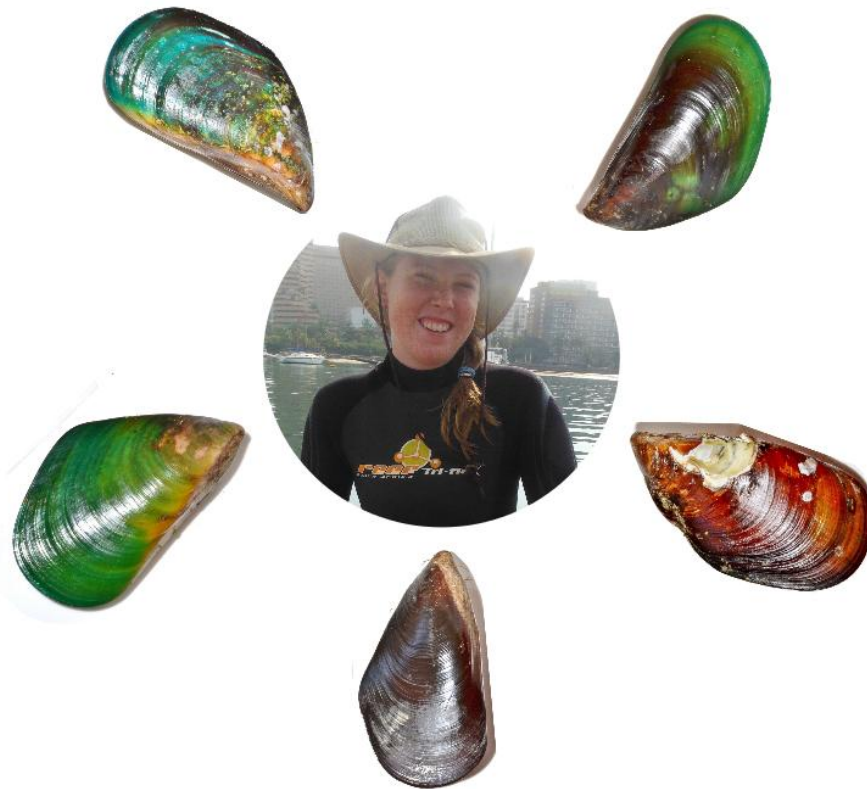




Invisible invasion: finding *Perna viridis* amongst South African green mussels

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Thesis presented in partial fulfillment of the BSc (Hons) degree in Marine
Biology, Department of Biological Sciences, University of Cape Town

Supervisor: Emeritus Professor Charles Griffiths

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Dedication

"In everything give thanks to the Lord for through him all things are possible"

To my parents who always believed in me and supported me throughout my life

Plagiarism Declaration

1. Plagiarism is to use another's work and to pretend that it is one's own. I know that plagiarism is wrong.
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Abstract

Non-indigenous marine organisms are a global cause for concern as they alter ecosystems, displace native species and cause damage to economic structures. *Perna viridis* is commonly called the Asian green mussel and is native to the Indo-Pacific region. By means of aquaculture, ship fouling and ballast water, *P. viridis* has successfully spread to eastern Asia, both North and South America in addition to many small Pacific Islands. Their ability to survive a large range of environmental conditions has resulted in successful establishment. Until recently, *P. viridis* was thought to be geographically isolated from other *Perna* species and consequently morphological differentiation was of least concern. Differentiation between species of *Perna* is difficult and therefore molecular methods have been developed for species identification. This research aimed to sample the harbours on the east coast and use a genetic approach to determine if *P. viridis* is in South Africa. In each of the six harbours, ten sites were chosen and all green mussels were collected. A phylogenetic analysis of the mitochondrial cytochrome c oxidase subunit 1 gene (mtDNA COI) using ten mussels from five of the harbours was performed using the Tamura-3-parameter model to create a Neighbour-Joining tree. A morphological analysis using genetically identified *P. perna* and *P. viridis* was performed to determine species level differences. Results showed only one *P. viridis* sample from Durban Harbour while the 30 *P. perna* samples were clearly defined into two clades. This grouping of *P. perna* did not show geographical trends as suggested in previous literature but this may be a result of a smaller sample size or between harbour transport. *P. perna* ranged in shell colour from brown to blue-green demonstrating the inaccuracy when determining species based on shell colour. The morphological analysis determined only two ways to distinguish between *P. perna* and *P. viridis*. Firstly, by examining the mantle papillae, which are enlarged in *P. perna*. Secondly, examining the pallial line, which is perfectly convex for *P. perna* whilst the adductor muscle scars in *P. viridis* extend beyond the pallial line. Further research is needed to allow accurate identification whilst in the field. There is a possibility of *P. viridis* spreading down the coast to Cape Agulhas and outcompeting the native species in that area and management should be aware of the *P. viridis* in South Africa, monitoring the area for any evidence of range expansion.

Keywords: *Perna viridis*, green mussel, bio-invasion, mtDNA COI

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1 Introduction

Non-indigenous marine organisms which become invasive are a global cause for concern as they alter ecosystems, displace native species and cause damage to economic structures (Masilamoni *et al.* 2002; Bax *et al.* 2003; Robinson *et al.* 2005; Sousa *et al.* 2009). One of the characteristics that make non-indigenous species invasive is their plasticity in new environments which aids their ability to thrive and spread. Phenotypic plasticity is the ability of an individual organism to adapt its physiology, behaviour and life-history patterns in a new environment (Smith 2009). A study of these adaptations will allow us to determine which species have the potential to become invasive, where they could spread to, what their ecological effects could be and how native communities might respond. One group of aquatic organisms that have shown their plasticity by invading new environments, dominating ecosystems and displacing native species, are bivalve molluscs (Johnson & Padilla 1996).

The Asian green mussel, *Perna viridis* (Linnaeus 1958), is a subtropical bivalve and has been identified as an invasive species due to its introduction to North and South Americas, Eastern Asia and Australia. *P. viridis* has a wide salinity range, a broad temperature tolerance and a long larval lifespan, which contribute to its ability to spread around the world (Vakily 1989; Segnini de Bravo *et al.* 1998). However, the quick identification of a *P. viridis* invasion and a good eradication program can control the spread and biodiversity impacts of this species (Hayes *et al.* 2005). The knowledge of the mussel's habitat and life history could highlight areas where future introductions could occur.

