therefore moving samples from one location to another would not result in hybridization depression.

2.5 Conclusion

For all species, the 16S gene region shows much lower levels of nucleotide and haplotype diversity than CO1. However this is to be expected as it is a less variable and more slowly evolving gene (Nicolas, et al., 2012). What was recognised as *S. cucullata* is now shown to be two distinct *Saccostrea* species: *S. cucullata* in Mtakatye and the northern, Umdloti and Port Edward, populations are *S. mordax*. The northern population samples show very low genetic diversity compared to Mtakatye *Saccostrea* and to *S. margaritacea*; this may be due to localized sampling, a population expansion or adaptive selection. All three of these possibilities require further study to determine which one is the cause. The reason that the two *Saccostrea* species were previously thought to be one is due to the many similarities between *S. cucullata* and *S. mordax*, which have led to frequent nomenclature confusion (Lam & Morton, 2006; Lam & Morton, 2009). *Striostrea margaritacea* does not show any barrier to gene flow and this may be natural or due to artificial movement of *S. margaritacea*. *Striostrea margaritacea* shows high levels of diversity and no distinct genetic populations along South Africa's coastline. It is unexpected to find that the northern population of *Saccostrea* has similarly low Fst values to *S. margaritacea* as they are in the mid- to upper-intertidal region where as *S. margaritacea* is in the lower intertidal; usually species in the higher inter- to sub-tidal zones show greater genetic structure than those in the lower zones (Kelly & Palumbi, 2010; von der Heyden, et al., 2013).

For *S. margaritacea*, there are no distinct genetic lineages along the coastline and populations may be inter-breeding; however, these are only the results from mitochondrial DNA. Further study would be necessary to determine whether free movement of *S. margaritacea* would be possible. *Saccostrea*, being two species, could be used for culture. However, the species identity would need to be confirmed for culture specimens. Since the morphological features mostly require being able to examine the internal surface of the right valve, which would be lethal to oysters, DNA barcoding should be used. This can be done by anesthetising the oysters, using a 50 $g.L^{-1}$ magnesium chloride ($MgCl_2$) - salt water mixture, and taking a small gill or mantle tissue sample; this concentration of $MgCl_2$ results in oysters being anesthetized in between 360 min and 720 min with a 100% recovery rate (Puchnick-Legat, et al., 2015)[3].

2.6 Future studies

Since two species of *Saccostrea* have been discovered, a taxonomic study should be undertaken to determine if there are phenotypic differences between the two *Saccostrea* species along-side a more extensive population genetics analysis which uses both mitochondrial and nuclear markers, AFLP genome-wide scans, and/or transcriptome sequence analysis; these analyses would create a robust consensus phylogenetic tree, determine whether or not there are hybrids or any form of hybridisation, and enable the examination of adaptation. The genetic study would also be able to answer the question of whether the single sample of *S. mordax* in Mtakatye indicates that there are in fact two different *Saccostrea* species with an overlapping range.

For the taxonomic study, samples should be taken from both sheltered and exposed rocky shore sites as these are the habitat distinctions for *S. cucullata* and *S. mordax* (Lam & Morton, 2009). The sheltered sites should include locations within estuaries on mangrove roots and rocky substrates in the upper and lower estuarine regions, and the exposed sites should either be completely exposed to wave action or in a tidal pool that is frequently refreshed with fresh salt water. Morphometric data and morphological features should be recorded and compared with those found in the literature. Comparative shell morphometry may not be able to distinguish *S. mordax* from *S. cucullata* (Lam & Morton, 2006). The samples used for the taxonomic study should also be used in the genetic analyses and the CO1 gene region should be used to confirm species identity. Sampling should occur in all three locations recorded in this study, between the three locations, and sites south-west of

[3] This study performed gonad sampling, not tissue extraction which may result in different recovery success.

Mtakatye; additional sites within South Africa and along the east African coast should also be included. Transplant experiments could also be performed to determine whether the habitat preference recorded by Lam and Morton (2006) holds true for South African *Saccostrea* samples as well as to determine growth performance and fitness of different broodstocks.

Chapter 3: Spat settlement trials, and the impact of a Harmful Algal Bloom (HAB) from December 2013 to February 2014

3.1 Introduction

The culture of *Striostrea margaritacea* was explored in the 1950's in Knysna estuary; but these attempts failed due to the fact that its natural habitat is rocky sub-tidal coastal reefs to depths of approximately 5m, rather than estuaries (Korringa, 1956; Kilburn & Rippey, 1982). A recent survey of three Southern Cape estuaries (Keightley, et al., 2015) indicates that *S. margaritacea* does not fare well in high sediment, brackish conditions of the estuary in which the historic growth trials were conducted. Since Korringa's growth trials in 1956, the method of oyster culture has been revised and has moved towards sub-tidal long-line culture. There is a well-established market for wild-harvested *S. margaritacea* from the southern Cape east- and north-wards into KwaZulu-Natal (Kyle, et al., 1997; Clarke, et al., 2002; de Bruyn, et al., 2009), but the current stock status is unknown. The augmentation of wild harvests with cultured local animals will relieve harvesting pressures.

3.1.1 Indigenous oyster species with culture potential

An oyster species with good culture potential would be one that is fecund, with a high growth rate, tolerant of a wide range of environmental temperatures and conditions, has spat that are easily collected, and is palatable. *Striostrea margaritacea* is endemic to South Africa and Mozambique, ranging from False Bay to Mozambique (Figure 3.1), and reaches a maximum size of 180 mm; it is a hardy oyster which occurs sub-tidally which makes this species well suited to long-line suspended culture which is the predominant culture method in South Africa (Robinson, et al., 2005; Kilburn & Rippey, 1982; Haupt, et al., 2010; Branch, et al., 2007; Watling, 1983). *Striostrea margaritacea* is a cupped oyster with a large and heavy shell, and is capable of changing sex from male to female as it ages but can change sex throughout its life time (Kilburn & Rippey, 1982). Reproduction occurs via broadcast spawning and the release of eggs and sperm into the water column can be triggered by the presence of sperm or eggs; this presence can induce an entire colony to spawn simultaneously (Branch, et al., 2007; Kilburn & Rippey, 1982). *Striostrea margaritacea* is the dominant species south of the Transkei and forms beds at and below the extreme low tide mark and is

described as the most economically important species in South Africa (Kilburn & Rippey, 1982).

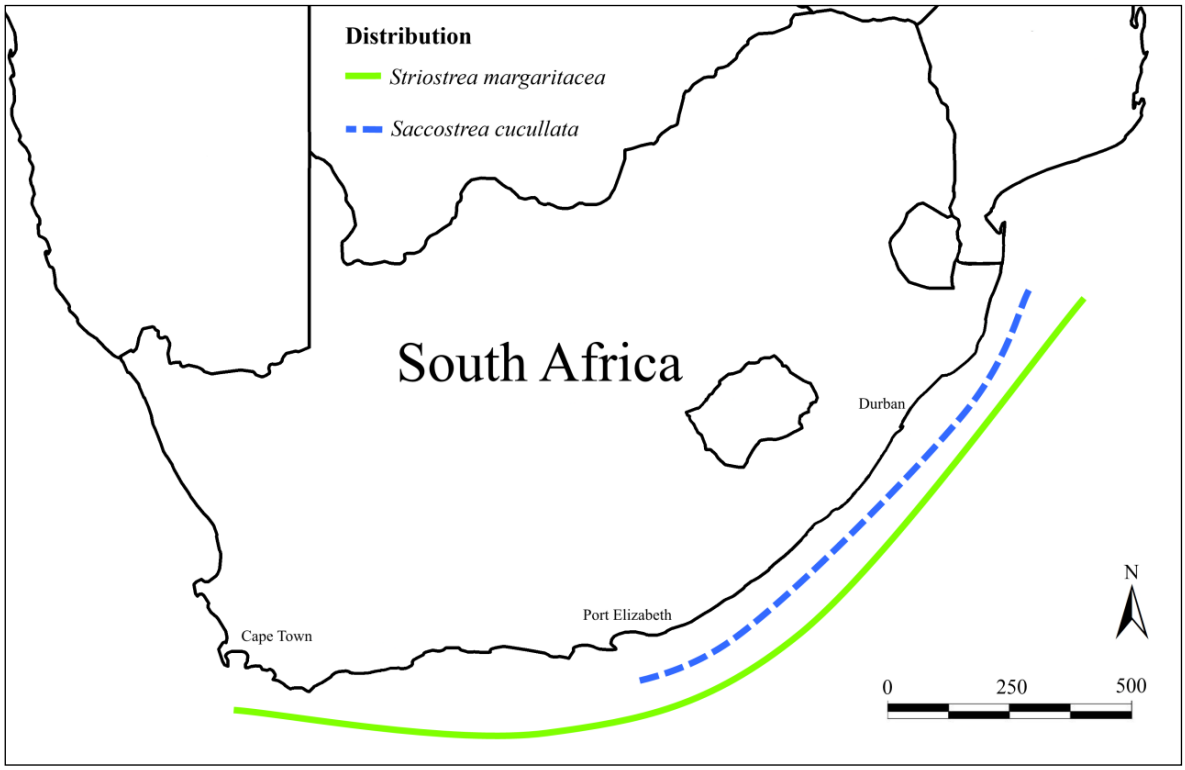

Figure 3.1 Distribution of *S. margaritacea* and *S. cucullata* along South Africa's coastline

Saccostrea cucullata ranges from Algoa Bay through the Indo-Pacific, and can be found in China, India, Thailand, and Madagascar (Kilburn & Rippey, 1982; Branch, et al., 2007; Klinbunga, et al., 2003; Jarayabhand & Thavomyutikam, 1995). This species has good potential for cultivation and has been developed as such in several tropical countries including Thailand, Guam, the Philippines, India, and Mauritius (Jarayabhand & Thavomyutikam, 1995; Braley, 1984; Angell, 1986; Joseph, 1998). *Crassostrea gigas*, which is the dominant oyster culture species globally, does not perform well in exposed coastal conditions, such as those found on South Africa's east and south coasts (Pieterse, et al., 2012); this could be a niche to be filled by *S. cucullata*. *Saccostrea cucullata* is harvested on a small scale in New South Wales, Australia (Nell, 2001) and is a very adaptable species. However, its potential highly outweighs the current utilisation (Mgaya, 2001). *Saccostrea cucullata* is harvested in KwaZulu-Natal and the Eastern Cape, mostly for subsistence purposes and a small portion for sale (Kyle, et al., 1997). Several communities in KwaZulu-Natal harvest *S. cucullata* for subsistence purposes as a food source; however it is not as highly targeted as red bait or mussels (Kyle, et al., 1997). Being a smaller species than *S. margaritacea*, *S. cucullata* only attains a maximum size of 80mm (Kilburn & Rippey, 1982).

Commercially oysters are sold when they attain a minimum size of 60mm, which may indicate that *S. cucullata* is not as good a candidate for cultivation purposes; however its culture in other countries improves its standing as there is more of a basis to build from. In South Africa, *S. cucullata* is found in the upper intertidal region which limits growth rates due to the fact that the oysters can only feed (and therefore grow) periodically which can only occur when submerged, at high tide (Kilburn & Rippey, 1982); *S. margaritacea* on the other hand can feed continually which results in this species having a higher growth rate and it attaining "market size" in a shorter time frame. *Saccostrea cucullata* is capable of surviving (and thriving) sub-tidally but there may be habitat competition that results in its remaining in the intertidal zone. Although *S. cucullata* naturally occurs in the intertidal region it is possible that cultivating it sub-tidally would result in a faster growth rate as is seen in *Mytilus* mussels which are sometimes moved from intertidal to sub-tidal areas to improve growth rates (Beaumont, et al., 2007).

The successful culture of either of these two species could relieve the pressures on harvested wild oysters by supplementing existing sales to well-established markets and contribute to the sustainable utilization of coastal resources by impoverished communities in the Eastern Cape and KwaZulu-Natal. Cultivation of indigenous species could also prevent *C. gigas*, and other associated epifaunal organisms, from becoming invasive. South Africa does not have functioning oyster hatcheries and therefor mariculture of indigenous oysters would have to rely on wild-spat settlement. Another reason to utilise wild-spat collection is that to set up and run hatcheries would also cost much more than would a set up for wild-spat collection (Nell, 2001). Oysters are considered "spat" once they have settled, during which time they transition from pediveliger larvae (Parker, et al., 2010). Environmental conditions play an important role in oysters surviving from eggs, to larvae, to settled spat.

3.1.2 Commercial spat collection

There are several countries around the world which utilise wild-spat for commercial culturing of indigenous oysters (Mgaya, 2001); some of which are India and the Philippines, where *S. cucullata* is cultured, and the USA where *C. virginica* is cultured (Joseph, 1998). Oyster culture begins with the acquisition of spat which are grown out to market size oysters (Joseph, 1998; Mgaya, 2001). Oyster hatcheries are used for mass production of oyster spat for mariculture and such facilities are heavily utilised in temperate countries (Mgaya, 2001). In the tropics, an alternative to hatcheries is the collection of wild-spat. To successfully harvest wild-spat the presence of the spat must be determined as well as having a suitable

substrate on which the spat may settle. To predict spatfall, spawning of the oyster species in question must be understood.

