Encapsulating yachts to manage the transfer of marine alien species

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Abstract

In marine systems, the introduction and spread of alien species occurs predominantly through shipping, with hull fouling a dominant vector. Vessel fouling is primarily managed through the application of antifouling paints. However, the most effective of these paints, those containing TBT, were banned in the early 2000s and no equally effective alternative has become commercially available. This study looked at the potential use of encapsulation, the wrapping of a structure in plastic to deprive fouling organisms of oxygen and food to ultimately cause their death, as a tool for managing hull fouling, with the aim of reducing the biosecurity risk posed by fouling on recreational yachts. The aims of this study were to: (1) assess encapsulation under laboratory conditions to determine a timeframe for encapsulation of yachts in the field, (2) test this timeframe in the field, and (3) provide guidelines for implementation of a national encapsulation programme. In the laboratory, ascidians, mussels and fouling communities were exposed to four treatments: an aerated control in seawater, encapsulation in seawater, aerated seawater with a 4% acetic acid solution and encapsulation in seawater with a 4% acetic acid solution. All organisms and communities in acetic acid died in 24 hours regardless of encapsulation, while in encapsulated seawater, mortality of all taxa occurred within three days. Due to the implications of disposing of acetic acid in the field, this treatment was not considered in the field experiments. An encapsulation berth was constructed and four yachts were encapsulated in the field before a storm destroyed the berth. Walkway pontoons were then encapsulated as proxies for yachts, providing an opportunity to consider the effect of high (80-100%) and low (30-50%) fouling cover on encapsulation. On average, yachts required 4.25 (± 0.5 SD) days for fouling biota to reach total mortality, while pontoons with high and low fouling cover required 3.7 (± 0.48 SD) days and 3.8 (± 0.42 SD) days respectively. Field tests showed that the three days suggested by laboratory experiments was not sufficient in the field. This likely reflects an unavoidable higher ratio of water to fouling biomass in encapsulation systems in the field. A national encapsulation program could be useful for addressing the biosecurity risk posed by foreign yachts entering South African waters. It is recommended that vessels be treated for five days at their port of entry. This could be aligned with customs processes that are already in place. Importantly, mortality of fouling biota should be confirmed before removal of the encapsulation system. It is concluded that the application of an evidence-

based management approach will support continual improvement of this emerging technique, and under these circumstances, encapsulation has the potential to considerably reduce the biosecurity risk posed by yachts visiting South African harbours.

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Table of Contents

Chapter 1: General introduction

Alien species are those which are introduced to a region beyond their native range as a result of human mediated movement. They are considered to be invasive once they successfully establish and spread beyond their initial point of introduction (Robinson et al. 2016). The introduction of species beyond their native ranges is occurring at an increasing rate (Kumschick et al. 2016) as a result of both direct and indirect human actions (Jeschke et al. 2014).

While many non-native species were initially seen simply as welcome additions to the local biota, people have increasingly started to recognise many non-native species as unwanted pests (Pysek & Richardson 2008). These non-native species can cause detrimental impacts in their new environment, such as changes to the surrounding ecosystems and native communities, economies and social systems (Kumschick & Nentwig 2010, Pysek et al. 2012, Kumschick et al. 2017).

From a biological point of view, invasive species have been implicated in the endangerment of native species as well as the degradation of habitats in both marine and terrestrial environments (Reaser et al. 2007). Invasive alien species can dominate native fauna and flora (Bax et al. 2003), reducing species richness and abundance of native biota and decreasing local species diversity (Pysek et al. 2012). Globally, invasive species have had negative impacts on native species and their ecosystem functioning (Reaser et al. 2007, Vila et al. 2009, Vila et al. 2011). They may, for example, hybridise with a native species, causing, in extreme cases, extinction of the native species (Reaser et al. 2007). In the marine environment, South Africa has suffered a number of invasions that have caused the displacement or change in abundance of native species (Mead et al. 2011). A great deal of the scientific literature considering marine invasions in South Africa has focused on bivalves, with most attention being placed on the established invasive mussel species *Mytilus galloprovincialis* (Alexander et al. 2016).