1.1 Habitat and life history

P. viridis evolved in a subtropical environment, adapted to tropical water temperatures, salinity fluctuations and a variety of substrates. The ideal temperatures are said to be 24 - 32°C and yet laboratory tests have found a 10% mortality below 10°C and above 37.5°C (Sivalingam 1977; Segnini de Bravo *et al.* 1998). The optimal salinity is 30 ppt but the salinity range of 0 - 64 ppt is even greater than that of temperature. This range was determined by the discoveries of mussels in a hypersaline lagoon in Venezuela, as well as surviving freshwater influxes in monsoon season in Asia (Sivalingam 1977; Segnini de Bravo *et al.* 1998). Another factor which varies a great deal is the substrate on which *P. viridis* settles. These mussels have been found to settle on hard rocky surfaces, artificial structures, conspecifics, red mangrove prop roots and even soft sediments (Agard *et al.* 1992; Buddo *et al.* 2003; Baker *et al.* 2012). One abiotic aspect which is often a limiting factor is depth. The abundance of mussels is highest in the first 2 m, although, some have been found to settle at depths of up to 12 m (Vakily 1989). This is due to light penetration which limits the

euphotic zone where the abundance of plankton is greatest. Whilst bivalves are usually non selective filter feeders, feeding on phytoplankton, zooplankton and detritus, *P. viridis* is unusual as it selectively picks food with a high organic content (Korringa 1976; Sivalingam 1977; Vakily 1989; Hawkins *et al.* 1998). Together, these abiotic factors influence the life history features of such a plastic species.

The growth of *P. viridis* is highly variable and is determined by a combination of factors, of which temperature is one of the most important. Temperatures below 17°C cause growth to stop, where as an increase in temperature has been linked to gonad development (Lee 1985; 1986). This indicates that mussels living in areas where the temperature drops below 17°C do not have constant growth and therefore shell length is a poor proxy for age. In areas where the temperature does not reach this threshold, growth over time decreases with age and follows a von Bertalanffy curve (Vakily 1989). The curve equation varies for different regions which results in separate areas having different maximum sizes. The largest Asian green mussels are found in the Philippines, where they attain a length of over 250 mm (Korringa 1976). Nevertheless, the maximum age is thought to be 3 y, with sexual maturity occurring 2 - 3 months after settlement and the mussels have grown to a length of 7 – 30 mm (Lee 1985; Rajagopal *et al.* 2006).

P. viridis is a gonochoristic broadcast spawner with highly variable spawning patterns and a long larval lifetime. The spawning season of *P. viridis* can be annual, biannual or sporadic throughout the year and it has been hypothesized that this variance is due to factors such as temperature, salinity, pH and food availability (Korringa 1976; Sivalingam 1977; Lee 1985; Lee 1988; Rajagopal *et al.* 2006). For the first 20 hours after fertilization, the embryos undergo a series of developments into different larval stages, after which they remain in the water column for 15 - 20 days (Siddall 1980; Vakily 1989). Settlement can occur at varying depths and is affected by water velocity (Rajagopal *et al.* 1998). Once settled, the mussels attach using byssus threads and still retain some locomotive abilities, able to move on the substrate to reach their optimum depth (Tan 1975). This long larval life span allowed them to travel large distances by means of currents and colonize many coastlines in the Indo-Pacific.

1.2 Native distribution

P. viridis, also known as the Asian green mussel, has a large native range in the Indo-Pacific region, extending from the Musandam Peninsula of Oman in the Persian Gulf, towards Papua New Guinea (Figure 1; Baker *et al.* 2007). Within this area, the mussels have naturally dispersed, yet the effects of many decades of anthropogenic introductions have not been accurately recorded. The origin of the Chinese populations is a source of debate, as Ye (1997) mentions a purposeful introduction for

aquaculture without stating if there was a prior natural population. Subsequently, wild populations established themselves in Hong Kong and Taiwan (Lee 1985; Han *et al.* 1997). As shipping increased and ballast water replaced dry ballast, *P. viridis* larvae have been able to travel larger distances, reaching other continents and using ocean currents to spread along the coastlines.



Figure 1: The global distribution of *Perna viridis*, showing the native region and the introduced (labeled) populations

1.3 Vectors of dispersal

The environment plays an important role in range expansion, both in naturally occurring regions and in introduced areas. This occurs by means of ocean currents carrying propagules along coastlines and past uninhabitable barriers. For example, in the Caribbean, *P. viridis* was introduced to Trinidad and propagules drifted along the coast and across the sea to Venezuela (Agard *et al.* 1992). Nevertheless, unlike anthropogenic introductions, range expansion is easily monitored and with oceanographic knowledge, can be predicted.

Other than natural dispersal, *P. viridis* has been transported by three human-caused vectors; aquaculture, ship fouling and ballast water (Minchin *et al.* 2009). Aquaculture was the main vector for dispersal of *P. viridis* throughout the Indo-Pacific islands, and is currently a source of income for many small scale farmers (Thangavelu *et al.* 2009). Ship fouling involves the attachment of adult organisms to the external surface of vessels and was the mode of invasion for *P. viridis* along the coastline of Venezuela (Rylander *et al.* 1996). However, since fouling causes a noticeable drag and leads to increased fuel consumption, international vessels maintain their hulls through the use of anti-fouling technology (Hewitt *et al.* 2009). Ballast water is water taken up to increase the weight of a less than ideally loaded ship, and includes all the organisms found in the water column. Most organisms die due to predation or lack of nutrients, however, the survivors are often released into or

just outside the destination harbour, depending on regulation. This practice is thought to have led to *P. viridis* appearing in Tampa Bay, Florida, as well as in Jamaica and probably South Africa (Benson *et al.* 2001; Buddo *et al.* 2003; Mead *et al.* 2011). However, the conditions in the destination harbour need to be ideal for settlement and survival to maturity before range expansion occurs. Therefore, climate change needs to be considered when predicting range expansion during larval transport as previously uninhabitable areas may become accommodating, leading to new invasions (Lonhart 2009).

1.4 Non-native distribution

P. viridis has become a major invasive species and a risk to biodiversity, causing increased management costs (Rajagopal *et al.* 2006). The most northerly occurrence of *P. viridis*, is an introduced population in southeast Japan (Baker *et al.* 2007). Larvae were introduced to the area by shipping prior to 1967 and populations have since survived in harbour thermal effluents (Umemori and Horikoshi 1991). Furthermore, in 1983, Philippine *P. viridis* were imported to Okinawa for aquaculture purposes (Hanyu and Sekiguchi cited in Baker *et al.* 2007). From 1972 - 1984, similar introductions from the Philippines, resulted in establishment of populations in New Caledonia, Fiji, Tonga, Tahiti, Western Samoa and the Cook Islands (Eldredge 1994). Although both the Fiji and Cook Islands populations failed, the Fiji population was re-established from New Caledonia (Baker *et al.* 2007). The first introduction outside the Indo-Pacific occurred before 1990 when *P. viridis* invaded Trinidad (Agard *et al.* 1992). The species has since spread to northeast Venezuela and Jamaica, where they are exploited for aquaculture (Agard *et al.* 1992; Ingrao *et al.* 2001; Buddo *et al.* 2003). Later, in 1999, individuals from the Chinese population were introduced to the Cape Verde Islands, but by 2004, had failed to establish a wild population (Baker *et al.* 2007). Additionally, *P. viridis* was identified in Tampa Bay, Florida, in 1999 (Benson *et al.* 2001). By 2010, established populations extended from Anclote Key, Florida, all the way around to St Mary's, Georgia (Baker *et al.* 2007; Spinuzzi *et al.* 2013). Lastly, in 2001, a *P. viridis* invasion was discovered in Cairns, Australia and was eliminated by an intense eradication program which lasted until 2005 (Hayes *et al.* 2005). Overlaps in the ranges of *P. viridis* and other species of the *Perna* genus have only been discovered in the last 30 years, before which they were thought to be geographically isolated.