Saccostrea cucullata and *Striostrea margaritacea* reproduce by releasing eggs and sperm into the water column (Kilburn & Rippey, 1982). Once oysters have spawned, the eggs are fertilised and hatched into pediveliger larvae. The swimming larvae spend several weeks in the water column before settling on a suitable substrate where they metamorphose into spat (Arakawa, 1990; Kilburn & Rippey, 1982). Oyster breeding season coincides with that of other fouling organisms, which results in them competing for available habitat for settlement and therefore determining which culch type to use for settlement is extremely important (Joseph, 1998). Spat are usually collected on various, common, hard materials (culch); some of these materials include broken tiles, pieces of cement or concrete, oyster shells (or other bivalve shells), branches of mangroves, coconut shells, or split bamboo just to name a few (Joseph, 1998; Mgaya, 2001). Different oyster culture methods use different settlement methods. There are four types of oyster culture methods: bottom culture, longline culture, stake-culture, and intertidal rack culture (Angell, 1986). Bottom culture is where oysters are grown on fixed apparatus which are slightly elevated from the substrate or seeding the ocean floor with oyster spat; long-line culture is where oysters are grown in mesh cages or "envelopes", suspended from long-lines and floated by buoys (usually used for *C. gigas*); stake-culture where poles (often bamboo) are driven into soft substrate and culch (for example of oyster shells strung on galvanised wires) are fixed to these poles; and intertidal rack culture where oysters are grown on racks which are fixed to the substrate within an estuary in the intertidal zone (Angell, 1986). When using bottom culture for *C. gigas* and *C. ariakensis* in Hong Kong, in the tropics, spat collectors are placed in the intertidal zone where, once sufficient spatfall has occurred, the culch plates are moved to deeper waters for grow-out (Joseph, 1998; Angell, 1986). In Mexico, spat collectors are suspended by being strung on ropes and placed in racks to collect *C. virginica* (Joseph, 1998). Longline culture usually uses hatchery-cultivated spat such as *C. gigas* and *S. cucullata* in several countries some of which include Thailand, South Africa, and Spain (Jarayabhand & Thavomyutikam, 1995; Pieterse, et al., 2012; Ruiz, et al., 1992). Stake-culture uses oyster shells strung on galvanised wires for spat settlement, the strung culch are then used for grow out of the oysters, and this method is used for *C. iredalei*, *C. melabonensis*, and *S. cucullata* in the Philippians (Joseph, 1998; Angell, 1986).

Regardless of the culch or settlement method utilised, the ultimate goal is to achieve economically feasible settlement of spat which can be grown out into adult oysters. However for the larvae to survive spawning and settlement, certain environmental conditions are required. Conditions such as temperature, pH, and salinity should remain within a certain range for ideal larval survival and settlement (Wilson, et al., 2005). An environmental occurrence which can affect many of these conditions affecting oyster larval survival and settlement would be Harmful Algal Blooms (HABs). There can be a negative or positive affect from an HAB depending on other environmental conditions. For example *Prorocentrum minimum* (bloom species) during day night cycles will produce extremely high oxygen levels at the end of the day cycle, while when in only dark cycles, hypoxic and anoxic levels will be reached within 4 days (Brownlee, et al., 2005). During the settlement trial of this study an extensive HAB, of predominantly *Lingulodinium polyedrum*, occurred around the study site. The settlement trial lasted from November 2013 to March 2014 and the HAB from December 2013 to February 2014.

3.1.3 Harmful Algal Blooms (HABs)

Algal blooms are not uncommon along South Africa's coast however the above mentioned bloom was unique as this was the first instance where *L. polyedrum* bloomed in South African waters (Bornman & Steyn, 2014). It was the largest bloom recorded in South African history in terms of both temporal and spatial extent; and this was the first instance where an HAB was recorded between Mossel Bay and East London (Bornman & Steyn, 2014). To visualise the extent of this HAB, Chlorophyll *a* concentrations (which are frequently monitored on a global scale) can be used to create a map; phytoplankton species utilise Chlorophyll *a* as a photosynthetic pigment which is why we can use this to track blooms. Figure 3.2 shows Chlorophyll *a* concentrations around South Africa over the period of 25 January to 1 February 2014; *L. polyedrum* concentrations peaked in late January through February 2014 and therefore this period can be considered to be the peak of the HAB on the southern coast between Mossel Bay and East London (Bornman, et al., 2014).

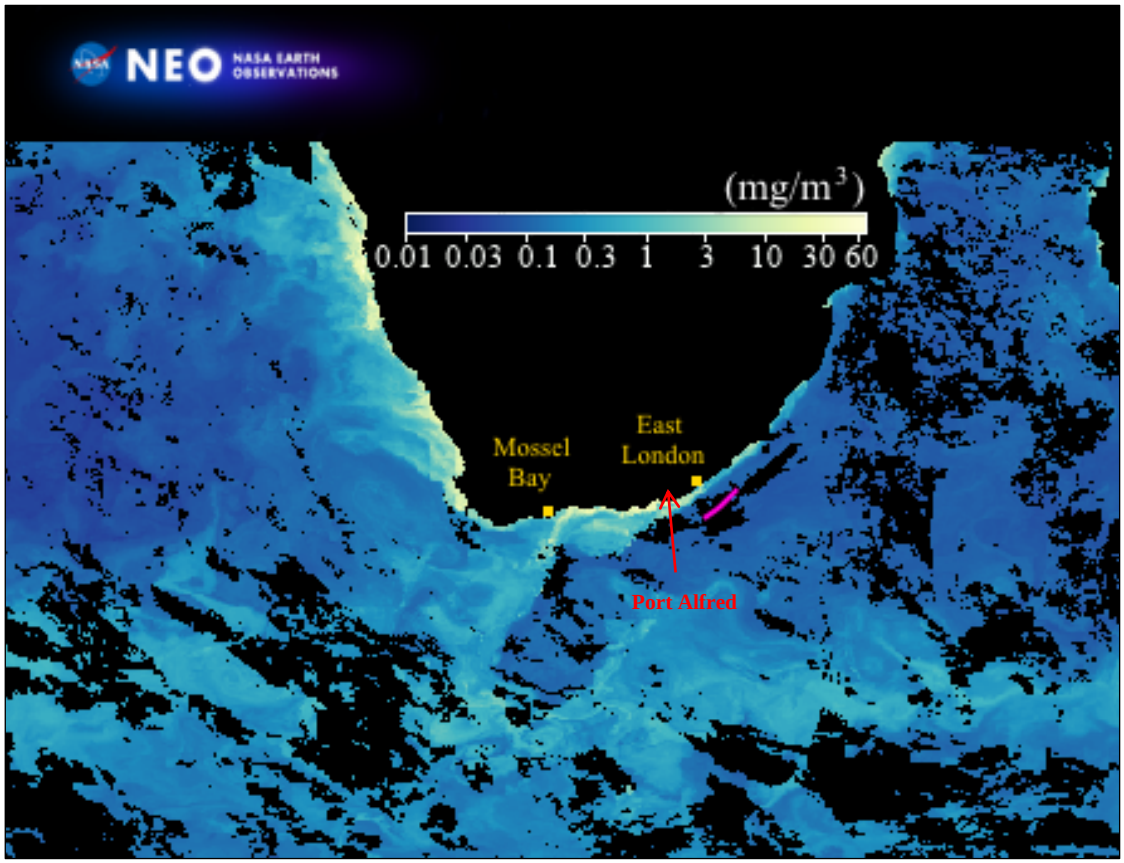

Figure 3.2 Satellite imagery of Chlorophyll *a* concentrations between 25 January and 1 February 2014. Port Alfred, spat settlement trial site, is shown by the red arrow and size distribution sample sites fall within the area spanned by the **pink** line (see Figure 3.12). Chlorophyll *a* concentrations can be analogous with algal blooms as phytoplankton species utilise this pigment for photosynthesis. Chlorophyll *a* concentrations are shown in milligrams per cubic meter where dark blue indicates a low concentration and white areas show a high concentration; black areas on the map show missing data (NASA Earth Observations, 2015).

An algal bloom is classified as a significant population increase of phytoplankton species (Smayda, 1997a). These blooms often contribute to nutrient availability which is beneficial to the upper trophic levels (Smayda, 1997a). Anthropogenic eutrophication has drawn much attention and is commonly assumed to be the leading cause of all algal blooms; but this is not always the case (Sneller, et al., 2003). Algal blooms can be caused by several processes such as circulation, upwelling, relaxation, river flow and eutrophication (due to both natural and/or anthropogenic loadings) (Sneller, et al., 2003). Development and persistence of HABs may be driven by degraded water quality from nutrient pollution (Heisler, et al., 2008). It is generally accepted that HABs are occurring more frequently globally, however the reason for this increase is debated; it should also be noted that there have been increased observation efforts (Sneller, et al., 2003). Algal blooms are common along the west coast of South Africa but fewer occur along the south coast, and blooms are rare on the east coast (Pitcher & Calder, 2000; Branch, et al., 2013). Phytoplankton blooms on the west coast, for the most

part, make a positive contribution to fisheries in this region (Pitcher & Calder, 2000). The development of blooms on the west coast has been strongly linked to wind-driven upwellings (Pitcher, et al., 1998).

Some algal blooms are, however, harmful or toxic (Pitcher & Calder, 2000). Harmful algal blooms can be characterised by the spread and sporadic dominance of a toxic or harmful species of alga and most blooms can be attributed to dinoflagellates (Pitcher & Calder, 2000). These HABs can have a considerable negative impact on the environment by altering many biotic and abiotic factors: reduced light penetration; decreased carbon dioxide (CO_2) and increased oxygen (O_2) levels during photosynthesis; and (vice versa) during the eventual decomposition of the bloom, leading to anoxic conditions (Gobler, et al., 2004; Spilmont, et al., 2009; Cloern, 1996; Landsberg, 2001; Bricelj & Lonsdale, 1997; Cockcroft, 2001). There are also environmental conditions that are not necessarily caused by HABs but are associated with them; these factors include alterations in the ammonium levels and increases and decreases in pH. The most notable impact of HABs are the mortalities caused by toxins from toxic bloom species; other mortalities are due to suffocation or starvation which can be caused either by anoxic conditions due to bloom decomposition or clogging or damage to the gills by dinoflagellate species (Branch, et al., 2013; Calbrese & Davis, 1966). Toxic dinoflagellates and their relationship with the shellfish industry have been widely studied and the main focus of said studies has been on toxin uptake, anatomic distribution and the depuration of bivalves (Brincelj & Shumway, 1998). However, there are 58 dinoflagellate species, which cause HABs, which are associated with bivalve mortality; one of these species is *Lingulodinium polyedrum* (Burkholder, 1998).

Before discussing *L. polyedrum* the life-cycle of dinoflagellates should be mentioned. The end and starting point for a dinoflagellate is the resting cyst which can be found in the substrate; cysts can lie dormant for several years and still be viable (Bravo & Figueroa, 2014). An environmental trigger, such as a change in temperature or increase in nutrients, would result in excystment; this is a vegetative stage where the dinoflagellate reproduces asexually at a rapid rate (Kaushal & Shukla, 1977). The exponential increase of dinoflagellates caused by vegetative reproduction will continue until environmental conditions deteriorate; thereafter sexual reproduction will occur which produces gametes that fuse to form a zygote which will in turn undergo encystment, returning to the sediment to await an improvement in conditions for the cycle to be repeated (Lewis & Hallett, 1997). *Lingulodinium polyedrum* is a planktonic dinoflagellate and one of few phytoplankton

species which has a wealth of laboratory studies dedicated to its behaviour and physiology (Lewis & Hallett, 1997). It reproduces using both forms of vegetative division, namely division either by sharing thecal plates or division after shedding said plates (Lewis & Hallett, 1997). *Lingulodinium polyedrum* is also capable of two different forms of sexual reproduction (encystment), an endysal sexual stage and a spiny resting cyst (Figueroa & Bravo, 2005). Cysts of *L. polyedrum* have an estimated half-life of between two and ten years under anoxic conditions with approximately 50% germination success (Keafer, et al., 1992; Lewis & Hallett, 1997). *Lingulodinium polyedrum* is an obligate phototroph and its nutrient uptake includes nitrogen metabolism, and it has a high half saturation constant for nitrate and ammonium (Thomas, 1955; Eppley, et al., 1969). This species blooms in nutrient–depleted surface waters but its ammonium and nitrate uptake capabilities allow it to outcompete other dinoflagellates and diatoms (Lewis & Hallett, 1997). Surviving in nutrient poor conditions make this species well adept to survive on South Africa's east coast, especially during the summer months. Ideal temperatures and salinity for *L. polyedrum* range between 20°-24° C and 30-35‰, respectively (Lewis & Hallett, 1997). These conditions are within the natural ranges in South Africa which make this coast appealing to *L. polyedrum*.

3.1.4 Aims

This study broadly aimed to determine whether any of the indigenous oyster species are viable candidates for commercial mariculture, using settlement trials. The first specific aim of this chapter is therefore to determine whether wild settlement of *S. margaritacea* or *S. cucullata* would occur in an estuary in Port Alfred (Kowie River) where *S. margaritacea* had settled the previous season. Due to the fact that an HAB occurred during the settlement trial, the second aim was to investigate the potential impact of this on settlement of oyster species. This was achieved by assessing size distribution and age structure of wild populations of *S. margaritacea* in three locations, in order from south to north: one within the bloom range (Gulu MPA), one on the border of said range (Gonubie MPA, East London), and one outside the bloom range (Kei Mouth MPA).

3.2 Methods

3.2.1 Spat collection

3.2.1.1 Study site

The Kowie River, Port Alfred, is an estuary located between East London and Port Elizabeth in the Eastern Cape, South Africa. While doing a preliminary survey and oyster sample collection in early August 2013 it was noted that there was settlement of juvenile oysters in Port Alfred's estuary. Given the size of the juveniles, a right valve length of approximately 20-30 millimetres, the oysters would have been approximately 7 months old (age determined using the modified von Bertalanffy function as calculated by Schleyer and Kruger (1991) for *S. margaritacea*, see Equation 1 below) which indicates that they had settled the previous season.

3.2.1.2 Spat collectors

Frames of 30 mm mild steel were constructed with the following dimensions: 2280 mm width by 875 mm height (internal measurements) and holes of 6mm were drilled every 190 mm along the top and bottom edge (Figure 3.3). The holes served as attachment points for the PVC coated, galvanised wire that was used to string culch plates (culch plates are expanded upon below) for settlement. Culch strung on galvanised wires has been used in stake-culture for settlement and suspension from frames or racks has been used in bottom and rack culture settlement (Angell, 1986). PVC conduit (40 mm long by 10 mm diameter) was used as spacers between each culch plate to allow room for water flow and growth on each plate. From here on PVC coated, galvanized wire with PVC spacers will be referred to as culch wires; each culch wire was strung with 10 culch plates. Two types of spat collecting frames were used in the settlement trials: two main spat collector frames and one indicator frame.