Ecological changes caused by invasive species are likely to impact ecosystem services and in turn the economy and human well-being (Vila et al. 2009). Economic effects from invasive species are clear in many parts of the world (Pysek & Richardson 2008). Generally, more species are known to have economic effects than ecological ones, simply because economic effects are much more apparent and are usually noticed and reported very soon after effects become obvious (Vila et al. 2009). Economic pests with direct impacts on humans are also more likely to attract attention in the scientific literature (Pysek et al. 2008). In a marine

context, the main economic effects caused by invasive species are negative effects on human health and the functioning of industries such as fishing, aquaculture and marine tourism (Bax et al. 2003). Decreases in productivity of these industries in turn impact the economy through a decrease in job availability (Bax et al. 2003). Marine invasive species can have negative social impacts such as decreases in human welfare, resulting from effects of invasive species on the surrounding environment (Bax et al. 2003). In South Africa, as of 2011 there were approximately 86 introduced species and 39 cryptogenic species (Mead et al. 2011). However, none have had detrimental economic effects; in fact the invasion of the South African coastline by mussel *Mytilus galloprovincialis* has had a positive economic impact as it forms the basis of the mussel culture industry in the region and has provided a habitat and food source for several species (Robinson et al. 2005).

The human-aided spread of species in a marine context occurs predominantly through a number of pathways and vectors (Ruiz & Carlton 2003) including shipping, aquaculture and the aquarium trade (Bax et al. 2003, Haupt et al. 2012), live seafood, and canals (Godwin 2003, Molnar et al. 2008). Shipping-related vectors have always been the dominant pathways (Minchin & Gollasch 2003, Coutts & Taylor 2004, Roche et al. 2015), as shipping accounts for approximately 80% of the world's trade (Bax et al. 2003). Traditionally, fouling by wood-boring species occurred on the hulls of wooden vessels (Bax et al. 2003, Griffiths et al. 2009), while dry ballast such as rocks and sand used in historical wooden ships often contained a suite of coastal and intertidal organisms (Griffiths et al. 2009). Organisms transported in solid ballast and on the hulls of wooden ships not only caused damage to the vessel itself, but also easily established on wooden docks and pilings in harbours where the vessel docked (Griffiths et al. 2009).

More recently, dry ballast has been replaced with ballast water, a major vector which led to a surge of invasions after first being used, due to its favouring planktonic organisms (Griffiths et al. 2009). Ballast water is typically loaded into vessels in shallow harbour environments, meaning significant amounts of sediment may be loaded into the vessels accidentally, taking with it a number of species (Hewitt et al. 2009). As a result of its importance as a vector, ballast water has received a significant amount of attention in the scientific literature (Coles et al. 1999; Awad et al. 2003; Hewitt et al. 2009). These increasing literature records and evolving regulations have led to the International Maritime Organisation (IMO)'s convention that has been in force since 8 September 2017, requiring most ships to have an on-board ballast water treatment system.

Hull fouling is a dominant shipping-related vector (Peters et al. 2014, Chan et al. 2015). The movement of vessels with organisms attached to them is problematic due to the risk of release of viable organisms outside of their native ranges during the vessel's journey (Floerl 2003). Drake and Lodge (2007) report biofouling as a greater risk for species introduction than ballast water based on the number of potentially introduced species, and Chan et al. (2015) found that the total abundance and richness of non-native species associated with hull fouling was higher than those associated with ballast water. Unlike with ballast water, a universally successful method has yet to be adopted for combating hull fouling (Floerl 2003). Anti-fouling paints containing tributyltin (TBT) were developed and available for use from the 1960s (Smith et al. 2008), and are seen as one of the most successful fouling-reducing methods to have been used (Minchin & Gollasch 2003, Floerl 2003). However, due to TBT having deleterious physical effects on non-target organisms (Bauer et al. 1995, Oehlmann et al. 1996, Floerl 2003, van Gessellen et al. 2018), coupled with the release of biocides into the surrounding environment, the use of TBT-containing paints was globally banned in early 2003 (IMO 1999). This ban, along with the lack of regulations for the management of hull fouling, resulted in a resurgence of hull fouling (Clarke Murray et al. 2011), which resulted in hull fouling being recognized as a significant and unmanaged risk to marine biodiversity (Godwin 2005).

It is not currently known whether large commercial vessels or smaller recreational vessels pose the greater risk of spreading biofouling organisms (Coutts & Taylor 2004). Large commercial vessels have received most of the attention in the literature, and most of the current mechanisms designed to decrease the introduction and transport of non-indigenous marine species have been focused on these large commercial boats (Clarke Murray et al. 2011). As a result, smaller recreational vessels such as yachts have been largely overlooked as a vector for invasions in several places across the world (Floerl & Inglis 2003b), but could be the biggest unregulated vector for the introduction and spread of non-indigenous marine species (Clarke Murray et al. 2011).