1.5 Taxonomy of the *Perna* genus

The four mussels in the genus *Perna* (Philipsson 1788) have been synonymized and misidentified for many years. These are *P. perna* (brown mussel; Linnaeus 1758), *P. viridis* (Asian green mussel; Linnaeus 1758), *P. canaliculus* (New Zealand green mussel; Gmelin 1791) and *P. indica* (Indian brown mussel; Kuriakose and Nair 1976). In 1980, Siddall cleared up most of the confusion between the

first three species by clearly defining them as *Perna* and not *Mytilus* as well as summarizing synonyms, distribution and morphological differences. For *Perna viridis*, the most common synonyms used in literature are *Mytilus smaragdinus* and *M. viridis*. In the case of *Perna perna*, there is still literature which lists an eco-morph, *P. picta*, as separate species found in the Mediterranean and North Africa (Siddall 1980). However, a genetic analysis supports Siddall's theory that it is, in reality, *P. perna* (Thankakkon and Edward 2013). A surprising find was that *P. indica* is a separate species and not just a population of *P. perna*, isolated to the southern tip of India. This misidentification came about because the differences between both brown mussels and *P. viridis* are said to be the same (Vakily 1989; Thankakkon and Edward 2013). Consequently, the single report by Sadacharan (cited in Vakily, 1989) that *P. perna* is found in Sri Lanka has not been confirmed through any later studies and a genetic analysis may find that it is, in fact, *P. indica*.

Other than the Sri Lanka case, the distributions of *P. perna*, *P. viridis* and *P. canaliculus* were geographically segregated before 1990. Since the recent identification of *P. indica*, there is limited literature on the co-occurrence of *P. indica* and *P. viridis*, both of which are found in India. *P. canaliculus* is restricted to New Zealand and the only co-occurrence is an introduction and quick eradication of *P. perna* in 2007 (Hopkins *et al.* 2011). *P. perna* is found patchily distributed all around the African coastline as well as in eastern Brazil and northern Venezuela (Berry cited in Baker *et al.* 2007). Since the introduction of *P. viridis* to Venezuela, the co-occurrence of these two species has led to a need to differentiate between the two.

1.6 Morphology

The morphology of *Perna* species is highly variable regarding both shell colour and shell shape. The shell colour of *P. perna* in South Africa is said to be yellow-brown with only traces of green as well as occasionally having a chevron (zigzag) pattern (Branch *et al.* 2010). Siddall (1980) admits to large variations in the shell colour, but never describes whether this variability is due to one species looking similar to the others, or all species having a range of all colour morphs. He merely states "light colored zigzag markings are most common in young *P. canaliculus* specimens. *P. perna* adults are typically brown to red-maroon with irregular areas of light brown and green. Brilliant green and blue-green predominate *P. viridis* juveniles while adult shells are less brilliant and have a greater proportion of brown" (Siddall 1980; pp 864). The variability extends to the shell shape as Seed (1968) argued that neither shell shape nor shell thickness were of any taxonomic value to identify species in the Mytilidae family, which include the genus *Perna*.

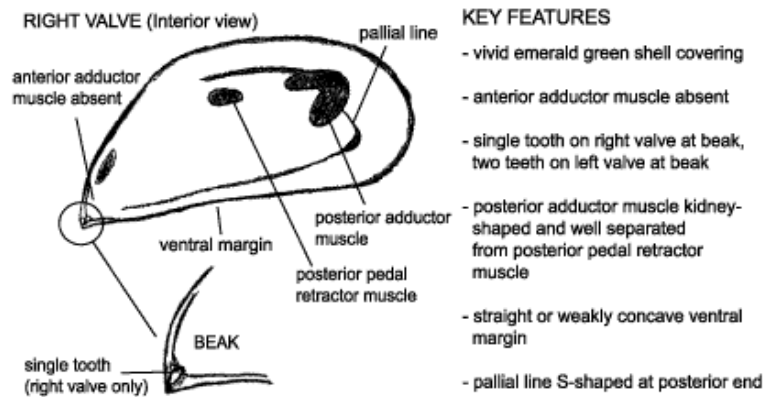


Figure 2: The morphology and key features of *Perna viridis*, sourced from Richard Willian, Museum and Art Gallery of the Northern Territory (NIMPIS 2014)

The distinctive features of *P. viridis* have been compared to that of *P. perna*, however, these comparisons are often only theoretical and based on ideal individuals (Figure 2). External characteristics used to distinguish *P. viridis* include a tapered, downward pointing beak, an arcuate middle dorsal region and a straight or weakly concave ventral margin (Velayudhan 2007; NIMPIS 2014). Internal features include a single tooth in the right valve and two in the left of its beak hinge (Velayudhan 2007; NIMPIS 2014). Another is a wavy or S-shaped posterior pallial line in *P. viridis*, whilst *P. perna* and *P. canaliculus* both have clearly convex lines (Siddall 1980; Velayudhan 2007; NIMPIS 2014). Looking at the soft tissue, Siddall (1980) mentions that *P. perna* have enlarged sensory papillae along the mantle margins, whereas those in *P. viridis* are reduced. He goes on to say that this is the only reliable method of species identification between the two. Since 1980, laboratory identification methods have become easy, reliable and more cost effective methods for species identification.

1.7 Molecular identification

The most accurate way to differentiate between the four species of *Perna* is through molecular studies. Interestingly, the chromosome numbers between the different *Perna* species differs. *P. viridis* and *P. canaliculus* have 15 pairs of chromosomes, *P. perna* has 14 pairs and that of *P. indica* is unknown (Ahmed 1974; Libertini *et al.* cited in Wood *et al.* 2007). Literature shows three different methods of species identification; electrophoretic analysis, cytological analysis and molecular markers (COI and ITS). The first two methods were used in studies by Agard *et al.* (1992) and Ingrao *et al.* (2001), respectively, but have been replaced by faster and more reliable genetic analyses. Mitochondrial cytochrome *c* oxidase subunit I gene (COI) has been shown to evolve slowly enough for DNA studies but fast enough to accurately determine differences between closely-related species (Hebert *et al.* 2003). Advantages of nuclear internal transcribed spaces (ITS) are that usable DNA can be amplified from very small samples and contains enough variation to determine species level

differences (Baldwin 1992). When doing COI or ITS analysis, a larger database of samples will increase the accuracy of results, which is why GenBank (NCBI), a free, global database, was developed.