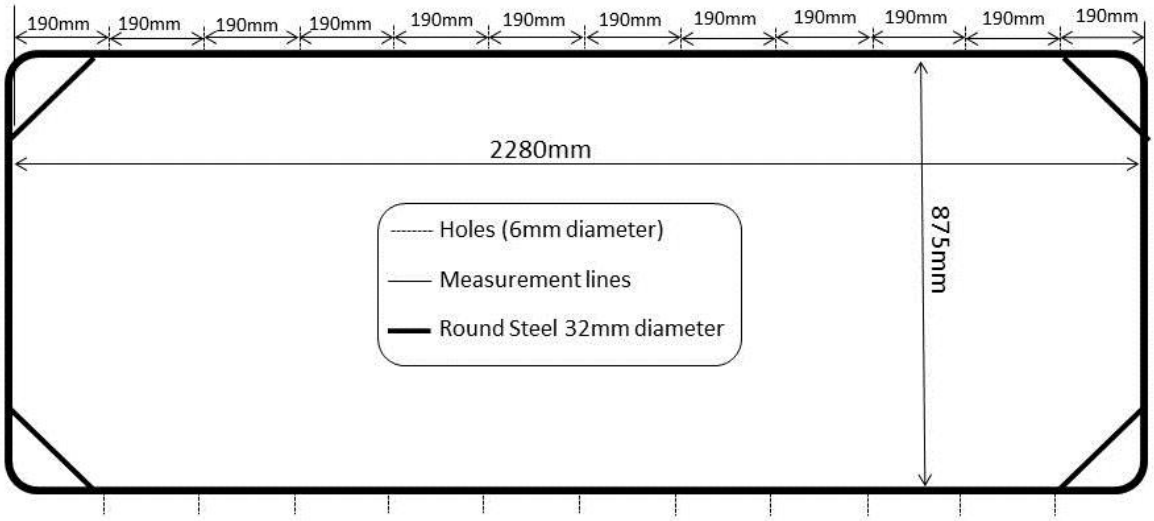

Figure 3.3 Design of frame for spat collectors. Round, mild steel with a diameter of 32 mm was used for the frame which had a width of 2280 mm and a height of 875 mm. Holes of 6 mm were drilled every 190 mm along the two longer edges of the frame. The holes were used as an attachment point for strung culch plates.

3.2.1.2.1 Main spat collector frames

Twenty culch wires were suspended within the two main frames (ten per frame) and frames were deployed on two separate occasions. The first settlement trial was from 6 November 2013 to11 February 2014 and the second from 21 February 2014 to 23 April 2014. For the first settlement trial the clutch types for each of the culch wires were as follows: five 130 mm unglazed plates and five 100 mm abalone shells were strung alternately on each wire (Figure 3.4). For the second settlement trial, after seeing no settlement of oysters, additional culch types were added to increase the possibility of settlement. Culch types utilised in the first settlement trial were 100mm abalone shells, two different species of oyster shells from both *Crassostrea gigas* and *S. margaritacea* (both left and right valves), and re-used 130 mm unglazed plates (Figure 3.5). "Re-used" unglazed plates refers to the fact that the plates used in this settlement trial were those from the initial settlement trial, which had been scrubbed clean of all organisms and biofilms, because artificial substrates should be weathered by allowing them to soak in salt water for a period of time to release all the chemicals from manufacture to make them more suitable for settlement by marine organisms (Krebser, pers. Comm.).

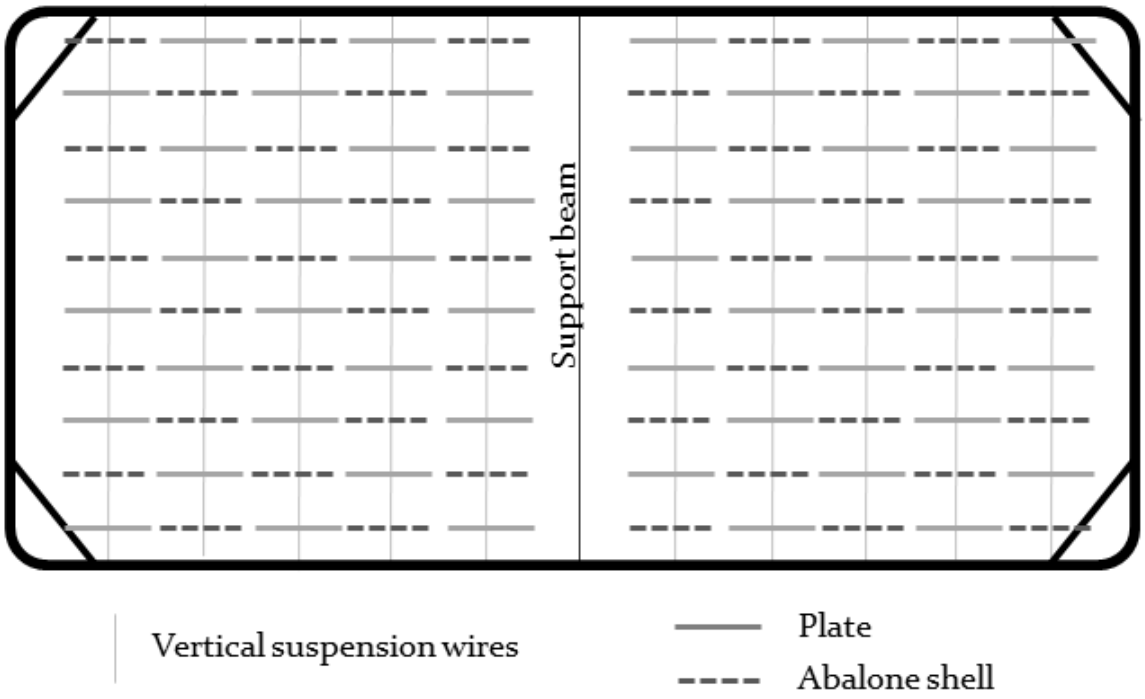

Figure 3.4 Configuration of the two culch types for the first settlement trial for spat the settlement trials. Unglazed ceramic plates were alternated with abalone shells to allow for even depth distribution of both culch types.

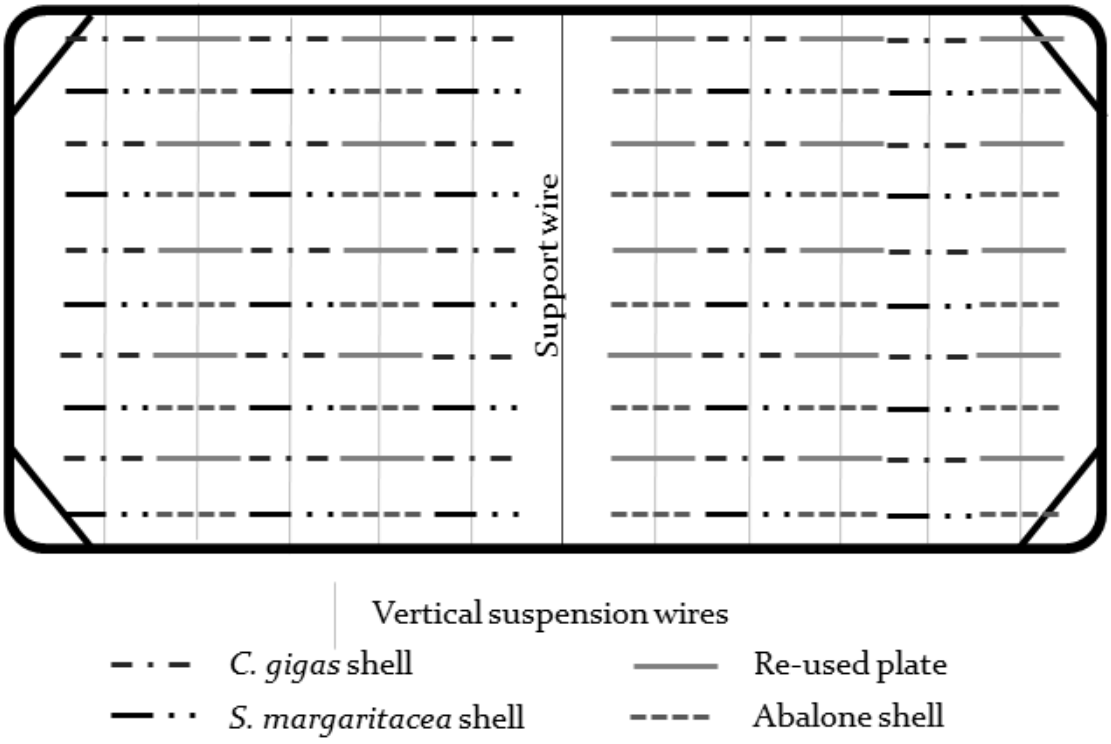

Figure 3.5 Configuration of the four different culch types used for the second settlement trial of frames for the spat settlement trials. Two new culch types were added (*S. margaritacea* and *C. gigas* shells), and the unglazed, ceramic plates from the previous settlement trial were re-used, after being scrubbed clean.

3.2.1.2.2 Indicator spat collector frame

The configuration of the indicator frame is different to that of the main frames, permitting one wire to be removed per week as an indicator of what settlement had occurred up to that point without disturbing the main frames (Figure 3.6). As the settlement trial periods were for several months, more culch wires were required and only one of each culch type was required for this inspection as it was preliminary. To increase the number of culch wires that could be attached within the frame, horizontal supports had to be made for attachment of the shorter culch wires. To make these supports PVC coated, galvanized wires were attached to the frame through holes drilled in the sides of the frame; 10mm PVC conduit surrounded the wire and 6 mm holes were drilled through this to create attachment points for the culch wires. As such a long section of wire was required to span the width of the frame vertical support wires were placed at various points; the support wires were either attached to the frame at one end and the attachment wire at the other, or both ends to the attachment wires (Figure 3.6). Culch plates were suspended with horizontal orientation, because this has been shown to improve spat settlement in the pearl oyster *Pinctada maxima* near the island of Bacon, Indonesia (Taylor, et al., 1998). For the first settlement trial: 19 wires with one unglazed plate and one abalone shell each were suspended from the indicator frame (two culch plates per wire rather than four per wire, as shown in Figure 3.6). For the second settlement trial: nineteen wires were suspended from the indicator frame with one unglazed plate, one abalone shell, one *C. gigas* oyster shell and one *S. margaritacea* oyster shell per wire (Figure 3.6). One wire was removed per week and the culch plates inspected..

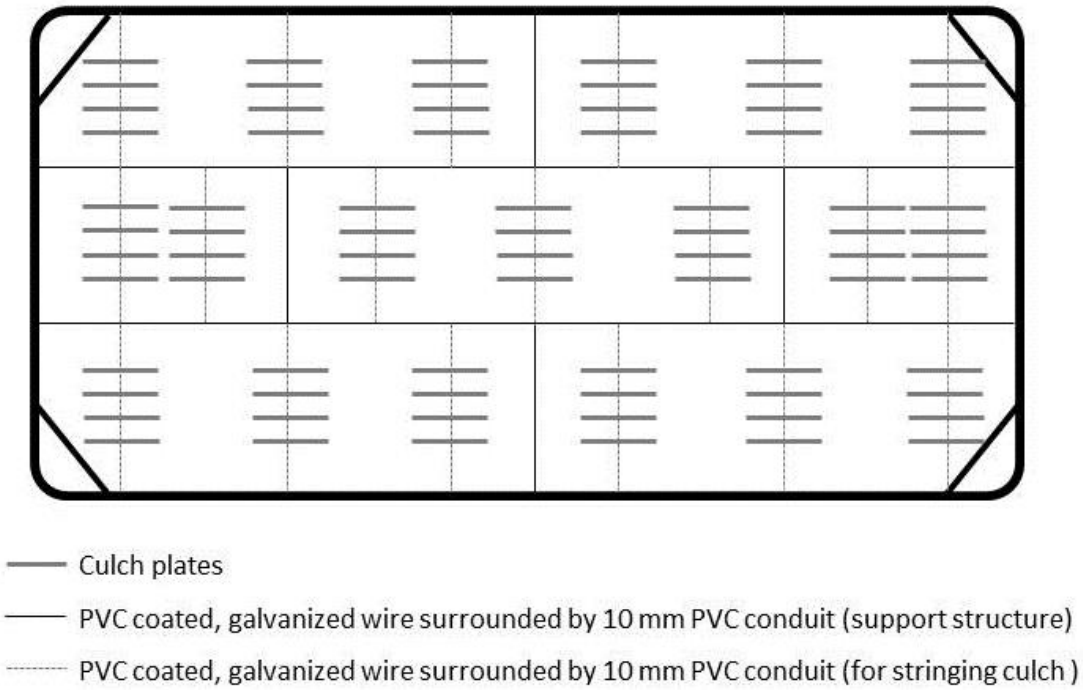

Figure 3.6 Configuration of the indicator frame with indicator culch plates. Horizontal wires were fixed to the frame so that more, shorter wires of strung culch plates could be suspended within the frame. These culch wires were removed on a weekly basis to monitor settlement.

3.2.1.3 Deployment of culch frames

The floating jetty from which the collector frames were suspended was located 800 meters from the river mouth. Spat collection frames were suspended below the jetty to ensure that they did not interfere with boat traffic and vice versa (Figure 3.7). Each frame was attached to the jetty by means of two pieces of 10mm nylon rope, one at each corner. To reduce movement and to maintain the stability of the frames they were braced against one another using 10 mm PVC conduit. A concrete weight of 10 to 15 kilograms was attached to each frame to ensure that it hung vertically beneath the jetty (Figure 3.7). The river bottom sloped beneath the jetty; once frames suspended from the jetty, they cleared the river bottom by less than 1 meter at the shallowest point at low tide to, at most, 4 meters at the deepest point at high tide. The frames were positioned so that they were parallel to the flow of the estuary (Figure 3.7).