Small recreational vessels often travel long distances at far slower speeds than larger commercial vessels. Additionally, the majority of recreational vessels are docked in marinas or harbours when they are not being used (Floerl & Inglis 2003a). As a result of the extensive exposure to marine waters when docked, these boats are very likely to accumulate fouling organisms (Floerl & Inglis 2003a) while moored. Once alien species have been introduced, several mechanisms may contribute to the spread of these species intra-regionally, one of which is the movement of recreational vessels, such as yachts, in and around the surrounding regions (Floerl & Inglis 2005, Peters et al. 2017b). Harbours where

many recreational vessels are docked often support a large extent of artificial structures which are favourable for colonization by fouling species and can potentially retain propagules as a result of the low flushing rates present in marinas (Floerl & Inglis 2003b, Roche et al. 2015). Moreover, harbours may assist in creating novel marine environments (Bax et al. 2003), through providing permanent, sheltered and shallow subtidal habitats (Arenas et al. 2006). Harbours also frequently incur disturbances like boat traffic and vessel maintenance (Bulleri & Chapman 2010). All these factors make harbours important entry points for non-indigenous marine species (Ros et al. 2013), as species introduced in sheltered areas may quickly establish self-sustaining populations. The combination of being stationary in sheltered harbours for long periods at a time and the slow movement of recreational vessels makes yachts ideal vectors for the accumulation of biofouling and the potential to spread marine alien species around the world (Coutts & Taylor 2004, Floerl et al. 2005, Clarke Murray et al. 2011, Ros et al. 2013). Yachts and other recreational vessels are also more likely to travel intra-regionally (Peters et al. 2017a), between ports in a country, and thus pose a risk for the spread of species within a region (Clarke Murray et al. 2011). In fact, Peters et al. (2017a) found that the number of yachts in a harbour was the main predictor of the number of alien species found in fouling communities in harbours, suggesting a strong link between yachts and the introduction of marine alien species.

Small, recreational boats are debatably the largest unregulated vector involved in introductions and the spread of alien species (Clarke-Murray et al. 2011). Although statistically Clarke et al. (2017) found yachts to have a lower biosecurity risk than other vessel classes such as commercial vessels, yachts have a higher potential for entrance into novel and unmonitored environments and regulations are lacking, posing the risk for introductions of non-native species into these novel environments (Clarke et al. 2017). One of the biggest fouling-related problems with these vessels is the lack of profit-related incentives for the removal of organisms from hulls or the renewal of anti-fouling paint compared to larger commercial vessels (Floerl & Inglis 2003b). This lack of incentive results in more variable renewal and removal intervals (Floerl & Inglis 2003b), which leaves gaps for new non-native species to attach to the hulls. Considering the lack of regulations for antifouling renewal on yachts and the potential that yachts have to enter novel environments, smaller private vessels such as yachts therefore pose two major risks: the introduction of new non-native species into an area and the spread of existing established marine alien invasive species (Floerl & Inglis 2003b).

Alien species associated with spread via fouling

A notable amount of physical force is exerted on fouling organisms as the vessels travels from one port to the next (Floerl 2003). It is common for organisms to become dislodged and fall off during the vessel's journey, which aids the spread of marine alien invasive species (Floerl 2003). Organisms which are most likely to survive the journey therefore need to withstand this physical pressure and are as a result are usually encrusting or rigid and attach firmly to the hull (Floerl 2003). The most favoured biofouling organisms would be those that are also able to withstand anti-fouling biocides (Floerl 2003). There are a number of invasive alien species which are thought to have been introduced via yachts (Floerl & Inglis 2003b).

The Japanese kelp *Undaria pinnatifida* is thought to have arrived in New Zealand in 1987 as a result of fouling on fishing vessels and has since spread extensively along the coast of New Zealand (Floerl & Inglis 2003b). While some natural spread has occurred, the long distances this species has spread along New Zealand's coastline is suspected to be primarily due to hull fouling by smaller domestic vessels such as fishing boats and recreational yachts (Floerl & Inglis 2003b). This species has been introduced to various locale across the globe (Silva et al. 2002, Valentine & Johnson 2003, Casas et al. 2004), and is specifically abundant in boating marinas (Floerl & Inglis 2003b).

Both the black striped mussel, *Mytilopsis sallei*, and the Asian green mussel, *Perna viridis*, are suspected to have been introduced and spread via the hulls of recreational vessels (Floerl & Inglis 2003b). *Mytilopsis sallei* has been successfully eradicated in Australia, but at a massive cost. Such management actions were, however, necessary, as it has caused significant damage to submerged manmade structures in invaded environments in India (Floerl & Inglis 2003b). *Perna viridis* has also invaded Australia and is being closely monitored, as it can have similar detrimental effects to *M. sallei* once established. Notably, this species has also been recorded in South Africa (Micklem et al. 2016), but it is unclear whether or not it has established viable populations.