1.8 South African mussel introductions

The South African shores have experienced major invasions of two mussels, *Mytilus galloprovincialis* and *Semimytilus algosus*. *Mytilus galloprovincialis* (Lamarck, 1819) were first reported in South Africa by Grant *et al.* (1984) and has since spread along 2000 km of coastline (Robinson *et al.* 2005). The established populations have changed the rocky shore community structure through their ecosystem engineering abilities and their ability to outcompete native molluscs (Branch and Steffani, 2004; Robinson *et al.*, 2007). The more recent invasion of *Semimytilus algosus* (Gould, 1850) along the South African northwest coast, was discovered in 2010 by Mead *et al.* (2011) and has since spread along 500 km of the west coast (de Greef *et al.* 2013). Even though both *Mytilus galloprovincialis* and *Semimytilus algosus* dominate the rocky shore, the two species are spatially segregated along the intertidal zone (de Greef *et al.* 2013). Furthermore, Mead *et al.* (2011) discovered *Perna viridis* in the East London Harbour in 2009 and classified them according to the morphology stated in Siddall (1980).

The aim of this research was to affirm whether the Asian green mussel, *P. viridis*, had invaded South Africa. This was done on a genetic basis rather than using shell colour alone. Samples were collected from harbours and adjacent shores rather than open coastline because ballast water from ships is the only viable method of introduction of this species in South Africa. The prediction is that there is an established population in East London Harbour where it was first found and that it may have spread out along the coast or to other harbours. This survey can function as a baseline study that will be used in subsequent research to determine how fast the invasion is spreading.

2 Materials and methods

2.1 Study sites

The 3000 km of South African coastline experiences three different biogeographic environments (Emanuel *et al.* 1992). Firstly, the cool temperate West Coast Province stretches from Namibia to Cape Point and is influenced by the Benguela Current, causing nutrient rich upwelling systems. Secondly, the warm temperate South Coast Province extends from False Bay to East London and experiences warmer water due to eddies from the Agulhas Current flowing offshore. Lastly, the subtropical East Coast Province ranges from East London up to Mozambique and the shortened continental shelf allows the Agulhas current close proximity to the shore.

Peters (2014) searched four harbours in the Southwest coast Province and two in the South Coast Province and found no *P. viridis*. This research extends Peters (2014) work by sampling another six harbours, four on the eastern edge of the South Coast Province and two in the East Coast Province (Figure 3).

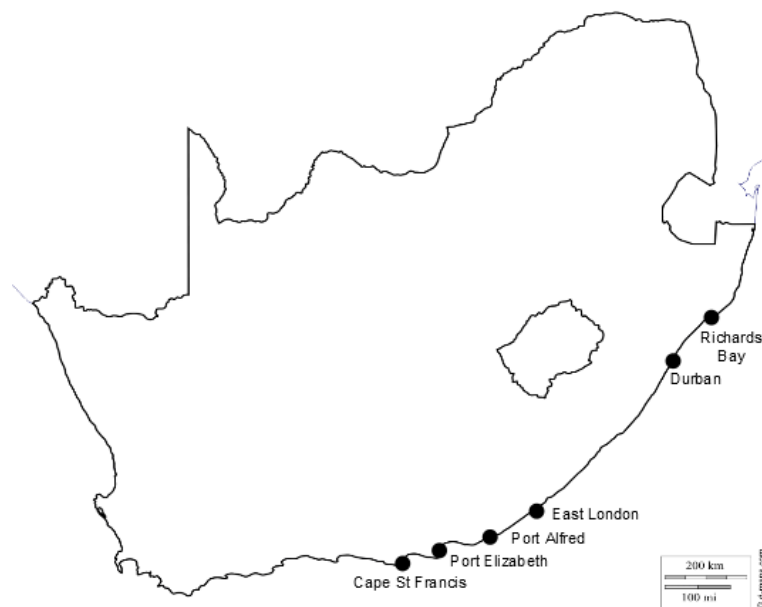


Figure 3: Locations of the six harbours sampled along the South African coastline.

The harbours were sampled from north to south in May and June 2014. Due to access restrictions and other technicalities, the entirety of each harbour was not sampled. However, sites within the harbours were spread out as much as possible to compensate for this.

All harbours were marine based, although East London and Port Alfred harbours are situated at the mouths of the Buffalo and Kowie Rivers, respectively. Richards Bay and Durban harbours are both neighboured by sandy beaches, but have groynes, piers and pipes along the coastline. The coastline outside the other four harbours is mostly rocky. The substrates sampled differed slightly between harbours due to accessibility and harbour design. All sites in Richards Bay, East London and Port Elizabeth were on hard, concrete surfaces, whereas some sites at Durban (10%), Cape St Francis (20%) and Port Alfred (100%) were on floating jetties.

2.2 Data collection

Within each of the six harbours, ten sites were chosen with a minimum of 10 m between sites. Approximately ten minutes were spent at each site, examining the substrate 5 m in both directions and between depths of 1 - 3 m depending on overhangs and visibility. Mussels were collected by divers using paint scrapers. The diving was done by qualified scientific commercial divers and overseen by a qualified dive supervisor from the UCT Research Dive Unit.

Since *P. perna* are commonly referred to as brown mussels and *P. viridis* as green mussels, all mussels with any degree of green were collected as *P. viridis* and any completely brown mussels were collected as *P. perna*. In each harbour, all green mussels found were collected as well as few brown mussels for reference. Specimens were preserved in 80% ethanol within 3 h after collection. The Durban samples were frozen for 24 h before alcohol immersion, due to a decreased availability of ethanol.

In harbours where green mussels were found, the shores outside the harbours were examined. The sampling method was similar to that used in de Greef (2013). The coastline was examined along 1 km stretches and samples of green mussels were collected. The coastlines extending away from the harbours were examined until either no mussels were found in suitable habitats for more than 2 km, or the tide prevented access to suitable mussel habitat (infratidal at low tide). For both Richards Bay and Durban, the western shore outside the harbour was inaccessible and only the eastern shore, which had groynes, piers or pipes extending into the water, were examined. Both the east and western shores outside the East London and Port Alfred harbours were examined. For Port Elizabeth, only the western shore was examined, as the eastern shore was inaccessible.

2.3 DNA extraction and amplification

From each of the harbours where green mussels were collected, a subsample of ten mussels, ranging in colour and shape, were chosen. These were labeled 1 - 10 with the abbreviations for each harbour as Richards Bay (RB), Durban (DB), East London (EL), Port Alfred (PA) and Port Elizabeth (PE). A photograph was taken of each cleaned and oiled mussel and included a label and scale bar.

The mussels were dissected and a 5-7mm section of muscle and mantle tissue from the left valve was removed, labeled and preserved in an eppendorf containing 95% ethanol. These samples were sent to the South African Institute for Aquatic Biodiversity (SAIAB) for DNA bar-coding.

The staff at SAIAB followed the methods as stated in Herbert *et al.* (2003). This included extracting DNA using the KAPA *Taq* ReadyMix protocol (KAPA Biosystems). First, a 658 base pair section of the COI (cytochrome *c* oxidase subunit I) gene was amplified using the LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') primers (Folmer *et al.* 1994). The PCR reaction was carried out in 25 ml volume which comprised of 2 ml of the DNA extract, 13.8 ml distilled water, 2.5 mM of KAPA buffer, 3 mM Mg Cl₂, 2.5 mM dNTP, 0.5 mM of each primer as well as 0.2 units of *Taq* polymerase. Each sample underwent a temperature regime of 63 cycles. These included an initial denaturation cycle (1 min at 94°C), 30 denaturing cycles (30 seconds at 94°C), 30 annealing cycles (1 min at 50°C), one extension cycle (1 min at 72°C) and a final extension cycle (10 min at 72°C) before being cooled to 16°C. The samples

were then sent to Macrogen, Korea where they were purified and sequenced. The gel purification was done using Qiaex II kit (Qiagen Inc.). An ABI 377 automated sequencer (Applied Biosystems, USA) and a Big Dye v. 3 sequencing kit were then used to sequence these products in one direction.