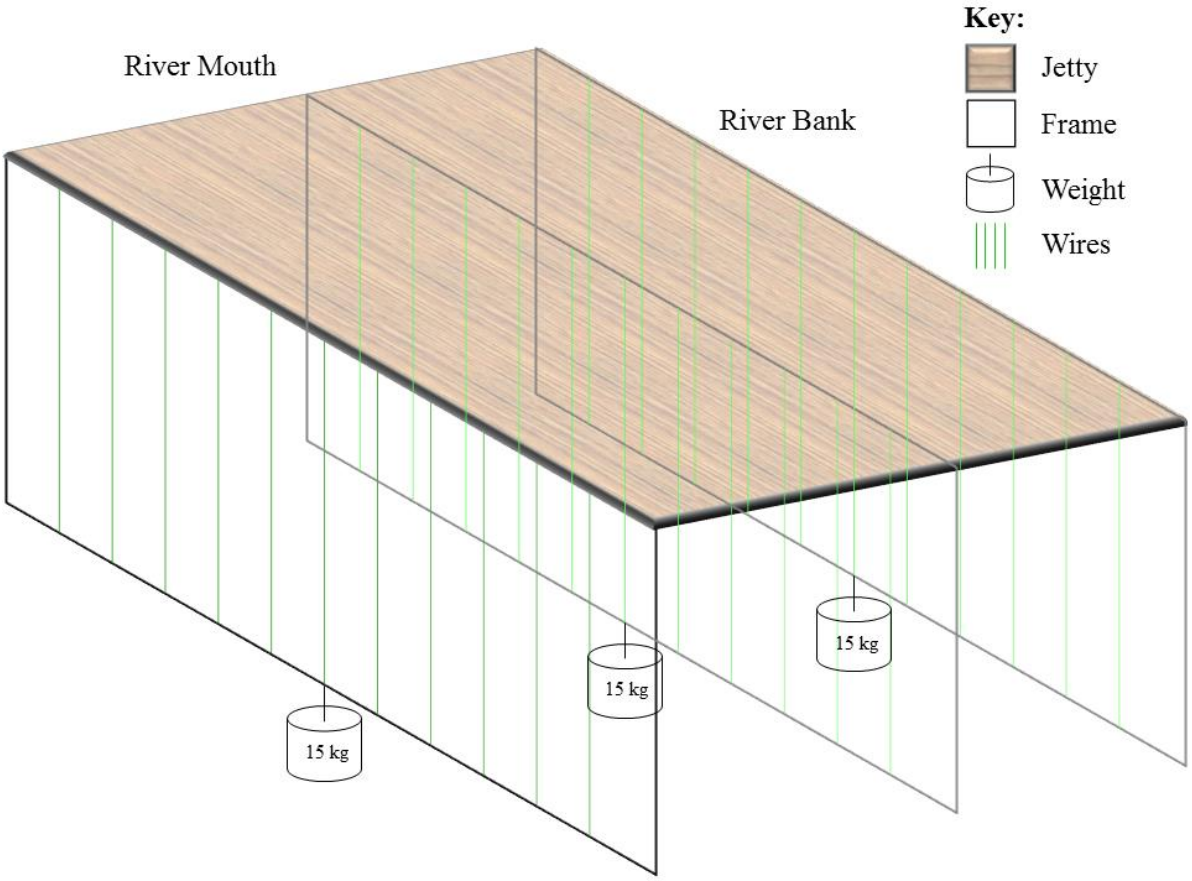

Figure 3.7 Configuration of the collection frames hanging below the Jetty. All spat collecting frames are depicted as though they are main frames for simplicity. Weight of the concrete is given within each weight and there are labels to indicate how the frames were positioned with regard to water flow by indicating the location of the river mouth and the bank.

3.2.1.4 Inspection techniques

Culch plates were removed and inspected on a fortnightly basis and all culch plates in the experimental frames were removed twice during the entire study period: frames were removed once after the first trial and once removed at the end of the study period. Due to the differences in shape of the culch, two different techniques were used for inspecting them. A step-wise circular search pattern was used for the unglazed plates and, since the oyster and abalone shells have a more oblong shape, a creeping line search was used for the inspection of the shells.

3.2.1.4.1 Circular search pattern

The plates were inspected under a stereo-microscope using 2X magnification using a step-wise circular search pattern (Figure 3.8). Starting at one point on the outer most point of the plate, a 20 X 20 mm section was inspected and any growth was recorded, thereafter a line was drawn (using a permanent marker) through the length of the field of vision (approximately the width of the rim). The plate was then turned in a circular motion while it

was inspected; note was taken of any bivalve species that had settled, along with a picture of said species being captured. Images were taken through the eye piece of the stereomicroscope using a Cannon PowerShot SX 240 HS digital camera. Once returned to the initial mark, the plate was moved, towards the centre, until the initial line could just be seen at the edge of the field of vision of the microscope. The process was then repeated until the entire surface area of the plate had been inspected. This process was used for both the front and back of the plate.

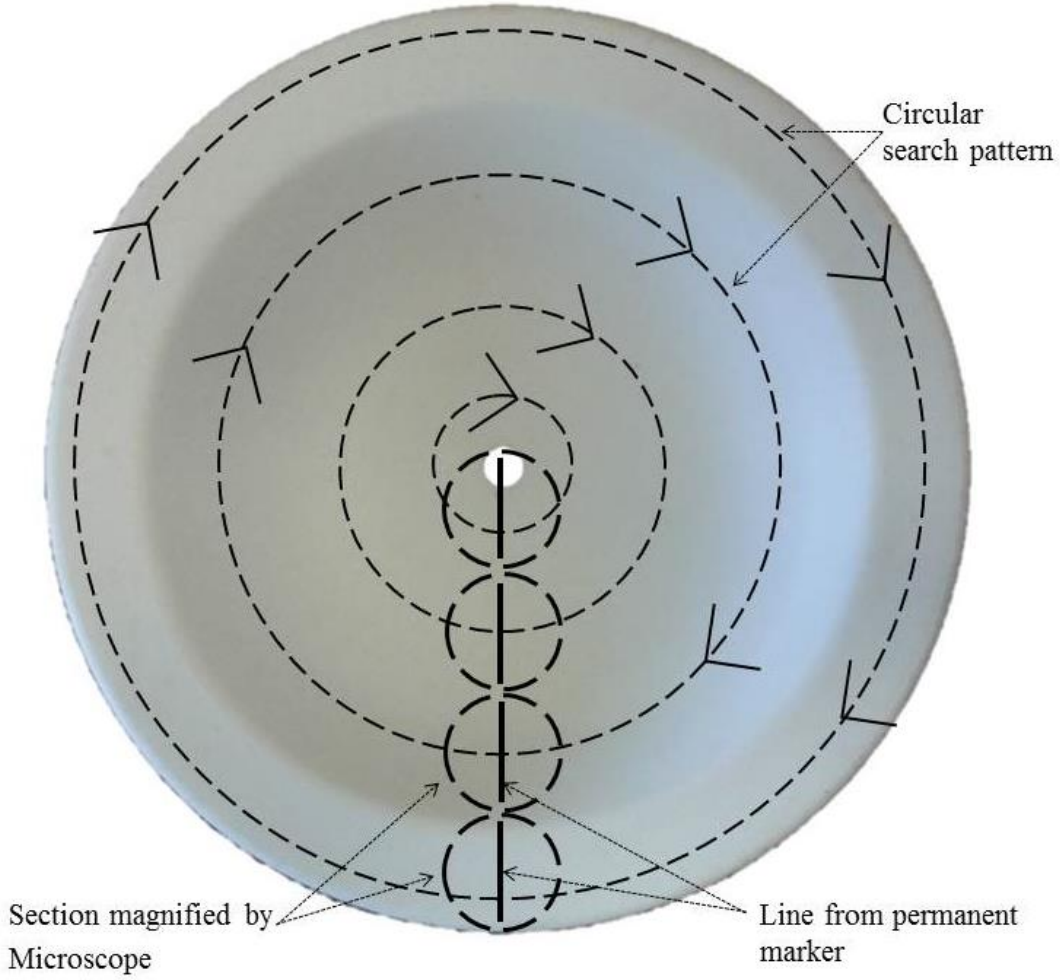

Figure 3.8 Step-wise circular search pattern utilised for inspecting unglazed ceramic plates.

3.2.1.4.2 Creeping line search pattern

A creeping line search pattern was used for the inspection of the shells (Figure 3.9). As with the circular search pattern, 2X magnification of the stereo-microscope was used. Inspection started at one side of the shell and moved in a linear fashion to the other side taking note and pictures of bivalves encountered. Once at the end of the shell, a mark was made through the field of vision with a permanent marker, the shell was then moved so that the edge of the

mark was at the edge of the field of vision and the shell was inspected in the opposite direction. This process was repeated until the whole shell had been inspected on both the ventral and dorsal sides (abalone) or inside and outside (oyster shells).

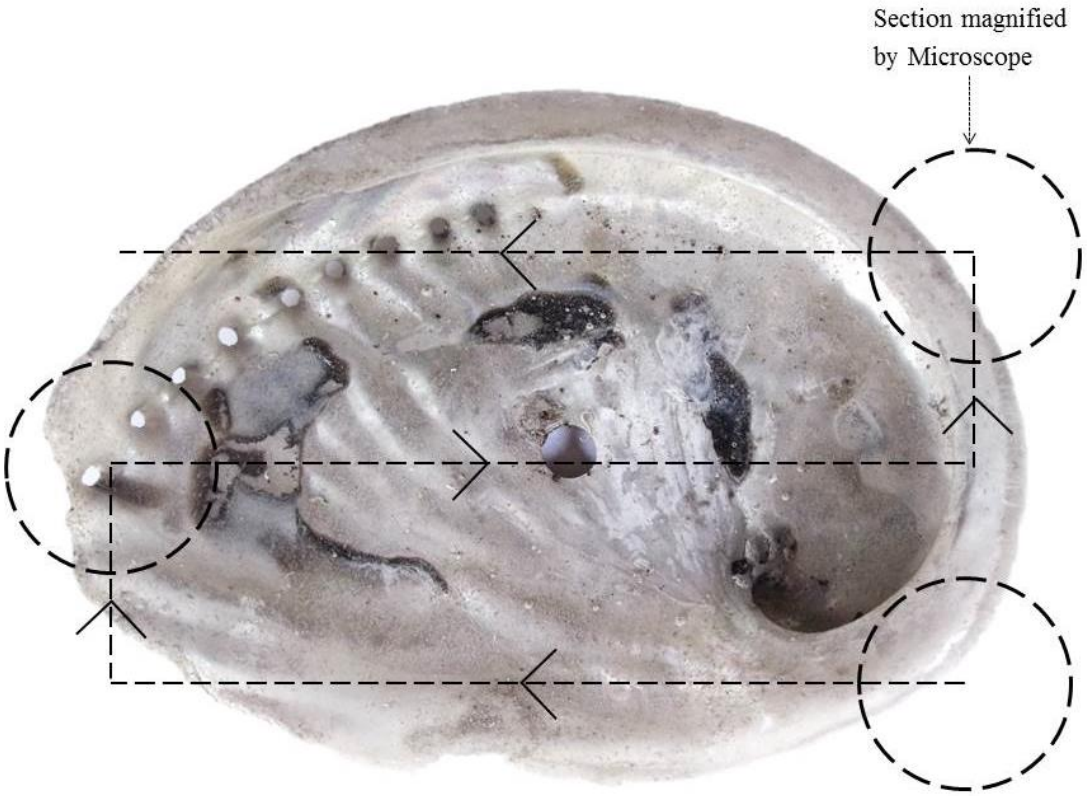

Figure 3.9 Creeping line search pattern used for inspecting shells This search pattern is shown on an abalone shell, however it was used for both abalone and oyster shells. Both ventral and dorsal sides (abalone) or inside and outside (oyster) of the shell was inspected.

3.2.1.5 Species identification and recording data

All bivalves found on culch plates were recorded. Mussels were identified guided by Bownes (2005) morphological identification of mussels. When the identity was indeterminate Dave Krebser was contacted for an identification of the individual in question. Dave Krebser guided me in the identification of oysters including saddle oysters (*Anomia achaeus*), *S. margaritacea*, *O. atherstonei*, and *O. algoensis*. Saddle oysters (which are not true oysters) can be easily distinguished from true oysters especially when they have recently settled; the lower valve has a smaller hinged valve through which the mollusc's foot can attach to the substrate; this can be seen when the saddle oyster is removed from the substrate. Most of the settled oysters encountered were saddle oysters. All other organisms were identified by the common name for the subphylum using Branch et al. (2007). Bivalves were counted, while the dominant species, the striped barnacle *Amphibalanus amphitrite*, and other fouling

organisms were noted by presence / absence. When recording data culch plates were tracked using a 3D co-ordinate system "frame : wire : plate – culch type" (Figure 3.10); frames were numbered 1-3 (one being furthest from the bank), wires were numbered 1-10 (one being furthest from the river mouth), and plates were also numbered 1-10 (one being closest to the water surface).

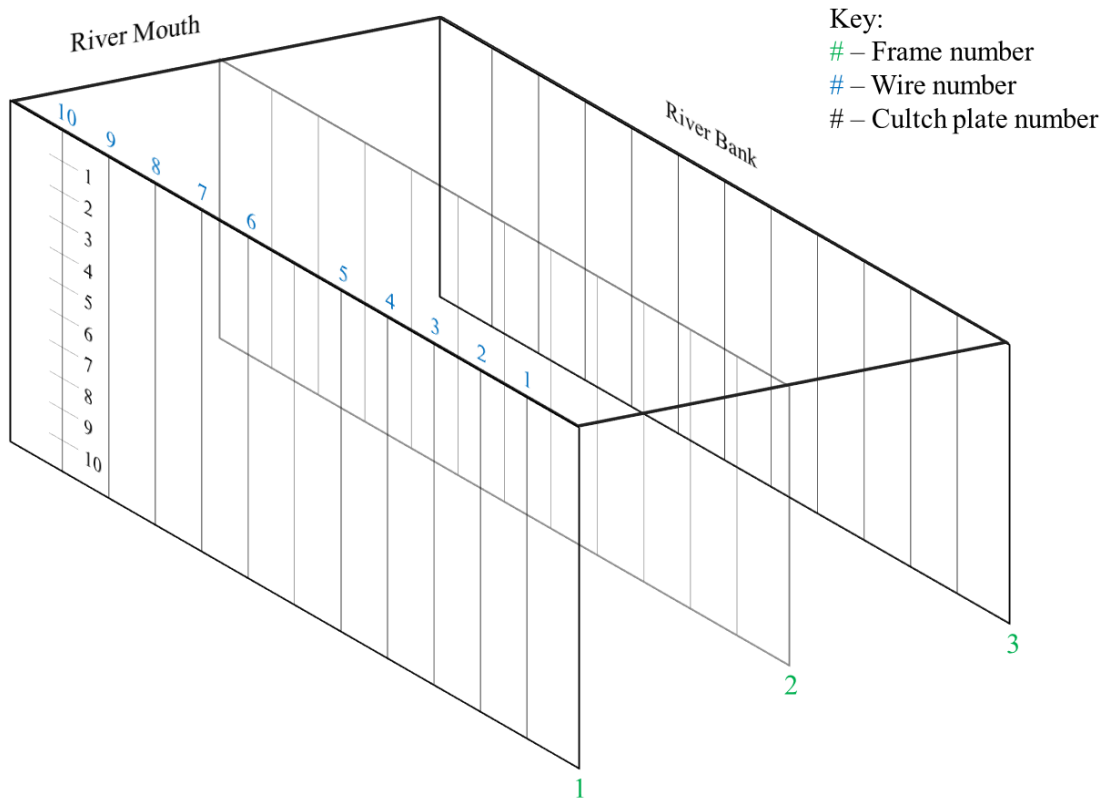

Figure 3.10 Frame co-ordinate system Each culch plate data point was recorded by the co-ordinate system "**frame:wire:plate** – culch type"

3.2.1.6 Statistics

As very few *S. margaritacea* settled during the settlement trial (approximately 20 individuals), no statistics were performed due to insufficient data.