The serpulid tubeworm, *Ficopomatis enigmaticus*, has spread across the world (Read & Gordon 1991, Schwindt et al. 2001, Schwindt et al. 2004, McQuaid & Griffiths 2014). Its presence among pontoons and recreational vessels in harbours indicates that the introduction and spread of this species is probably a result of fouling on recreational vessels (Floerl & Inglis 2003b). This species has become a pest in New Zealand where it fouls water pipes in power and flood protection stations (Read & Gordon 1991, Floerl & Inglis 2003b). In

recent years, there has been a rapid increase of *F. enigmaticus* in South Africa, where it has invaded the Zandvlei estuary in Cape Town (McQuaid & Griffiths 2014).

Small recreational boats have also been implicated in the introduction of macroalgal species such as *Ulva flexuosa* (Mineur et al. 2007), algal species such as *Caulerpa taxifolia*, and the broccoli weed, *Codium fragile* spp. (Floerl & Inglis 2003b), as well as the bryozoans *Watersipora subtorquata* and *Bugula neritina* (Floerl & Inglis 2005). These species may also aid the fouling and survival of other organisms by offering them primary settlement substrate with no direct contact with the hull (Clarke Murray et al. 2011).

It is notable that not only sessile organisms are associated with vessel fouling. Mobile species, such as amphipod *Caprella mutica*, have also been discovered in hull fouling communities, and may be found on the hulls of small vessels where macro-fouling species provide shelter (Frey et al. 2009). This amphipod species has invaded numerous regions, and is especially successful in artificial habitats in North America and Europe (Ashton et al. 2007, Cook et al. 2007) and has recently been found to be abundant on the hulls of yachts in South Africa (Peters et al. 2017b).

Management of hull fouling as a vector

Acknowledging the impact of alien fouling species (Bax et al. 2003, Molnar et al. 2008, Fitridge et al. 2012) and the ease with which they are transported (Gollasch 2002, Coutts et al. 2010, Clarke Murray et al. 2011, Sylvester et al. 2011), there is an obvious need to manage this vector and reduce its effects where possible. The main two established ways of managing fouled hulls are through the use of anti-fouling paint and the manual removal of the biofouling (Floerl 2003).

Antifouling Paint

Antifouling paints are used on a variety of underwater structures to provide protection from the attachment of fouling organisms (Katranitsas et al. 2003). Copper has been used as a biocide on ships for centuries (Yebra et al. 2004), but antifouling paints containing copper have been used since the 1860s, when the first was developed using copper oxide (Yebra et al. 2004). These paints were replaced with TBT-containing paints after the Second World War, when new developments occurred with regards to antifouling techniques (Yebra et al. 2004). After the development of antifouling coatings containing TBT, few studies on hull fouling took place (Minchin & Gollasch 2003), leading to a lack of alternative techniques once TBT was banned. As a result, copper-based antifouling paints regained favour

following the ban of TBT-based paints (Katranitsas et al. 2003), and copper has become the main biocide used in antifouling paints (Chen et al. 2013).

However, recently, concerns have been raised regarding the effects of copper antifouling paints on the marine environment (Srinivasan & Swain 2007). These concerns are founded on the detection of high copper levels in areas of heavy boat traffic (Srinivasan & Swain 2007) and the fact that high levels of copper in a biologically available form can be toxic to aquatic organisms (Katranitsas et al. 2003). Antifouling paints containing copper can cause contamination of aquatic systems in many ways (Srinivasan & Swain 2007). The most common way is through the continual leaching of biocides into the water. The rate at which biocides are released depends on paint formulation, age and condition of the paint, as well as the operation of the vessel (Srinivasan & Swain 2007). Hull maintenance can also provide another source of copper contamination through underwater hull cleaning, high-pressure washing of boats, abrasive blasting, hull repair, painting, overspray, and paint spills (Srinivasan & Swain 2007).

Organic booster biocides are compounds which may be added to antifouling paints in order to improve their effectiveness (Thomas 2001). Worldwide, there are approximately 18 booster biocides which are in use (Thomas 2001), of which 9 are approved for use by the Health and Safety Executive (Konstantinou & Albanis 2003). Although these booster biocides are in use and data is available for those commonly used in areas such as Japan, North America and Europe (Konstantinou & Albanis 2003) recent studies and data on others, as well as the degradation of these biocides are lacking (Thomas 2001, Konstantinou & Albanis 2003).

Self-polishing copolymer paints were first introduced in the 1970s (Callow & Callow 2002). These paints work by dissolving the polymer away over time as seawater smoothes over the hull's surface, continually allowing a fresh paint surface to be revealed (Callow & Callow 2002, Loschau & Kratke 2005). Self-polishing paints consist of linear polymers which have a side group attached to them, and this side group is released from the linear polymer during interaction with seawater, as are the fouling organisms attached to the top layer of paint (Loschau & Kratke 2005). However, self-polishing paints can also contain biocides or heavy metals which leach off with the paint layers, linking their effectiveness to their toxicity to fouling organisms, which also poses a risk to non-target organisms (Loschau & Kratke 2005). They are also only effective on fast-moving vessels, as the release of the paint layers depends the speed of the vessel as well as environmental factors such as water temperature and pH level (Kiil et al. 2002).