Sequences from the GenBank (www.ncbi.nlm.nih.gov) database were added as reference material. One sample for each species for *P. canaliculus*, *P. indica* and *Mytilus galloprovincialis* was included to identify other possibly misidentified species. For *Perna perna* and *P. viridis*, one reference sample from every available country was added. Unverified samples were not used and the only verified sample of *P. viridis* from Japan was too short and was excluded from the final analysis. In Table 1 a complete list of each sample and its origin can be seen.

Table 1: List of reference samples taken from GenBank to supplement genetic analysis

Species	Country	GenBank number (COI)	Reference	
<i>Mytilus galloprovincialis</i>	South Africa	DQ351478	Zardi <i>et al.</i> (2007)	
<i>Perna canaliculus</i>	New Zealand	DQ917607	Wood <i>et al.</i> (2007)	
<i>Perna indica</i>	India	EU543992	Divya <i>et al.</i> (2008)	
<i>Perna perna</i>	Angola	KC692001	Cunha <i>et al.</i> (2014)	
	Mauritania	DQ917597	Wood <i>et al.</i> (2007)	
	Morocco	DQ917603	Wood <i>et al.</i> (2007)	
	Mozambique	KC692009	Cunha <i>et al.</i> (2014)	
	Namibia	KC692005	Cunha <i>et al.</i> (2014)	
	New Zealand	HG005374	Pochon <i>et al.</i> (2013)	
	Oman	KC692013	Cunha <i>et al.</i> (2014)	
	South Africa	DQ917616	Wood <i>et al.</i> (2007)	
	Tunisia	KC961986	Cunha <i>et al.</i> (2014)	
	Venezuela	DQ917588	Wood <i>et al.</i> (2007)	
	<i>Perna viridis</i>	Australia	DQ343578	Blair <i>et al.</i> (2006)
		China	JN179059	Gilg <i>et al.</i> (2013)
		India	DQ917612	Wood <i>et al.</i> (2007)
Jamaica		GQ497838	Gilg <i>et al.</i> (2013)	
Philippines		DQ917599	Wood <i>et al.</i> (2007)	
Singapore		JN179066	Gilg <i>et al.</i> (2013)	
Thailand		DQ917590	Wood <i>et al.</i> (2007)	
Trinidad		GQ497835	Gilg <i>et al.</i> (2013)	
USA		JN179053	Gilg <i>et al.</i> (2013)	
Vietnam		DQ917584	Wood <i>et al.</i> (2007)	
Venezuela	DQ343590	Blair <i>et al.</i> (2006)		

2.4 Genetic analysis

The COI sequences were aligned using the default options for ClusterW in MEGA6 (Tamura *et al.* 2013). Each available model was run and the best model chosen by looking at the Aikaike (AIC) and Bayesian Information Criterion (BIC), for both of which a lower number is better. Using this model, a neighbour-joining phylogenetic tree was computed to show the evolutionary distances between

samples (Saitou and Nei 1987). This comparison allowed us to infer the species of each unknown sample by comparing it to reference material from known samples.

Using the phylogenetic tree, samples were grouped according to species level, however, if a strong inclination to a lower level grouping was seen, these were grouped separately as clades. The estimated evolutionary distances within and between the groups were computed using the same model used to create the phylogenetic tree.

2.5 Morphological analysis

The morphology of *P. viridis* and *P. perna* were examined according to previous literature. Any clear differences found were photographed. The mussels collected from each harbour were re-examined to determine if any *P. viridis* were missed in the subsamples sent for genetic analysis. This was done by looking for the previously identified differences found between genetically identified *P. viridis* and *P. perna* (Siddall 1980; Rajagopal *et al.* 2006).

3 Results

3.1 Data collection

Together, green and brown mussels were found in five of the six harbours samples. Approximately 50 - 100 were found in East London, 20 - 50 in Durban and Port Elizabeth, 10 - 20 at Richards Bay and no mussels in the St Francis yacht harbour. The green mussels were patchily distributed in between brown mussels and were outnumbered. Baker *et al.* (2007), found blankets of mussel colonies, but this was only seen outside of harbours and the green mussels were always patchily distributed within the brown mussels.

3.2 Genetic analysis

Species identification was possible for 32 of the 50 samples, as the DNA of the other 18 samples did not amplify. The best model to use was the Tamura-3-parameter model together with a gamma distribution (+G=0.26) as well as a homogenous pattern amongst lineages. This model had low BIC (3662) and AIC (2722) values along with 121 parameters. The Neighbour-Joining phylogenetic tree computed using this model can be seen in **Error! Reference source not found.**



Figure 4: The evolutionary relationship between samples

The Neighbour-Joining method was used to compute the phylogenetic tree (Saitou and Nei 1987). The tree shown above is the best possible fit and has a sum branch length of 1.38030717. The number shown at each branch is the percentages of the bootstrap (1000 replicates) tests that showed the branching event (Felsenstein 1985). The branch lengths are drawn to a scale whereby each unit of length represents a unit of evolutionary time and only those >50% are shown. These units are in units of the number of base substitutions per site and was computed using the Tamura-3-parameter method (Tamura 1992). The Tamura-3 parameter model was used, together with a gamma distribution (shape parameter = 0.4), to determine the rate of variation among sites. The number of nucleotide sequences used was 54 and all sets of incomplete data were excluded. The final number of positions analyzed in the final dataset was 361. This analysis was performed using the program, MEGA6 (Tamura *et al.* 2013).

The samples included one *Mytilus galloprovincialis* (PA02), one *Perna viridis* (DB08), and the rest were all identified as *P. perna*. The five species shown are clearly distinct from one another and only *P. perna* was defined into separate clades. Yet there are two outliers, *P. perna* from Oman and a brown mussel from Durban (DB02) which was identified as *P. perna* and part of clade A in other models. These two are spaced close to *P. indica*, but are not grouped together.

Within the *P. viridis* group, there is a strong inclination (72%) to separate the *P. viridis* from Durban, Philippines and the USA from the rest and yet the divergence within *P. viridis* globally is only 0.4% (**Error! Reference source not found.;** **Error! Reference source not found.**). The *P. perna* are separated into three clear clades. The first is labeled as *P. perna* clade A and includes mussels from Port Elizabeth to Mozambique. This large clade shows no further differentiation and has a low (0.5%) divergence within the group. The second clade, *P. perna picta*, includes *P. perna* from North Africa which were previously called *P. picta* and were strongly separated from the Brazilian mussel 76% of the times the model was run **Error! Reference source not found.**. This group only consists of four samples and contains the lowest within group divergence of 0.2%. The third group, *P. perna* clade B, has a higher divergence within it (1.2%) than all the other groups. Within the group, there is a strong partition between the Port Alfred, New Zealand and Namibian (all from cold water habitats) mussels from the Richards Bay, Venezuelan and Angolan mussels (all from warm water habitats; **Error! Reference source not found.**).