3.2.2 Oyster size class distributions in Marine Protected Areas: determining the influence of the HAB

3.2.2.1 Study sites

To follow up with an investigation of the potential influence of the HAB on spat settlement along the coast in the area around the Kowie River mouth, I chose three Marine Protected Areas (MPAs) on the northernmost border of the area affected by the Harmful Algal Bloom,

(East London or Gonubie) which was approximately 115 km NE of the spat settlement trial location. The southwestern-most of these sites (Gulu) was within the range of the HAB; Gonubie was on the border of the HAB, and the northeastern-most site, at the Kei River mouth, was outside the range of the HAB. The MPAs are described in the Government Gazette (DEA, 2011) by GPS co-ordinates and each one has a range between two towns: Gulu MPA (on the South West) ranges from Gulu to Christmas rock, Gonubie MPA from the suburb of Gonubie to that of Nahoon in East London, and Kei MPA (on the North East) from Kei Mouth to the Nyara River (Figure 3.12). Accessible, rocky sections of each MPA were surveyed for all size classes of *S. margaritacea* in the low intertidal zone. The surveys were conducted over an 8 day period (6 September to 14 September 2014) during a spring low tide whose low water height ranged from a maximum of 0.43 meters to a minimum of 0.05 meters. Due to the low density of oysters visible above the low water mark, directed random sampling was used to most effectively utilize the duration of the low tide, i.e. oysters were targeted and those falling within the same 200 x 200 mm quadrat were measured and grouped. The right valve (top valve) length and width were measured using digital and Vernier callipers for exposed and submerged oysters respectively (Figure 3.11).

Figure 3.11 *Striostrea margaritacea* showing right valve length and width. Right Valve Length (RVL) is measured between the hinge, on the dorsal side of the oyster, and the ventral margin. Right Valve Width (RVW) is measured between the anterior and the posterior margin of the dorsal side.

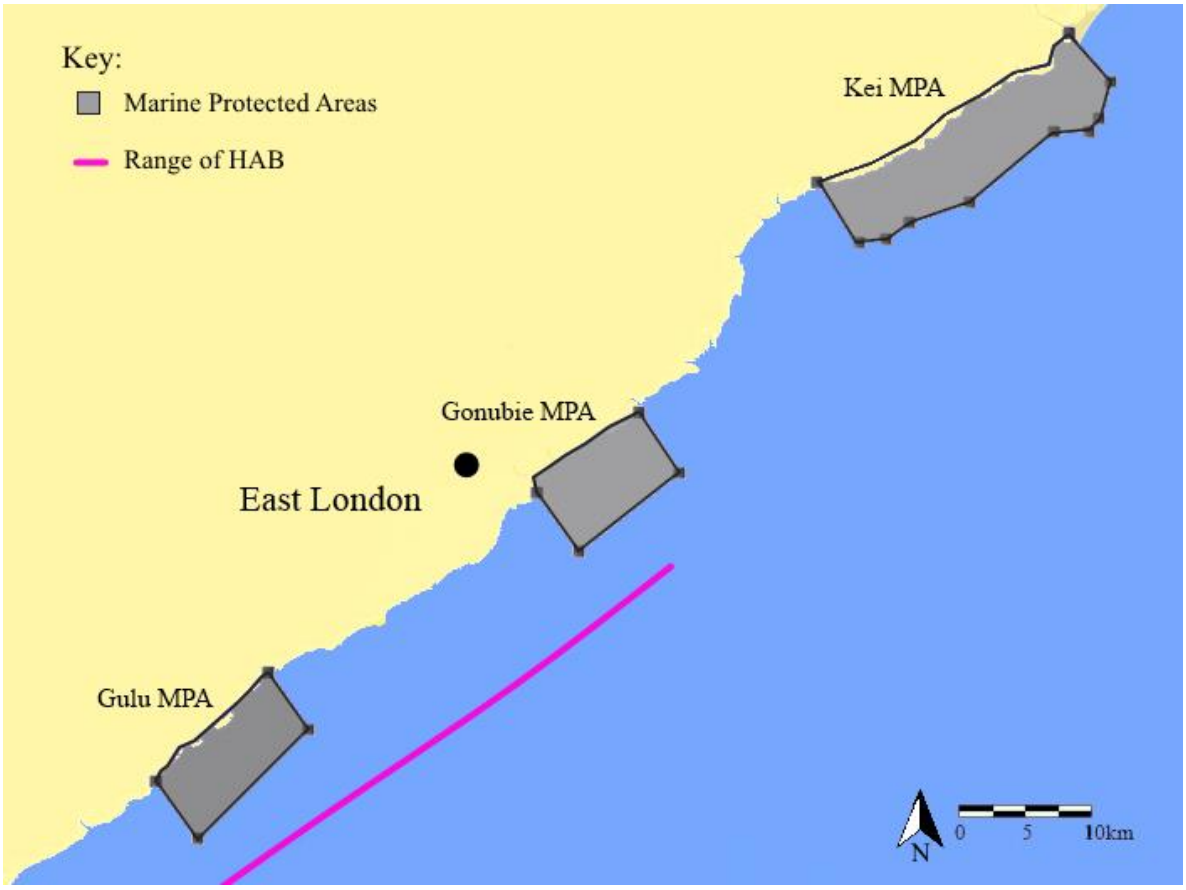

Figure 3.12 Gulu, Gonubie and Kei MPAs. Light grey squares are the areas encompassed by the GPS co-ordinates given in the Government Gazette (DAFF, 2011). The pink line show the eastern-most extent of the HAB.

3.2.2.2 Data analysis

Oysters were grouped into estimated age classes using right valve length according to Equation 1 (Schleyer & Kruger, 1991). Schleyer and Kruger (1991) recorded oyster growth for 33 months and their equation can only be assumed to be accurate within that time frame; therefore, the maximum age group was "more than 3 years" to minimise extrapolation. Five age groups were defined: less than seven months, seven months to one year, one to two years, two to three years, and older than 3 years. The age group "less than seven months" was chosen as these oysters would have settled in the previous season's settlement occurring between November 2013 and May 2014, which was during the HAB period.

Equation 1 Modified von Bertalanffy equation for oyster length vs age (Schleyer & Kruger, 1991).

$$RVL_t = RVL_\infty(1 - e^{-K(t-t_0)})^p$$

RVL_t	- Right valve length at time t	
RVL_∞	- asymptotic length	117.4 mm
K	- age growth constant	0.014 $months^{-1}$
t_0	- age at zero length	1.75 months
p	- power factor	0.654

3.2.2.3 Statistics

To determine age group distributions, Equation 1 was used to determine the maximum and minimum RVL for each age group. All samples were then sorted into age class bins as either present or absent (1 or 0, respectively). As quadrats were used to group samples when collecting data, a count per quadrat could then be determined for each age class; counts are analogous with frequency distributions for each age class. Within each age class, instead of using a nonparametric ANOVA, a Kolmogorov-Smirnov two-tailed test (K-S) was used as this analysis is more accurate than the ANOVA. To correct the p-value for a three-way comparison from the two-tailed test, the Bonferroni correction was applied which resulted in a significant p-value being less than 0.017 (Dunn, 1959).

3.3 Results

3.3.1 Settlement trials

Over the duration of the settlement trial a total of 19 *S. margaritacea* settled on the culch plates; one settled during the first settlement trial and the other 18 during the second (Table 3.1). The majority of the *S. margaritacea* spat settled on the unglazed ceramic plates, however with such low numbers nothing conclusive can be said about settlement with regard to culch type. Table 3.2 below shows a breakdown of surface area for each culch type, and each settlement trial (given in m^2). The first trial the settlement had 0.14 oysters per m^2, and the second trial had 3.71 oysters per m^2.

Table 3.1 Settlement of *Striostrea margaritacea* 6 November 2013 to11 February 2014 and the second from 21 February 2014 to 23 April 2014

	Culch substrate	Total settled
First settlement trial	Plate	1
Second settlement trial	Plate	9
	Abalone	3
	Oyster Shell	6

Table 3.2 Culch surface area. All surface areas are given in m^2. The total surface area is given for each settlement trial and culch type; the total culch surface area was 11.9 m^2.

Culch type	Surface area per plate/shell	Number used	Total surface area
Ceramic plates	0.027	100	2.66
Abalone shells	0.044	100	4.40
		First trial	**7.05**
Ceramic plates	0.027	50	1.33
Abalone shells	0.044	50	2.20
Oyster shells	0.013	100	1.32
		Second trial	**4.85**

Other fouling organisms which settled on the culch plates included barnacles (Cirripedia), sea squirts (Ascidiacea), sponges (Porifera), lace animals (Bryozoa), and various bivalves as well as other oysters; oysters included 54 *Ostrea algoensis* (the weed oyster which does not attain a very large size) and 1 *Ostrea atherstonei* (the red oyster); *Anomia achaeus* (saddle oyster) dominated with a total of approximately 380 during the first settlement trial, and almost 1070 during the second trial. Other bivalves that settled added up to approximately 10 000 individuals; these included *Perna perna* (brown mussel), *Mytilus galloprovincialis* (Mediterranean mussel), *Choromytilus meridionalis* (black mussel), as well as two *Pinctada*

capensis (Cape pearl oyster, which can be identified by its distinctive shape: having a flat hinge and attachment via byssus threads), and other unidentified mussels and clams. The dominant fouling organism, however, was not a bivalve but the striped barnacle, *Amphibalanus amphitrite*, whose progression of fouling over an eight week period can be seen in Figure 3.13 below.

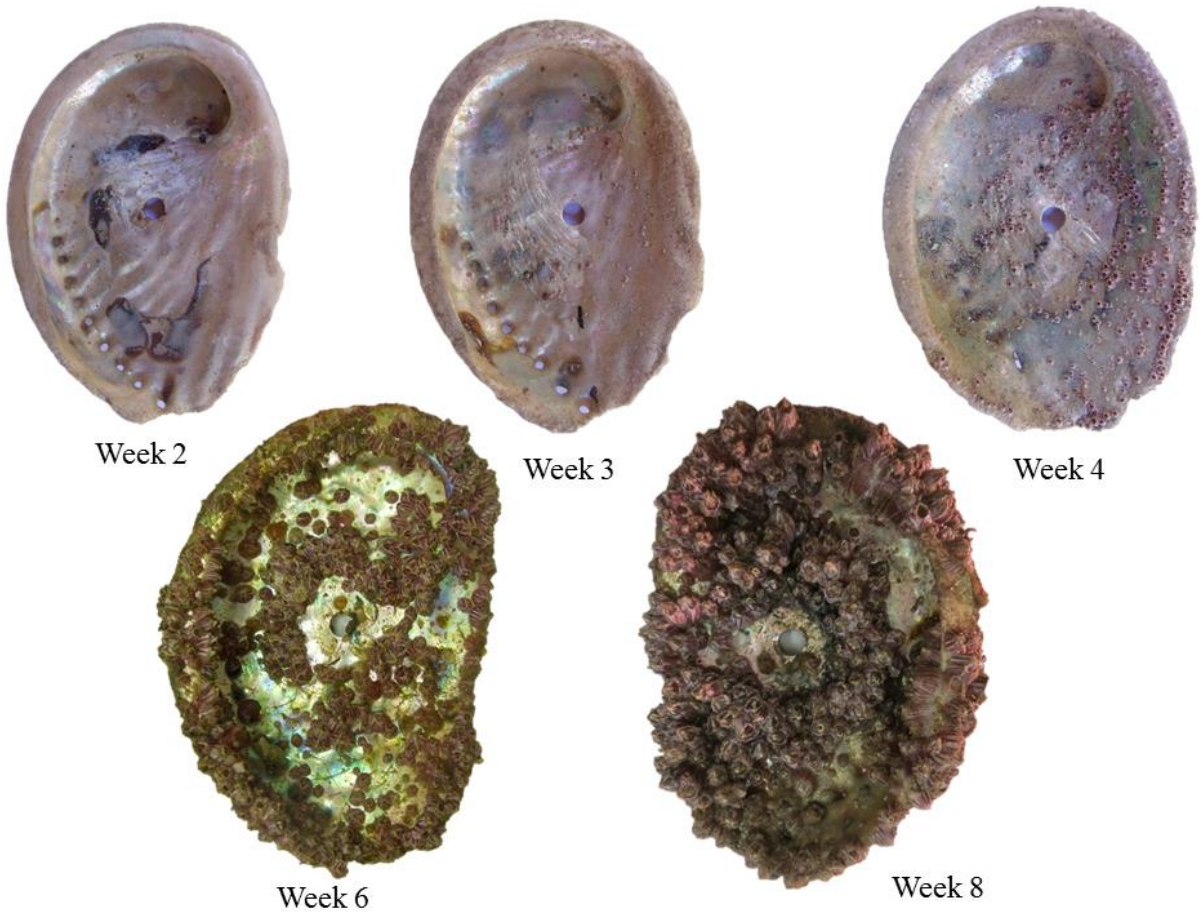

Figure 3.13 Progression of settlement on abalone spat collectors. Abalone shells were approximately 100 mm in length by 70 mm width; the striped barnacle, *Amphibalanus amphitrite*, dominates the available surface area.

3.3.2 Size class analysis

3.3.2.1 Age group distributions compared between three MPAs

The box and whisker plot output of Kolmogorov-Smirnov (K-S) tests were combined for each age class so that the results for all three populations fall on the same plot. Raw RVL data were used to show size class distribution using a histogram and a K-S test was used on raw RVL data to show mean size per population; the Bonferroni correction was used for this K-S test (significant $p < 0.017$); and data are displayed in the same manner as for the age group distributions.