Developed as a substitute for antifouling paints containing biocides, silicone fouling-release coatings work through limiting the attachment strength of fouling organisms (Callow & Callow 2002). When first developed, these coatings were costly and prone to tearing and were therefore only applied in specific cases, such as in areas where biocides are banned (Callow & Callow 2002). These days, however, fouling-release coatings are well established and widely used (Clare pers. comm.)

Besides their negative impacts on the surrounding marine environment, one of the biggest problems that arises with the use of a paint to prevent hull fouling is the need for reapplication. In order to inhibit organisms from settling on the hull surface, a critical level of biocide has to be present in the boundary layer surrounding the hull. The leaching rate of biocide toxins lessens with time, creating the need for hulls to be repainted regularly (Floerl 2003). This upkeep of the vessels' paint is vital, as along with the decrease in the leaching rate of toxins from paint, the paint itself tends to wear off along weld seams or may be applied insufficiently in some instances, making the hull surface susceptible to attachment of new organisms when the paint is not touched up frequently (Godwin 2003). Recreational vessels such as yachts that are left moored in marinas for extended periods of time, are especially susceptible, as microbial slimes and hydrolysed paint material may gather on the surface of the paint, lowering the leaching rate and therefore overall effectiveness of the anti-fouling paint (Floerl 2003). In addition, private vessels that are coated with any common anti-fouling paint and not one which is specific to their travel patterns are more likely to become fouled before the paint's recommended service life expires (Floerl 2003). Thus, even with the use of antifouling coatings, hull fouling still occurs, specifically on worn-out, damaged or unpainted areas of the vessels' hulls, for example gaps in coatings where structures have been while vessels are kept in dry docks or maintenance facilities (Minchin & Gollasch 2003).

Manual removal

Labour-intensive manual removal methods such as scraping, the use of high pressure water cleaning, and brushes are used to augment the use of antifouling paints. Even though these manual removal methods can be cheaper than paint renewal and upkeep, they are time consuming and may also result in the release of organisms as they are removed from the hull (Hopkins & Forrest 2008). Many devices which are used to clean hulls are also unable to retain defouled material, thus potentially resulting in the organisms being dropped into the harbour. In addition to this flaw, many of these devices do not effectively clean the entire hull, leaving some organisms behind (Hopkins & Forrest 2008, Floerl 2003). The removal of fouled organisms from the vessel's hull often requires the removal of the vessel from the

water. Vessel haul-out is a costly process (Inglis et al. 2012) and is also only an option when dry docks or maintenance trailers are available for large and small vessels respectively, making it an expensive and limited solution. Grooming, the frequent cleaning of vessels' hulls when in port or at idle to remove fouling, is a proactive method of managing hull fouling (Tribou & Swain 2010). While manual removal methods of hull cleaning are usually aggressive, grooming is a gentle method of cleaning, using underwater vehicles containing brushes which gently wipe away fouling and particulate debris without harming vessels' antifouling coatings (Tribou & Swain 2015, Hunsucker et al. 2017). However, in order to be sufficiently effective at managing biofouling, grooming needs to be done as frequently as weekly (Tribou & Swain 2015).

Other management approaches

Other antifouling approaches have been considered, but little is available in mainstream academic literature. Approaches such as the use of biochemical stimuli (Morse 1984), radiochemical and ultrasonic technologies (Matsunaga et al. 1998) have been investigated but with little success (Terlizzi et al. 2001). Other approaches to antifouling include immersion or docking in freshwater (Brock et al. 1999), the use of hybrid polymer or gold nanoparticles (Boyer et al. 2009), the production of bubbles using electrochemical treatment to prevent the attachment of organisms to surfaces as well as to remove existing fouling and proteins from surfaces (Wu et al. 2008). Zosteric acid, which is a sulphoxy phenolic acid derived from eelgrass (*Zostera marina*), has also been tested and used to prevent the adhesion of fouling organisms to surfaces (Callow & Callow 2002). The use of microorganisms associated with macro-organisms such as seaweeds, ascidians and marine invertebrates to prevent larval settlement through the production of bioactive compounds has been examined (Satheesh et al. 2016), but requires more attention as the collection of large amounts of these compounds is difficult (Terlizzi et al. 2001). The use of ultraviolet radiation to prevent biofouling also has potential, as it is less harmful to the surrounding environment than biocides and has minimal space requirements (Patil et al. 2007). However, this technique is not well documented in the literature and also has high cost implications and high energy usage (Patil et al. 2007).