In Table 3, the difference between the *Mytilus* and *Perna* is clear. The largest difference between species of *Perna* is between *P. viridis* and *P. canaliculus*. The smallest difference between species is between *P. perna* and *P. indica*. However, the difference within the three clades of *P. perna* with *P. perna picta* and *P. perna* clade B is the least of all groups.

Table 2: The estimated average evolutionary divergence over sequence pairs within the groups.

The numbers shown are the number of base substitutions per site, computed through averaging all the sequence pairs within groups. Analysis was done using the Tamura-3-parameter model (Tamura 1992). The gamma distribution (shape parameter = 0.4) was used to model the rate of variation among sites. The analysis used 54 nucleotide sequences and included a total of 361 positions in the final dataset. Incomplete positions were excluded from the analysis. The evolutionary investigation was performed in MEGA6 (Tamura *et al.* 2013).

Group	d
<i>Mytilus galloprovincialis</i>	0.011
<i>Perna viridis</i>	0.004
<i>Perna perna picta</i>	0.002
<i>Perna perna</i> clade A	0.005
<i>Perna perna</i> clade B	0.012

Table 3: The estimated evolutionary divergence over sequence pairs between the groups.

The numbers shown are the number of base substitutions per site, computed through averaging all the sequence pairs between groups. Analysis was done using the Tamura-3-parameter model (Tamura 1992). The gamma distribution (shape parameter = 0.4) was used to model the rate of variation among sites. The analysis used 54 nucleotide sequences and included a total of 361 positions in the final dataset. Incomplete positions were excluded from the analysis. The evolutionary investigation was performed in MEGA6 (Tamura *et al.* 2013).

	1	2	3	4	5	6
1 <i>Mytilus galloprovincialis</i>						
2 <i>Perna viridis</i>	2.155					
3 <i>Perna indica</i>	2.026	0.365				
4 <i>Perna canaliculus</i>	1.699	0.509	0.318			
5 <i>Perna perna picta</i>	2.152	0.415	0.103	0.295		
6 <i>Perna perna</i> clade A	2.171	0.398	0.074	0.335	0.050	
7 <i>Perna perna</i> clade B	2.606	0.422	0.093	0.279	0.039	0.042

3.3 Morphological analysis

The morphological characteristics between the two species and within clades were examined and no differences in shell colour were found. *P. perna* ranged from black to brilliant green and blue. Even those with chevron patterns had variable colouring (Figure 5). The genetically identified *P. viridis* (f) sample was in fact less green than some of the *P. perna* (a-e). There was no difference in colour between the two *P. perna* clades, both including mussels with a range of colour from brown to green.

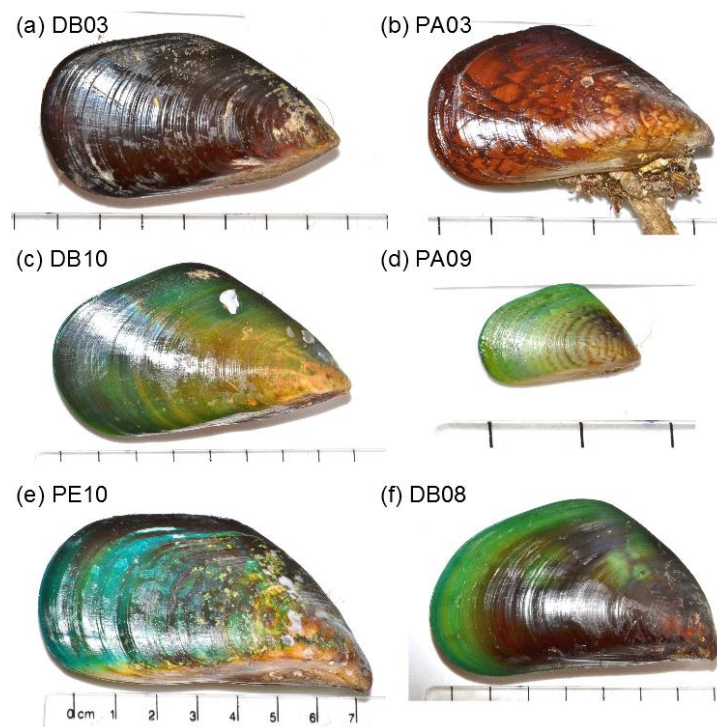


Figure 5: The range of colours found in *Perna perna* include (a) brown, (b) brown chevron, (c) green, (d) green chevron, (e) blue and (f) *Perna viridis*

The shell shapes were also highly variable. *P. perna* were found to occasionally have a distinct dorsal angle, as seen in Figure 6(c). The beaks were straight in almost all (~ 95%) the *P. perna*, whilst *P. viridis* had a downward pointing beak (Figure 6).

Of all the distinguishing features of *P. viridis*, only two clearly show a difference between *P. viridis* and *P. perna*. The first is the mantle papillae, which are enlarged in *P. perna* to the point of being clearly visible with the naked eye, whereas *P. viridis* has a smooth mantle with no extensions (Figure 7). Using this method of identification, two more *P. viridis* were identified, both from Durban harbour.

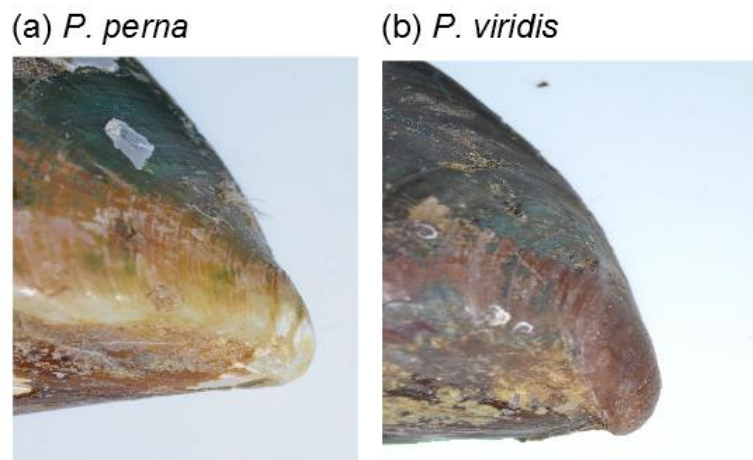


Figure 6: The anterior beaks which point straight for *Perna perna* (a) and downward for *Perna viridis* (b)