In Figure 3.14to Figure 3.19 boxes depict the 25-75th percentile, whiskers show minima and maxima, and open squares indicate the median; sites are arranged from left to right in order of presumed decreasing influence of the HAB, from SW to NE.

3.3.2.1.1 Oysters less than 7 months old

Kolmogorov-Smirnov two-tailed tests represented in Figure 3.14 showed this age class was better represented, with a higher count, in the population at Gonubie (on the northernmost fringe of the HAB) than at Gulu (well within the HAB range) ($p<0.01$). Gulu, in turn, showed a higher count of juvenile oysters than did the Kei Mouth population, which is north of the area affected by the HAB ($p<0.001$). A higher count of oysters younger than 7 months old was also observed at Gonubie than at Kei Mouth ($p<0.001$).

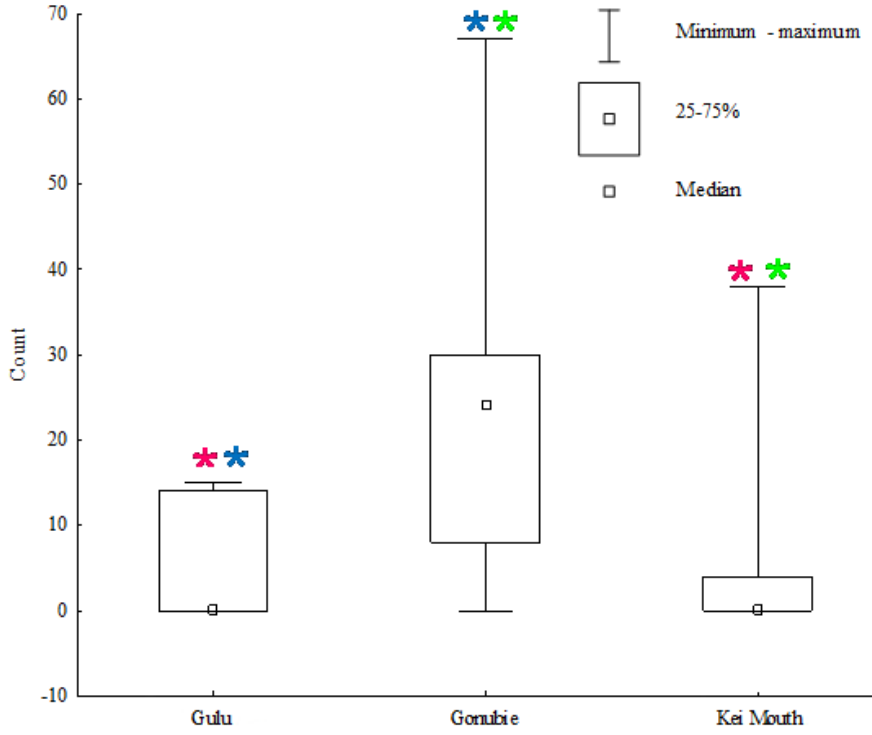

Figure 3.14 Weighted counts of oysters less than 7 months old. * ($p<0.001$), * ($p<0.01$), and * ($p<0.001$) show locations that are significantly different from each other.

3.3.2.1.2 Oysters seven months to 1 year old

The K-S tests in Figure 3.15 show that Gonubie had a significantly higher count of oysters aged 7 months to one year old than did Gulu ($p<0.001$) and a higher count than Kei Mouth ($p<0.001$). Gonubie's mean population count was approximately 1.0 where Gulu's and Kei Mouth's mean population count was 0.

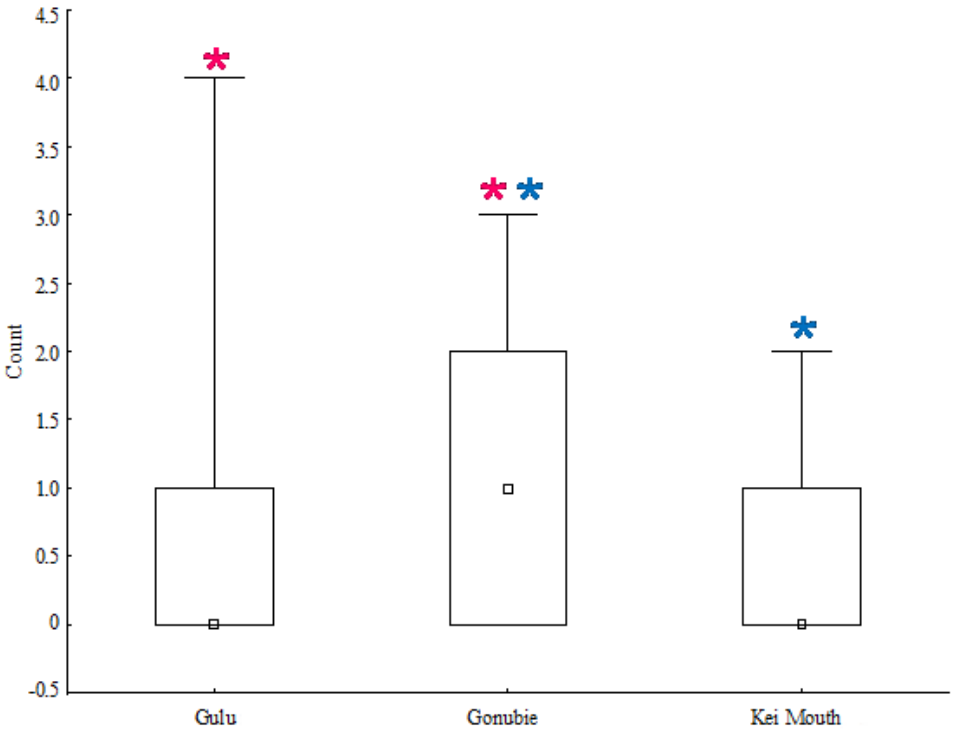

Figure 3.15 Weighted counts of oysters between 7 months and 1 year old. * ($p<0.001$) and * ($p<0.001$) show locations that are significantly different from each other.

3.3.2.1.3 One to two year old oysters

Sexual maturity is reached in this age class (de Bruyn, 2006). Sexually mature oysters were more poorly represented in the Gonubie population than in either the Gulu ($p<0.001$) or Kei Mouth populations ($p<0.001$) (Figure 3.16). There is no significant difference between the counts of mature oysters in Gulu and Kei Mouth ($p>0.017$).

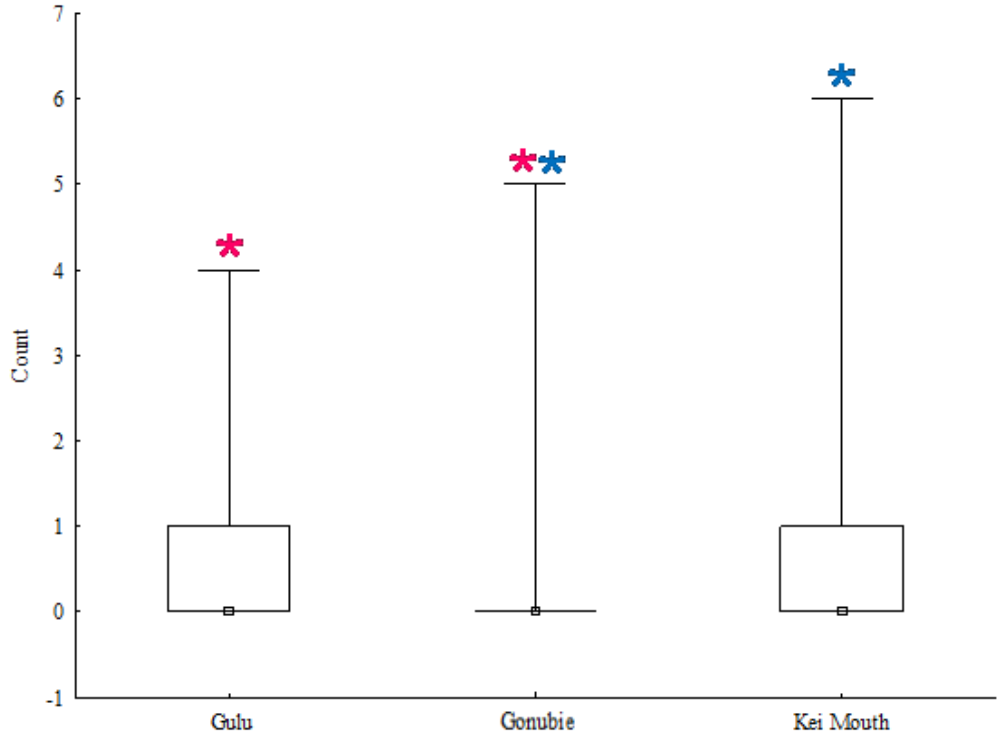

Figure 3.16 Weighted counts of oysters 1 to 2 years old. * ($p<0.001$) and * ($p<0.001$) show locations that are significantly different from each other.

3.3.2.1.4 Two to three year old oysters

The Kei Mouth population better represented the two to three year old oysters, reproductively active (de Bruyn, 2006), than did Gonubie ($p<0.001$). There was no significant difference between Gonubie and Gulu or Gulu and Kei Mouth ($p>0.017$) oysters of this age range.

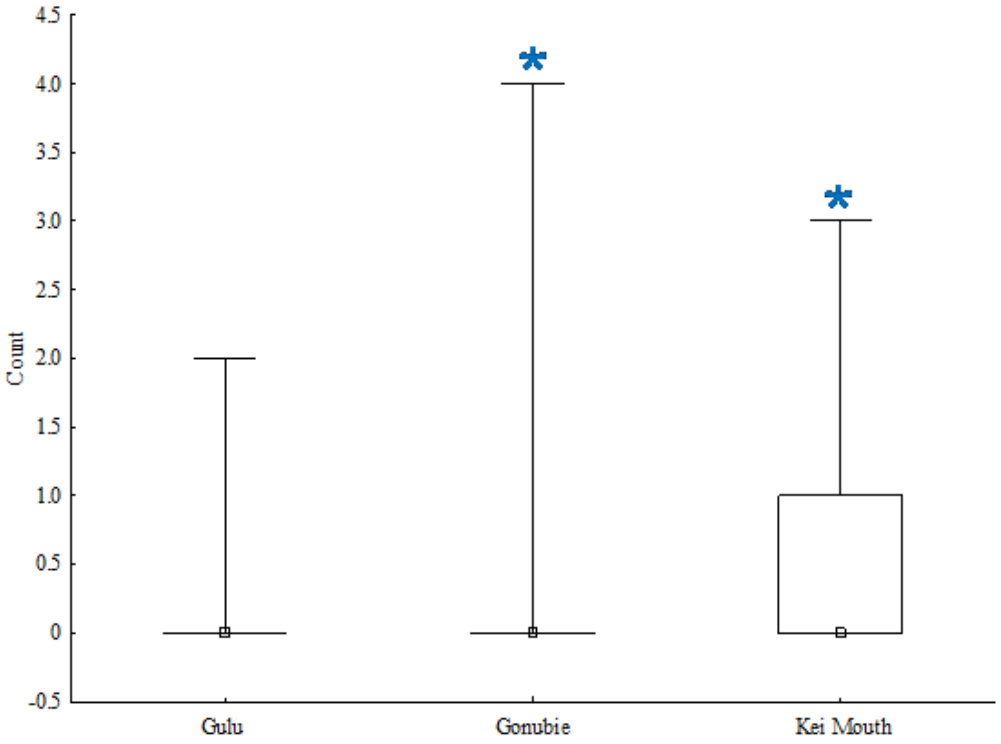

Figure 3.17 Weighted counts of oysters 2 to 3 years old. * ($p<0.001$) shows that Gonubie and Kei Mouth are significantly different from each other.

3.3.2.1.5 Oysters three years or older

The greater than 3 years age class was not significantly better represented at any of the populations when compared to one another. This may indicate that oysters are harvested or removed before they reach this age class.

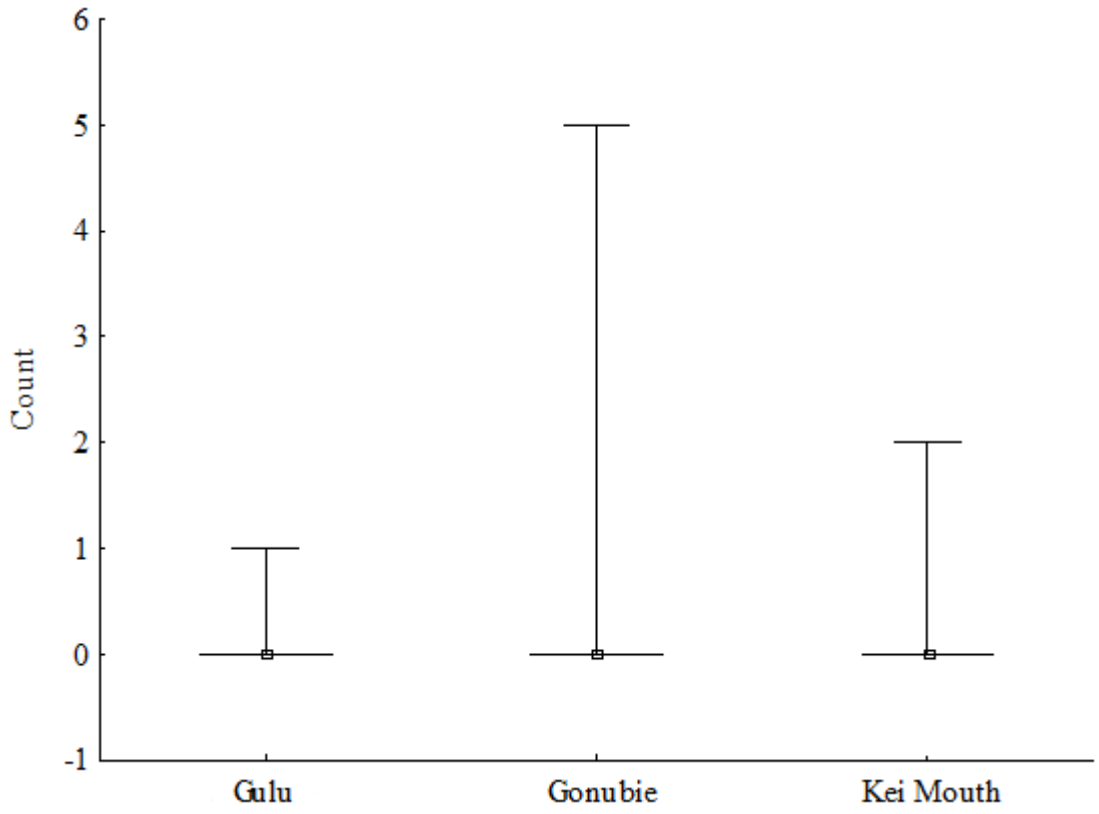

Figure 3.18 Weighted counts of oysters older than 3 years. None of the populations are significantly different from one another ($p>0.017$).