Encapsulation

In-water encapsulation of vessels is a relatively new and potentially viable approach to managing fouling (Coutts et al. 2010) but requires scientific investigation. Encapsulation refers to the wrapping of a structure in plastic or fabric, depriving fouled organisms of light, oxygen and food (Coutts & Forrest 2007). This method is promising, as it allows fouled organisms present on the vessel hull to be treated in situ, negating the need to take vessels

out of the water for cleaning (Roche et al. 2015). The encapsulation system may be enhanced by creating an anoxic environment within the seawater enclosed around the boat (Roche et al. 2015). Anoxic conditions create a toxic environment by favouring the conversion of sulphur to hydrogen sulphide as encapsulated organisms respire (Coutts and Forrest 2007). Anoxic conditions enhance the encapsulation technique by increasing the likelihood of death through sulphide toxicity in addition to the potential starvation and suffocation caused by encapsulation (Atalah et al. 2016). The process of killing fouling biota through encapsulation can be sped up by the addition of chemicals to the encapsulation system (Roche et al. 2015). The efficacy of chemicals such as acetic acid and chlorine as biocides have been tested in preliminary studies (Forrest et al. 2007, Roche et al. 2015).

Thesis aims

Despite the above approaches for managing vessel fouling, the introduction of marine alien species via fouling remains problematic. In recognition of the importance of yacht fouling as a vector of marine alien invasive species, and the need to develop more effective management strategies, this thesis aimed to:

1) Use laboratory experiments to develop a protocol for encapsulating yachts as a fouling management tool.

2) Test this protocol on yachts in the field.

3) Drawing on conclusions from the laboratory and field results, provide recommendations for the implementation of a national encapsulation program.

Chapter 2: Establishing the susceptibility of fouling biota to encapsulation under laboratory conditions

Abstract

Encapsulation, the wrapping of a vessel to deprive fouling organisms of oxygen and food, has been highlighted recently as a potential tool for managing hull fouling and the resultant introductions of alien species. Within the encapsulation system, respiration and metabolic processes by fouling organisms result in the depletion of oxygen and the build-up of waste products ammonia and sulphide. Several factors have been shown to speed up the process of encapsulation, including an increase in temperature and the addition of chemicals such as acetic acid and chlorine to the system. The aim of this chapter was to determine the susceptibility of a range of common fouling organisms to encapsulation to provide insight into the required timeframe for encapsulation to be effective for yachts in the field. The invasive ascidian *Ciona robusta,* the invasive mussel *Semimytilus algosus* and four-month-old fouling communities were exposed to four treatments. These included an aerated control, encapsulated seawater, aerated seawater with a 4% acetic acid solution and encapsulated seawater with a 4% acetic acid solution. This was done at 15°C and 23°C to consider the effect of various temperatures that typify the South African coast. Water samples demonstrated the encapsulation process and recorded a decrease in dissolved oxygen levels and an increase in ammonia and sulphide. In acetic acid treatments, all organisms reached total mortality within 24 hours, regardless of encapsulation. *Ciona robusta* died within 24 hours in all treatments. At 23°C, fouling assemblages died within 24 hours, while *S. algosus* survived for up to 48 hours. At 15°C, both fouling assemblages and *S. algosus* survived for up to three days. These results indicate that an increase in temperature results in faster mortality. Although this means that a shorter encapsulation period may be required on the warm east coast of South Africa than the cool west coast, one standardised national approach is easier to implement. As such, from the results of these experiments, an encapsulation period of three days was recommended for testing on yachts in the field.

2.1 Introduction

Hull fouling is one of the most important vectors responsible for the introduction and spread of marine alien species (Thresher 1999, Minchin & Gollasch 2003, Godwin 2005, Floerl et al. 2010, Lacoursière-Roussel et al. 2012). From as early as the 18th century, coatings containing biocides such as copper and arsenic were applied to vessels in an attempt to prevent the growth of fouling organisms on hulls (Dafforn et al. 2011). Paints containing tributyltin (TBT) were used as antifouling coatings from the 1960s (Smith et al. 2008), however, the harmful effects of TBT on non-target organisms resulted in the ban of TBT-containing paints in early 2003 (Champ 2003). Since the ban of TBT, although alternative antifouling techniques have been investigated, there has been a lack of well-developed, effective antifouling techniques that can be applied while vessels remain in the water (Piola et al. 2009). Furthermore, the regulations surrounding the application of antifouling to recreational vessels are lacking. As a result, vessels have to be routinely dry docked in order for fouling to be removed from the hulls. This process is expensive and often inconvenient, as cleaning can only happen when dry docks are available (Inglis et al. 2012). However, recent work has highlighted encapsulation as a promising method for removing fouling from a range of structures, including vessels (Coutts & Forrest 2007, Roche et al. 2015, Atalah et al. 2016). One of the reasons for this is that encapsulation allows vessels to be treated *in situ*, thus negating the need to remove them from the water for cleaning (Roche et al. 2015). This approach, however, requires scientific consideration as no standard operational protocol currently exists, with the method being adapted *ad hoc* to each application.