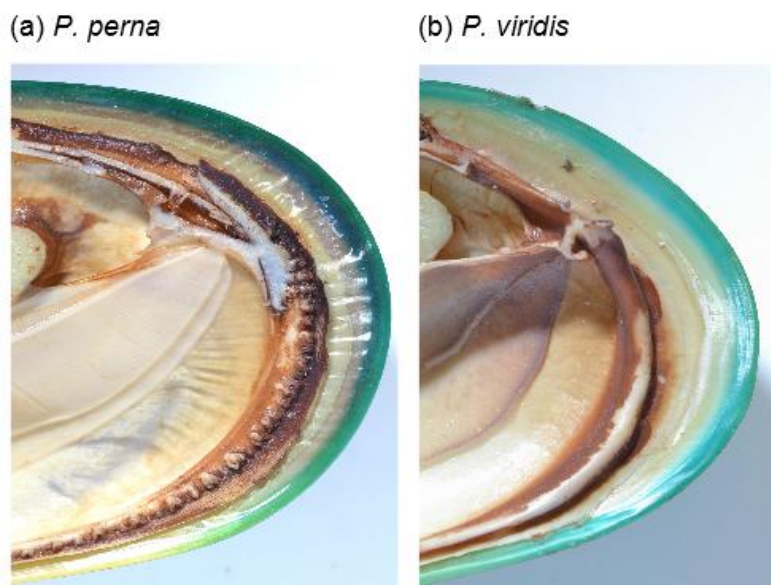
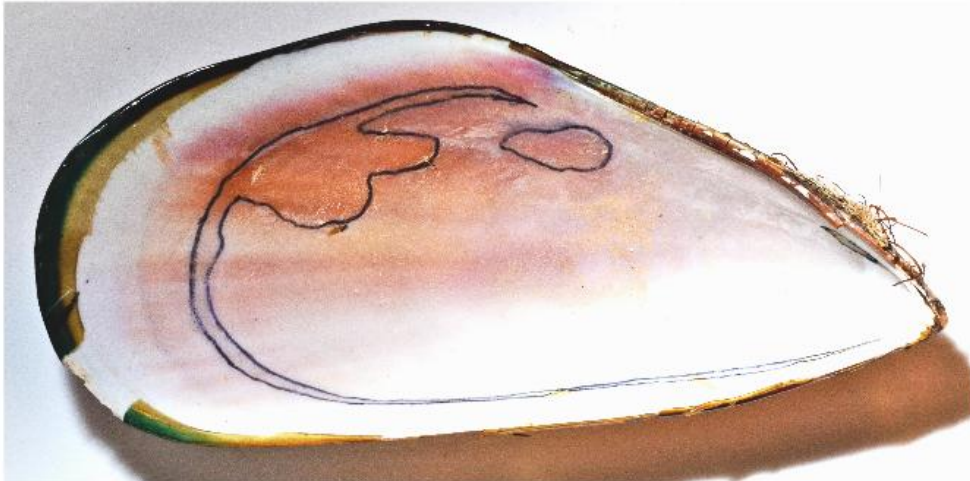


Figure 7: The posterior internal morphology of (a) *Perna perna* and (b) *Perna viridis* showing the presence of distinct mantle papillae in the former and absence in the latter.

The second proposed distinguishing feature is the scars left on the inside of the shell due to tissue attachment. In *P. perna*, the adductor muscle scar is always within the perfect convex shape of the pallial line (Figure 8). *P. viridis* is said to have a wavy or S-shaped posterior pallial line. This shape is not due to the variability in the mantle attachment to the shell, but rather to the posterior adductor scars extending beyond the pallial line. Looking at mussels whose tissue had been removed, another two *P. viridis* were identified using pallial line identification.

In total, five *P. viridis* were found, all of them from Durban Harbour and all approximately the same size (~ 80 mm). Green *P. perna* were found in many harbours in South Africa and the only way to tell them apart in the field is by looking at the mantle papillae or, if the tissue has been removed, the pallial line.

(a) *P. perna*



(a) *P. viridis*



Figure 8: The scars on the inside of the left valve which are convex for *Perna perna* (a) and wavy for *Perna viridis* (b).

4 Discussion

Of the six sites studied, *Perna viridis* was only found in Durban Harbour. However, green mussels were found in five of the sites, and genetic analysis identified them as *P. perna*. The distribution and abundance of *P. viridis* is not as substantial as previous research thought, however, there is a possibility of dispersal and outcompeting the native species.

4.1 Genetic analysis

The genetic analysis showed that one of the eight mussels found in Durban Harbour was *P. viridis* while all the other green mussels were *P. perna*. The low level of genetic variation in *P. viridis* between global samples prevented the determination of the origin of the invasive species. Wood *et al.* (2007) supports this finding and suggests this could be because *P. viridis* underwent a population bottleneck before expanding its range to other countries and continents.

P. picta was identified as a clade within the *P. perna* species as suggested by Vakily (1989). The Brazilian population might be a relatively recent invasion from the Mediterranean and possibly occurred during the early time of transoceanic shipping, when invasions were not recorded. Similarly, the Venezuelan *P. perna* population may have originated from South Africa as the reference sample was found among the South African *P. perna* clade B. This too, could be the result of centuries of intercontinental shipping, with mussels attaching to wooden ship hulls. However, further analysis would be needed to tell which population was the source and which was the introduction.

This research found the South African *P. perna* split into two clades. According to an in depth study on *P. perna* lineages in South Africa (Zardi *et al.* 2007), there are two distinct lineages made up of 50 unique haplotypes. The *P. perna* clade A appears to represent the eastern lineage, which should include samples from Kosi Bay to Kenton-on-Sea, just west of Port Alfred. However, Zardi *et al.* (2010) mentions a clear geographic barrier between the lineages with a 200 km overlap from Kenton-on-Sea to Haga Haga. Nonetheless, all five Port Elizabeth samples, which should be restricted to clade B as they are not in the area of lineage overlap, were found in clade A. Similarly, *P. perna* clade B includes a sample from Richards Bay which does not follow the geographic limits for the southern lineage reported in Zardi *et al.* (2007). The differences between the results of this paper and those of Zardi *et al.* (2007) could be because this paper was limited to sampling harbours and the coast adjacent to them as well as having a smaller sample size. The use of shipping as a vector for dispersion is still valid within a species range and therefore harbours might include individuals from different populations, which would skew any biogeographical analysis.

This study showed that *P. perna* were most closely related to *P. indica*. This may have led to the many morphological similarities and previous misidentifications (Kuriakose 1980; Vakily 1989). However, the close proximity of the two *P. perna* samples (one from Oman and one from Durban) to *P. indica* indicates the need to clear up the confusion between the two species which can be done using a large sample size and genetic studies.

4.2 Morphology

The large number of *P. perna* ranging from brown to blue-green highlights the variability in shell colour. The common names of *P. viridis* and *P. perna* are the green mussel and the brown mussel, respectively. These names are used quite often in both literature and scientific websites despite a few key papers having mentioned that the shell colour is highly variable (Siddall 1980; NIMPIS 2014). This variability is never defined and only two papers mention its extent. The first is Rajagopal *et al.* (2006) who stated that the shell colour of *P. viridis* can be found on a spectrum from blue-green to completely brown. The second is McDonald (2012), who noted that juvenile *P. viridis* found were not the bright green as stated in literature, but were a dull olive-green colour and displayed the chevron pattern which is also found on *P. perna*. In each of these cases, it was *P. viridis* that was variable and looked similar to the brown *P. perna*. However, this study found that in South Africa, *P. perna* was found to range from brown to blue-green which has never been clearly stated before. Consequently, there is a possibility that all previous discoveries of *P. viridis*, not in Durban Harbour and only identified through shell colour, were misidentified.