3.3.2.2 Size distribution

The Gonubie population had smaller, younger, oysters than the Kei Mouth ($p<0.01$) and Gulu populations ($p<0.01$) (Figure 3.19). The Kei Mouth populations had a higher number of oysters within the 10-20mm RVL than of other size classes; Gonubie had a far greater portion of the population with a RVL of less than 20mm; and Gulu had a population spanning all size classes quite evenly (Figure 3.20).

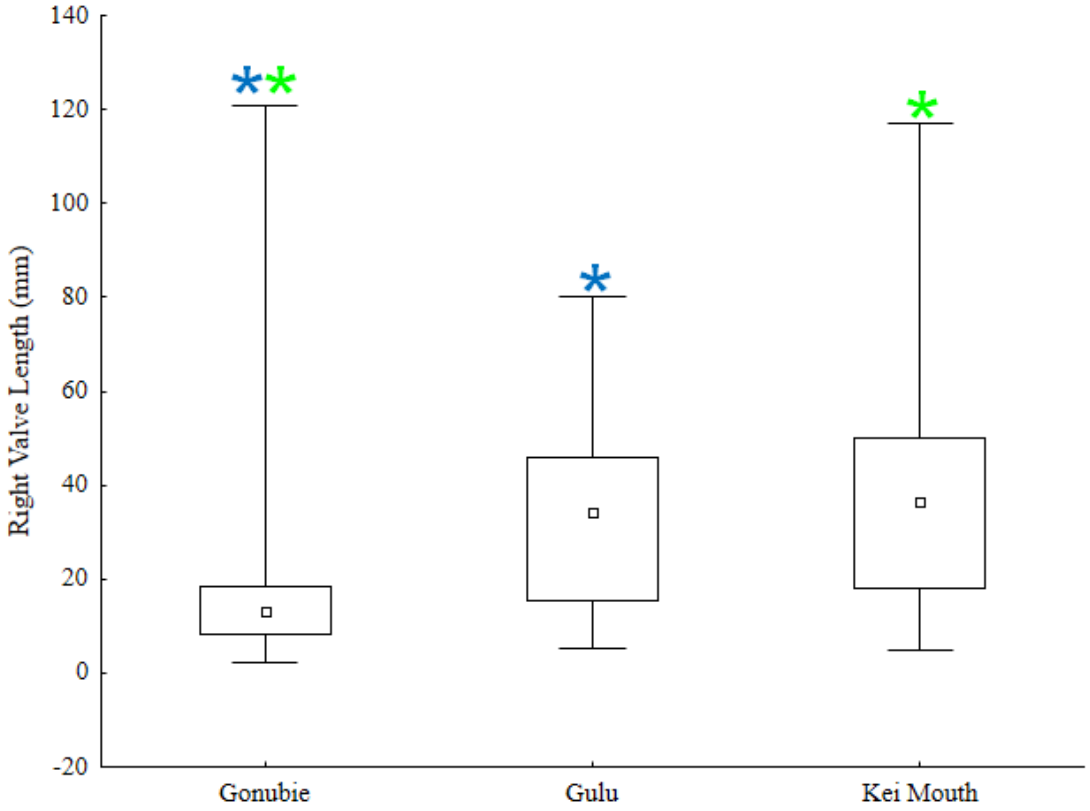

Figure 3.19 Box and whisker plot of Kruskal-Wallis ANOVA for RVL of oysters in all three populations. * and * ($p<0.01$) indicate populations that are significantly different from one another.

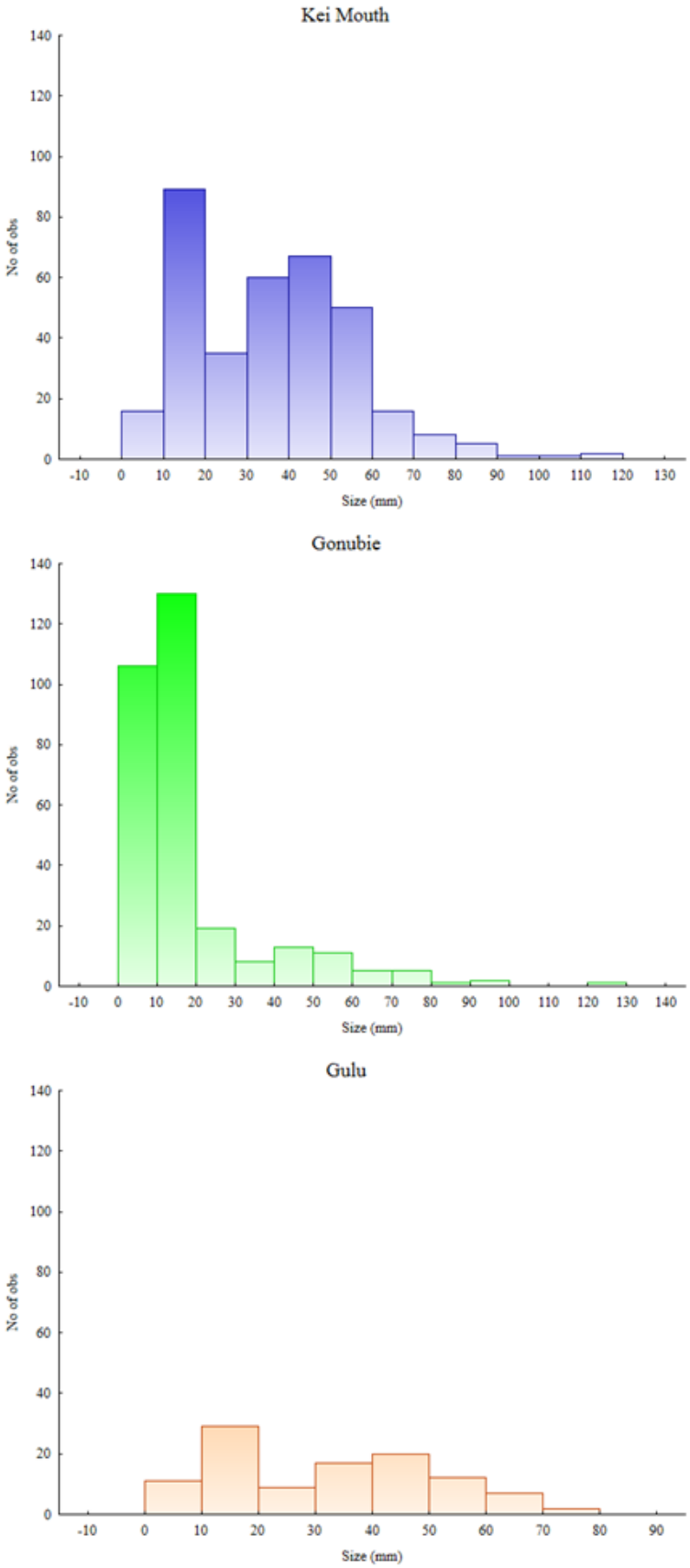

Figure 3.20 Histograms of oyster size frequency distributions for all three populations, Populations are displayed from north to south.

3.4 Discussion

3.4.1 Settlement trials

Settlement trials resulted in only 19 *S. margaritacea* oysters settling throughout the entire trial period of approximately 5 months. Other true oysters (Ostreidae) that settled included 54 *O. algoensis* individuals and one *O. atherstonei*. *Ostrea algoensis* attains a maximum size of 45mm and is a physically fragile species, making it unsuitable for commercial use (Kilburn & Rippey, 1982). Settlement of Ostreidae occurred on all culch types, indicating that there was suitable substrate for settlement. Although many other bivalves settled, none were true oysters. True oysters have different methods of attachment to substrate and may have different environmental requirements for settlement than do other bivalves. This could suggest that the chosen culch types were suitable but other, environmental, factors had an effect on settlement. The most notable environmental factor during the settlement trial period was the HAB which began in November 2013 and proliferated through February 2014 around Port Alfred; the bloom stretched from Mossel Bay to East London and was concentrated in Port Elizabeth (Bornman, et al., 2014). *Lingulodinium polyedrum* was the dominant dinoflagellate species of the HAB.

There are 58 dinoflagellate species, which cause HABs, associated with bivalve mortality; *Lingulodinium polyedrum* is one of these many species (Burkholder, 1998). Some dinoflagellates cause extensive damage and even mortality to both trochophores and D-shaped larvae; these deleterious effects include reduced activity, prevalence of damage, delay or inhibition of shell mineralisation, and severely reduced survival (Basti, et al., 2011). The effects of the toxic dinoflagellate *Heterocapsa circularisquama* on bivalve larvae were examined by Basti et al. (2011) and the impact of this dinoflagellate occurred within 3-6 hours of exposure and at densities exceeding 5 X 10^3 cells/mL; the HAB experienced in Port Alfred, during the settlement trials, had densities in excess of 29 X 10^3 cells/mL of the dominant dinoflagellate *L. polyedrum* (Basti, et al., 2011; Bornman, et al., 2014).

There are several ways in which HAB species can affect oysters and oyster larvae. The dominant contributing factor to mortality would be low pO_2 caused by decomposition of the algal bloom (Bricelj & Lonsdale, 1997; Cockcroft, 2001). Other factors include: mechanical damage and clogging of feeding mechanism or gills; high or low pH levels caused by photosynthesis of algal bloom species and altered levels of ammonium; anoxic and/or hypercapnia (low CO_2 levels) resulting from eventual decomposition of algal bloom species;

and toxins which have fatal or sub-fatal effects (Branch, et al., 2013; Calbrese & Davis, 1966).

Oyster eggs develop normally within a pH range of 6.75-8.75, however, above a pH of 9 normal larval development is greatly reduced to approximately 15% (Calbrese & Davis, 1966). High ammonium levels also increase the pH and elevated levels of ammonium were present during the 2014 bloom (Bornman, et al., 2014). Ammonium levels may also be the cue that prompts oyster settlement behaviour and ultimately recruitment (Coon, et al., 1990). According to Bornman et al. (2014), ammonium levels during the 2014 bloom differed from the 2010-2013 mean ammonium levels. Differences in ammonium levels between the 2014 settlement season and previous years may have altered the timing of settlement and resulted in oysters metamorphosing to settling before finding suitable substrate.

According to Timmins-Schiffman et al. (2012) as a bloom decomposes, respiration results in a high pCO_2. High pCO_2 also leads to low pH levels, and at a pH of less than 6.5 less than 30% of oyster eggs will develop normally (this percent continues to drop drastically as pH drops and below a pH of 6.25 less than 2% of eggs will develop normally) (Calbrese & Davis, 1966). Increased pCO_2 results in increased metabolism of oyster larvae, and increased metabolism results in larvae transitioning more quickly from non-feeding (lecithotrophic larvae) to feeding larvae (Timmins-Schiffman, et al., 2012). Once in the feeding larval stage, oysters become reliant on environmental resources for nutrition and growth. During an algal bloom where *L. polyedrum* is highly prevalent and is able to outcompete other phytoplankton, the increased metabolism that oyster larvae experience may result in mortalities due to lack of suitable nutrition and general food availability (Timmins-Schiffman, et al., 2012). Low food availability results in greatly reduced larval growth and far fewer larvae successfully undergoing metamorphosis, settling and becoming spat (Rico-Villa, et al., 2009).

As explained, there are many ways in which HABs can affect oyster larvae growth, survival, and settlement. One or more of these factors may have come into play during the 2014 HAB and disrupted larval survival, development, and settlement in Port Alfred. Although there was an extensive and sustained HAB which drew much attention for this study with regard to low settlement, other natural factors should not be overlooked. Krebser (pers. com., 2013-2014) investigated oyster spat settlement in the Knysna estuary in the 1980's and found that once the government began releasing water and overflow from the sewage treatment plant into the estuary, the oyster spat settlement rate dropped dramatically due to larvae failing to develop

properly. Belmont Valley waste water treatment plant releases treated water into the Kowie Catchment, flowing into the Kowie River (Chadzingwa, et al., 2013). In March 2013, high levels of *E. coli* were recorded in the discharge of treated water and there was also raw sewage being released into the tributaries due to two leaks in the main sewer line (Mngxitama-Diko, 2013). It is unknown when the leak was repaired and when the *E. coli* levels returned to normal.

Another issue may be a lack of larvae released into the water column. Spatfall may be attributed to other environmental conditions prior to adult oysters spawning. Ulanowicz et al. (1980) created a mathematical model to determine conditions that resulted in oyster settlement variability in the Patuxent River (Maryland, USA). However, the factors that they investigated were abiotic factors such as air and water temperatures, salinity, hydrologic drought index, rain fall, and freshwater input. If adult oysters are in poor condition prior to spawning, their output of eggs and sperm may be much lower.

3.4.2 Size class analysis

The Gonubie MPA had a higher number of juvenile oysters (less than one year) than the other two MPAs (Figure 3.14 and Figure 3.15). This trend was unexpected as the Gonubie MPA is on the border of the 2014 HAB range whereas Kei Mouth MPA falls outside of said range. This result suggests either that settlement in these three MPAs was not affected by the HAB, or that other, pre-existing conditions were a stronger driver for settlement. The peak in settlement in Gonubie may be linked to the reduced abundance of medium sized oysters which could leave more available habitat, or dead shells (oysters that were improperly harvested could leave behind cemented shell valves) for the settlement of recently metamorphosed oyster larvae. Another, more likely, factor that may have influenced age structure of intertidal oyster communities is human predation.