Encapsulation refers to the wrapping of a structure in a material which traps water inside an airtight system. Organisms inside this airtight wrapping are effectively deprived of light, oxygen and food (Coutts & Forrest 2007). As these organisms use up the existing oxygen in the system and release waste products, the conditions inside the enclosure deteriorate, and the death of the organisms becomes inevitable. Thus, the mechanisms through which the encapsulation process likely acts are induced hypoxia, the build-up of waste products such as ammonia (NH_3) and sulphides and ultimately the synergistic interactions of these adverse conditions. As a result of its regulatory effect on physiological and chemical processes, temperature is an important driver of mortality in encapsulation systems (Roche et al. 2015, Atalah et al. 2016).

Respiration by organisms within the encapsulation system results in the rapid depletion of oxygen (Coutts & Forrest 2007) and ultimately the development of anoxic conditions inside the encapsulation system (Atalah et al. 2016). Temperature affects the rate of dissolved

oxygen depletion by biota, as both respiration and decomposition occur at faster rates at high temperatures (Theede et al. 1969). The implication of this for the encapsulation process has been highlighted before, with encapsulation at high temperatures resulting in considerably lower dissolved oxygen levels than at lower temperatures (Atalah et al. 2016).

Under anoxic conditions, the conversion of sulphur to hydrogen sulphide is favoured during decomposition (Atalah et al. 2016). In addition, the bacterial reduction of sulphides along with the putrefaction of proteins results in high levels of hydrogen sulphide (Theede 1973). Hydrogen sulphide can be toxic as it forms sulphides with ions of heavy metals, resulting in the interrupting of cellular respiration (Theede 1973). As such, high sulphide and low oxygen conditions have the same effect on organisms, ultimately resulting in suffocation (Bagarinao 1992). Hydrogen sulphide toxicity can also be affected by temperature, as under high temperatures the metabolism of organisms is enhanced, which in turn elevates oxygen demands, heightens the production of hydrogen sulphide and hastens the suffocation of biota. Additionally, pH affects sulphide toxicity as it affects the form in which sulphide can occur.

Ammonia is an unusual toxic substance in that it is produced by and yet poisonous to animals (Ip et al. 2001). Ammonia can be especially dangerous to aquatic organisms as it is taken up easily through gills and cell membranes (Boardman et al. 2004). It is toxic to organisms as a result of the formation of nitrates, which enhance the conversion of oxygen-carrying pigments to forms that are incapable of carrying oxygen, thus resulting in oxygen deficiency in the organism's system, and ultimately suffocation (Camargo et al. 2005). When ammonia is present in water at high enough levels, it is difficult for aquatic organisms to sufficiently excrete the toxicant, leading to toxic build up in internal tissues and blood, and death (Boardman et al. 2004). Ammonia toxicity increases as nitrate concentration increases (Camargo et al. 2005). In biological systems this occurs when nitrogen waste is produced by organisms, a process that increases with movement and feeding (Ip et al. 2001, Randall & Tsui 2002). Ammonia levels are minimised by aquatic animals through direct excretion of this compound (Ip et al. 2001). Some organisms, such as fish, utilise various physiological mechanisms to combat and avoid ammonia toxicity (Randall & Tsui 2002, Ip et al. 2004), including maintenance of high levels of ammonia excretion (Randall et al. 1999) and the conversion of ammonia to forms which are not toxic, for example free amino acids (Peng et al. 1998). Environmental factors, such as pH and temperature, can affect ammonia toxicity in aquatic animals, as ammonia toxicity, expressed as total ammonia ($[NH_3]+[NH_4^+]$, mg N/L), increases with water pH (Randall & Tsui 2002), as well as with temperature (Wurts 2003).