The findings of this research confirms the work of Siddall (1980) who stated that the only way to tell *P. perna* and *P. viridis* apart is by identifying the enlarged sensory papillae in *P. perna*. This study also found a clear difference in the pallial line scar, however, this was only done using three *P. viridis* samples and not confirmed with individuals from more than one location. Further research into morphological differences would aid in alleviating misidentification in the field.

4.3 Current *Perna viridis* population in South Africa

Each of the five mussels was greater than 80 mm length and probably part of the same cohort. This indicates that settlement occurred at least 5.5 months prior (using the fastest growth rate estimates from the Andaman Islands; Soundararajan *et al.* 1988). However, pollution has been shown to retard growth rate and subtropical, rather than tropical, water temperatures mean that any predicted age is a severe underestimate (Lee 1985). The *P. viridis* mussels were sexually mature but no younger individuals were found. This could be attributed to the entirety of the harbour not being sampled, or because successful reproduction did not occur.

4.4 Further invasion risks

Using these limits, it is predicted that *P. viridis* will be restricted to harbour invasions or, if they were to invade the open coast, they would only continue west to Cape Agulhas. Since the Agulhas Current moves southwards down the coast, it would carry propagules southwest, not northeast. However, if attached to vectors such as local vessels, a northeast invasion is possible, given the upper thermal tolerance levels are only found in the tropics (Segnini de Bravo *et al.* 1998). A concern for management should be the possibility of an introduction into the St Lucia estuary. This protected area is home to a large amount of biodiversity, only approximately 250 km up the coast from Durban and a *P. viridis* invasion could cause a great deal of damage. For example, the zebra mussel, *Dreissena polymorpha*, invaded the USA in 1988, spreading to 18 states in seven years and the cost of management by the year 2000 was US \$1.8-3.4 billion (Johnson and Padilla 1996; Ruiz *et al.* 1997).

Low temperatures will halt the southwest expansion as the warm Agulhas Current moves offshore as a result of the Agulhas Bank. Current physiological thermal limits include $>14^{\circ}\text{C}$ average winter temperature for survival, $>17^{\circ}\text{C}$ for growth and around 24°C for spawning (Lee 1985; Lee 1988; Urian *et al.* 2011). The average temperature at Cape Agulhas is around 17°C and the average winter temperature is around 15°C (Smit *et al.* 2013). This means that for approximately half the year, growth will be halted and winter die-offs will occur. However, in Japan, *P. viridis* populations survived in areas outside of their temperature range by settling in the vicinity of thermal pollution (Umemori and Horikoshi 1991). Nevertheless, it is unlikely that *P. viridis* will settle in areas beyond the current range of *P. perna*. Although *P. perna* exists outside of harbours, *P. viridis* may not be able to, instead, may use ship traffic to move from harbour to harbour as many other invasive species have. Griffiths *et al.* (2009) noted that most invasive marine species in South Africa are restricted to harbours, lagoons and estuaries. This is due to most of the South African coastline experiencing high wave energy and Vakily (1989) states that *P. viridis* prefers sheltered water. Regular studies into the breadth and depth of any future spread is recommended.

4.5 Impacts

Currently, the impacts of *P. viridis* in Durban Harbour would be minor on account of a variety of reasons. Firstly, large mussel beds of either *P. viridis* or *P. perna* were found only on navigational buoys and not covering large surfaces. Secondly, any impacts specific to bivalves would already be incorporated into the ecosystem as the harbour is colonized by the native mussel, *P. perna*, as well as by oysters. These impacts include fouling structures, affecting the phytoplankton assemblage and the nutrient cycle (review in Rajagopal *et al.* 2006). Lastly, the *P. viridis* population would not be colonizing a habitat devoid of natural predators. One of the major predators, the mud crab *Scylla*

serrata, is found along the east coast (Vakily 1989; Branch *et al.* 2010). Other predators include fish such as snappers, bream and blacktail, which are also present (Vakily 1989). *Thalamita* crabs and *Thais* gastropods have been observed to induce predator avoidance behaviour in *P. viridis* and species within these genera are common along the South African east coast (Cheung *et al.* 2004a; 2004b). Together, these factors will result in a unnoticeable impact in Durban Harbour.

The impact of *P. viridis* on the native species is a priority for concern. Fishermen from Venezuela have noted a decrease in *P. perna* numbers after the invasion of *P. viridis* (Rylander *et al.* 1996). Penchaszadeh and Velez suggested that *P. viridis* would outcompete *P. perna* due to the green mussel having a higher level of adaptability due to multiple life history traits (cited in Segnini de Bravo *et al.* 1998). These characteristics include a higher temperature tolerance, a wider salinity tolerance and an ability to survive longer periods of air exposure than *P. perna* (Segnini de Bravo *et al.* 1998; Rajagopal *et al.* 2006). Both species have a predator avoidance behaviour whereby they secrete additional byssus threads in response to predators, however, more so in the case of *P. viridis* (Rajagopal *et al.* 2006). The Asian green mussel grows faster, bigger and can filter twice as much water as *P. perna*, as well as having a higher feeding rate and food ingestion rate than any other filter feeding bivalve (Hawkins *et al.* 1998; Rajagopal *et al.* 2006). Looking at these life history traits, it seems inevitable that *P. viridis* would outcompete *P. perna*. However, Rylander *et al.* (1996) found a case of red tide which killed only *P. viridis*, while *P. perna* survived, indicating a more complex outcome for the competition. Even though all the life history traits make a strong case for *P. viridis* outcompeting *P. perna*, only the reproductive output determines whether one species will outcompete another and therefore more research needs to be done, comparing the reproductive output and settling behaviour between the two (Barber *et al.* 2005). Nevertheless, any research on the competition between these two species needs to be done carefully, as colour cannot be used to identify the two species.

5 Conclusion

This study found five *Perna viridis* in Durban Harbour, South Africa. They possibly invaded the harbour as larvae transported in ballast water and although they have reached sexual maturity, no younger cohorts were found. The morphology of *P. perna*, the native species, was found to be highly variable and shell ranged from brown to blue-green. Therefore, previous reports of *P. viridis* in South Africa could have been misidentifications and were, in fact, green *P. perna*. Another observation is that there is more genetic variation within the native species than within the global *P. viridis* population. However, the geographical separation of the two South African *P. perna* clades is not as clear as described by Zardi *et al.* (2007). If *P. viridis* spreads along the South African coast, there is a strong case that it may displace the native species. Nonetheless, the competition between *P. viridis* and *P. perna* is not fully understood. Future research is needed to determine whether the two South African *P. perna* lineages are truly geographically separated as well as determining the possibility of the *P. viridis* distribution increasing and impacting the biodiversity.

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