Oysters between 1 and 3 years have a size range of approximately 30-60mm, which is the size at which locals would harvest them for sale and/or consumption; the average size for small scale commercially wild-harvested oysters in KwaZulu-Natal is between 50 and 70 mm (Steyn, 2015). The Gonubie MPA has fewer 1-2 year old oysters than both the Gulu and Kei MPAs and fewer 2-3 year old oysters than the Kei MPA (Figure 3.16 and Figure 3.17). The fact that the Gonubie MPA has fewer oysters in this age/size range suggests a higher harvesting pressure in the Gonubie area than in the Gulu or Kei MPAs. This harvesting

pressure may be due to the fact that the Gonubie MPA is in a more densely populated and built-up area than the Kei and Gulu MPAs; Gonubie (Buffalo Municipality) has a population of 755 200 (density of 298 persons.km^{-2}), Kei (Great Kei municipality) has a population of 38 991 (22 persons.km^{-2}) and Gulu (Ngqushwa Municipality) a population of 72 190 (32 persons.km^{-2}) (Statistics South Africa, 2011). Kei MPA and Gulu MPA encompass holiday- or resort-areas with seasonal population increases. It is reasonable to assume that areas with a larger and more permanent population density would result in a heavier harvesting pressure on indigenous oysters. Although all of the chosen MPAs in this study are declared "no-take" zones, meaning no fishing or harvesting is allowed in these MPAs, this status was only put into effect as of 2011 (DAFF, 2011); the degree of enforcement in these recently established, no-take MPAs is unknown and harvesting in the no-take zones may still be occurring.

3.5 Conclusion

The 2014 HAB may have impacted the settlement of oyster larvae in the Kowie River (Port Alfred) due to one or more of several possible factors. These factors include mechanical damage caused by dinoflagellates, anoxic conditions, variations in pCO_2 levels, depleted source of nutrients, and altered ammonium levels, amongst other effects, which severely reduce survival of oyster larvae. The effects of the HAB on the settlement of oysters on the fringes of the HAB range seem to be less pronounced. These sites were more heavily impacted by their pre-existing community structure, which has roots in anthropogenic activities. If wild settlement were to be implemented for indigenous oyster culture, it is recommended that a larger, long-term study is performed over several breeding seasons and in multiple locations. Several environmental conditions should be monitored over the course of this study, including water temperature, salinity, and rainfall. This would aid in determining variations of wild-spat settlement due to varying environmental conditions. If the study were sufficiently long-term, these data could be used to eventually create spat-fall prediction models similar those made by Ulanowicz et al. (1980) and Kimmel and Newell (2007).

Chapter 4: Conclusions and further study

There are several indigenous oyster species along South Africa's coastline, and of these there are two palatable species *Striostrea margaritacea*, the Cape Rock oyster, and *Saccostrea cucullata*, the Natal Rock oyster (Kilburn & Rippey, 1982). To minimise the impact indigenous oyster culture would have on wild populations' natural genetic structure, population structure and diversity were determined for both *S. margaritacea* and *S. cucullata*, and natural settlement experiments and size class analyses were performed for *S. margaritacea*. It is important to have the genetic structure of a species determined before moving samples around.

4.1 Genetic diversity

4.1.1 Genetic diversity: CO1 versus 16S gene region

The CO1 and 16S gene regions show differences in genetic diversity; CO1 is a more diverse gene region than is 16S due to CO1 evolving at a faster rate than 16S (Salvi, et al., 2014; Nicolas, et al., 2012).

4.1.2 *Saccostrea cucullata*

It was previously thought that there was a single *Saccostrea* species along South Africa's coastline, *S. cucullata*. Both CO1 and 16S gene regions show that this is, in fact, not the case and there are two species (*S. cucullata* and possibly, *S. mordax*). Samples from Mtakatye group with *S. cucullata* while the two northern populations (Umdloti and Port Edward) group more closely with *S. mordax* (Figure 2.9 and Figure 2.10). Studies have shown that *S. mordax* and *S. cucullata* are frequently misidentified as the other due to similar morphology and the plasticity of colour and shape (Lam & Morton, 2006). The two species appear to have a geographic separation at some point between Mtakatye and Port Edward. The given biogeographic break in this region is at Mbashe which is south or south-west of all three populations (Sink, et al., 2012). However, different species show this break at different points and one such point is at Port St John's which falls between Mtakatye and Port Edward (Lombard, et al., 2004). Port St John's may be the point at which *S. cucullata* and *S. mordax* are separated; however, one sample from Mtakatye grouped with the two northern population samples (Umdloti and Port Edward). The sample may be anomalous, the result of human error, or there may be overlapping ranges of the two species due to sampling being performed by different individuals who may have sampled a larger or smaller area. Human error is

unlikely as the samples from Mtakatye were not handled at the same time as the samples from the northern population. The sample may be anomalous and would not normally survive in this region but for an unknown reason this individual has or the two species may have overlapping ranges but are separated due to habitat preference. *Saccostrea cucullata* and *S. mordax* are found in different habitat types; *S. cucullata* can be found on sheltered rocky shores, in estuaries, and on mangrove roots (Braley, 1982), whereas *S. mordax* can be found exclusively on exposed rocky shores (Lam & Morton, 2004).

Since *Saccostrea cucullata* and *Saccostrea mordax* are frequently subject to nomenclature confusion (Lam & Morton, 2006), species identity should be determined before cultivation and, in the case of mature *Saccostrea* samples, being taken to begin a hatchery. Lam and Morton (2009) showed that there are few morphological features that can be used to distinguish between *Saccostrea* species, those features are mostly on the internal side of the right valve of the shell, and that morphometrics do not show significant differences between species. Accessing the internal side of the right valve would require killing the oyster; therefore, breeding oysters should be identified using CO1 barcoding. It is possible to take tissue from oysters without causing mortality by using a magnesium chloride – salt water mixture (50 $g.L^{-1}$ $MgCl_2$) to anesthetise the oysters (Puchnick-Legat, et al., 2015). Once anesthetised, the adductor muscle of the oyster relaxes, opening the valves and allowing access to the mantle tissue. It is recommended that a very small amount of mantle tissue is taken to minimise damage to the oyster and improve the likelihood of recovery.

The northern population of *Saccostrea* shows exceptionally low levels of genetic diversity (Table 2.2), even though the samples are taken from two locations (Umdloti and Port Edward) that are approximately 175 kilometres apart. There are several possible explanations for the low diversity however, the most likely is that there has been a recent population expansion. Several genetic markers, nuclear and mitochondrial, would need to be used to confirm this statement and further study should be undertaken.

4.1.3 *Striostrea margaritacea*

Striostrea margaritacea has much higher diversity than the *Saccostrea* samples which is unexpected as species found in the upper-intertidal usually show more structure than those in the lower- to sub-tidal (Kelly & Palumbi, 2010; von der Heyden, et al., 2013). The differences in genetic structure between *Saccostrea* and *S. margaritacea* may be due to differences in life histories (tidal zone), anthropogenic movement of *S. margaritacea* for

mariculture purposes, or the northern *Saccostrea* population undergoing a population expansion which results in it having much lower than expected genetic diversity. *Saccostrea* can be found in the upper- to mid-intertidal zone, whereas *S. margaritacea* can be found in the low-intertidal to subtidal zone (Kilburn & Rippey, 1982). As for anthropogenic movement, *S. margaritacea* has been translocated along South Africa's coastline since the 1600s (Thompson, 1913), across multiple biogeographic regions. Such movements would result in hybridization between populations and blur any pre-existing genetic structure (von der Heyden, 2009). *Striostrea margaritacea* has low fixation rates for the CO1 and 16S gene regions, and these gene regions are not ideal when investigating fine-scale genetic patterns (Teske, et al., 2011). To investigate more recent patterns in genetic structure, microsatellites should be used. However this would be a time-consuming task as a microsatellite library would first have to be developed for *S. margaritacea* as one does not currently exist. Due to time constraints, microsatellites were not used in this study. To determine whether or not *S. margaritacea* could be moved around the coastline freely, nuclear markers, in addition to the mitochondrial markers, would have to be examined.

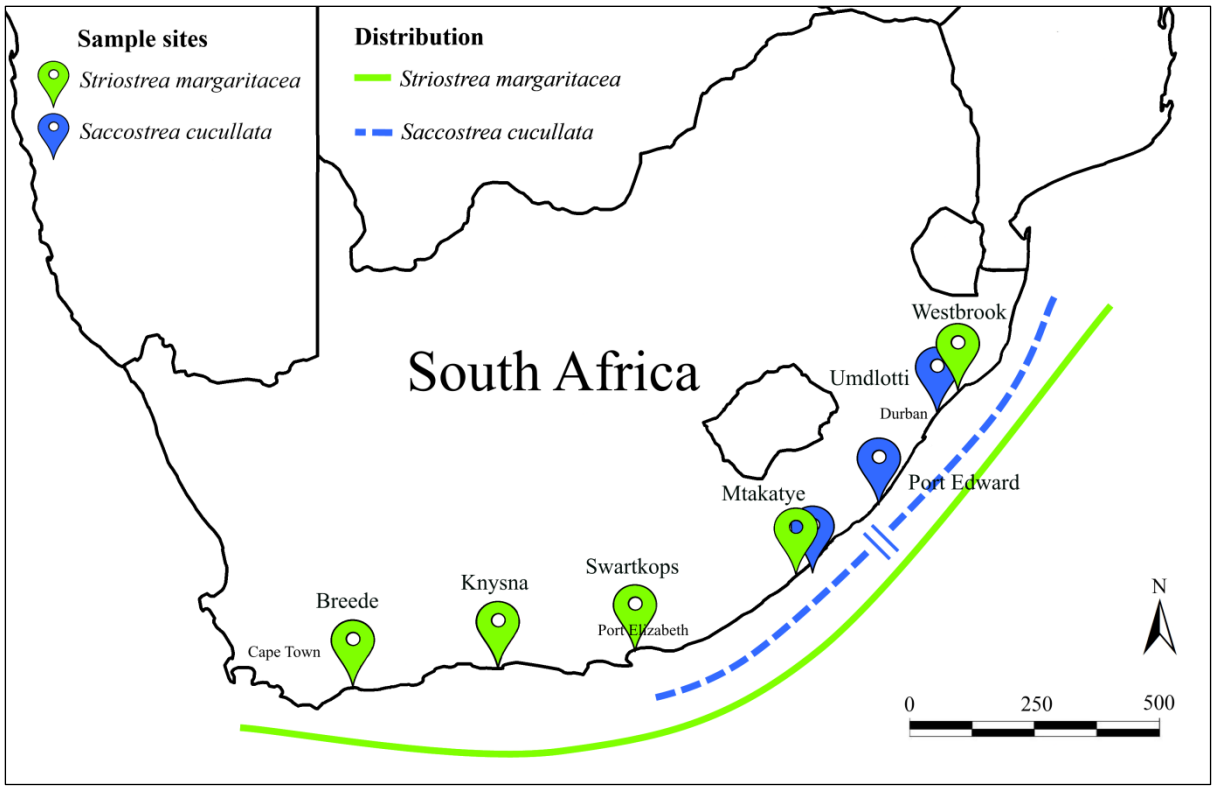

Figure 4.1 Genetic disjunction displayed in *S. cucullata* and not *S. margaritacea.* *Striostrea margaritacea* and *S. cucullata* distribution and sample ranges are shown along with the sampling sites. The biogeographic break is shown as it falls on the distribution range for *S. cucullata*.

4.2 Spat settlement

Settlement of *S. margaritacea* was not successful in this study, which may be due to several factors. Variation in settlement could have been caused by lack of available larvae in the water column due to low output by the breeding stock. There are several factors which contribute to low output, such as poor conditions prior to spawning which could cause fewer adults to spawn due to low excess energy or body fat. There may also have been raw sewage leaking[4] into the tributaries leading to the Kowie River during the settlement period, which would hamper the settlement of the larvae (Krebser, pers. comm, 2013-2014). Another factor that may have been a strong contributor to the poor settlement could be the extensive HAB which occurred during the settlement period. There are many environmental factors associated with HABs, either factors that cause the bloom to occur or that are present due to the bloom's occurrence, which would be detrimental to larval development, survival, and metamorphosis. Essentially, wild settlement is an unreliable method of spat collection for this species and the possibility of hatcheries should be investigated for indigenous oyster culture. Alternatively, larvae could be collected from the water column but this too would not be a reliable or commercially viable method.

4.3 Size class analysis

Rather than the HAB affecting number of recruits in the three chosen locations, pre-existing population structure seemed to be the driving force behind spat settlement. The pre-existing population structure was most likely a result of anthropogenic harvesting which also created available habitat upon which larvae could settle. Perhaps the low impact of the HAB in these locations was due to the fact that they were not affected for as long a period as the epicentre of the HAB in Port Elizabeth. At the central point of the HAB, there were reports of fish and bird mortalities (Bornman & Steyn, 2014). A study could be performed closer to the centre of the HAB radiating outwards to determine if recruitment at sites with high mortalities of other species were affected.

4.4 Oyster culture

This study is just the first step in determining whether indigenous oyster culture is likely to be commercially viable in South Africa. Growth trails need to be performed, more extensive spat settlement trials, and monitoring of larval presence are all important factors that need to be studied further. To determine whether spat settlement is naturally variable and can be

[4] A raw sewage leak was recorded from March of 2013 (Chadzingwa, et al., 2013) but I was unable to establish an end date to this.

predicted using models, annual recruitment would need to be recorded along with several environmental factors. Settlement at several different sites should be monitored to determine those most suited to recruitment. Translocation studies could be performed, however this should only take place after assessing nuclear genetic markers. Additionally, oysters may be harvested and moved to more appropriate facilities, where experimentation to breed them for hatcheries can occur after nuclear marker analysis. *Saccostrea* species grow to a smaller size than *Striostrea margaritacea* and the commercially cultured Pacific oyster (*Crassostrea gigas*) (Kilburn & Rippey, 1982). However, *S. cucullata* are harvested and sold in Australia and there are often markets for smaller oysters. To optimize an oyster mariculture operation, multiple species could be cultivated side-by-side to have larger and smaller oysters being cultured simultaneously, such as *S. margaritacea* and *S. cucullata* or *S. mordax*. Basing commercial culture on wild-spat settlement is not advisable due to unpredictable spatfall. Further study is required and creating an experimental hatchery would be recommended as the next step to investigate indigenous oyster culture.

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