Chemical additives

While the primary mechanisms driving successful encapsulation have been explained above, the addition of biocides has been found to be useful for accelerating the rate of mortality in encapsulation systems (Forrest et al. 2007, Roche et al. 2015, Atalah et al. 2016). There are a number of readily-available chemicals which have been used in controlling biofouling including acetic acid (Forrest et al. 2007, Roche et al. 2015, Atalah et al. 2016), chlorine (granulised (Coutts & Forrest 2007) or as sodium hypochlorite (Carver et al. 2003, Roche et al. 2015)), brine solutions and hydrated lime (Carver et al. 2003). In a laboratory environment, both sodium hypochlorite and acetic acid have been found to reduce the biomass of fouling organisms by causing size regression after as little as one week of treatment (Roche et al. 2015). Application of these chemical treatments in the field has demonstrated similar results, with significant decreases in the surface area of fouling on vessels after treatment (Roche et al. 2015).

A number of recent studies have highlighted acetic acid as the best option for adding to encapsulation systems to reduce biomass of a variety of common fouling organisms (Forrest et al. 2007, Roche et al. 2015, Atalah et al. 2016). This compound is particularly useful when a quick-acting solution is required, for a number of reasons. Firstly, it is able to overcome the defences of hardy encapsulation-resistant taxa such as mussels and bryozoans, by dissolving their calcareous exoskeletons (Forrest et al. 2007). Secondly, an acetic acid concentration of just 4-5% is able to effectively eliminate fouling species (Forrest et al. 2007, Piola et al. 2009, Roche et al. 2015). A 4-5% solution is equivalent to household vinegar and therefore does not pose a significant environmental or occupational risk, as long as appropriate measures are taken with regards to waste disposal (Forrest et al. 2007). Lastly, acetic acid concentrations are known to remain stable over time in the presence of organic matter and where necessary, levels in the field can be determined using simple titration-based approaches (Forrest et al. 2007), making this a logistically feasible option for use in the field.

Chapter aims

Despite the urgent need for managing fouling, few studies have considered encapsulation and none have developed formalised recommendations for implementation of this technique in the field. Those that have been undertaken have largely applied a species specific approach with the susceptibility of fouling communities receiving little attention (Coutts & Forrest 2007, Forrest et al. 2007, Roche et al. 2015, Atalah et al. 2016). In light of this, this chapter firstly aimed to experimentally determine the susceptibility of: (1) different taxa

representing the extremes of vulnerability of fouling biota (i.e. hard-shelled molluscs and soft-bodied ascidians) to encapsulation and (2) complex fouling communities that may demonstrate a different response to individual taxa. Secondly, it aimed to use these experimental results to develop a protocol for testing under field conditions. These laboratory experiments compared the effect of encapsulation with and without the use of acetic acid at temperatures representative of the South African cool temperate west coast and the warm subtropical east coast. Based on the literature, the following *a priori* hypotheses were tested: (1) soft bodied biota would be more susceptible to treatment via encapsulation than shelled molluscs (Atalah et al. 2016); (2) the time required for encapsulation to effectively kill fouling organisms would decrease with increasing temperature (Atalah et al. 2016); (3) the use of acetic acid would shorten the treatment time required to kill all fouling biota (Forrest et al. 2007, Roche et al. 2015, Atalah et al. 2016).

2.2 Methods and materials

Collection of specimens

Model species were chosen to represent different groups of fouling biota that are likely to represent the extremes of vulnerability to encapsulation. These were the hard-shelled mussel *Semimytilus algosus*, and the soft-bodied solitary ascidian *Ciona robusta* (previously referred to in this region as *Ciona intestinalis*). Both these species are alien to South African waters (Robinson et al. 2016). Mussels of 2-3cm were collected from Gordon's Bay Yacht Club (34°09'52"S; 18°51'42"E). This size class was chosen as it is representative of mussels previously recorded fouling yachts (Robinson pers. comm.). *Ciona robusta* individuals were collected at the Yacht Port Marina (33°01'36"S; 17°57'40"E) in Saldanha Bay (Figure 2.1) and transported to the laboratory at Stellenbosch University (33°93'28"S; 18°86'44"E). The size of *C. robusta* used varied between 2-4cm tunic length when measured out of the water. Individuals were transported in cool conditions and were exposed to experimental conditions immediately after arrival in the laboratory.

Fouling communities were allowed to settle on 20cm x 20cm PVC plates deployed at Yacht Port Marina for sixteen weeks. This enabled the development of dense fouling assemblages to develop at depths of 2 to 3m, the typical depth rage of dense fouling on yacht hulls in this region (Peters pers. comm.). To avoid the edge effect confounding the results, assemblages were only considered within the central 15cm x 15 cm of each plate. Percentage fouling cover was estimated visually using a grid consisting of 100 1.5cm x 1.5cm squares. Here, fouling cover ranged from 85% to 100% (92.3 ± 4.2 SD). These communities were diverse

with a mean of 8 (± 5 SD) species present per plate, representing a mix of indigenous and alien species (See Appendix 1 for details).

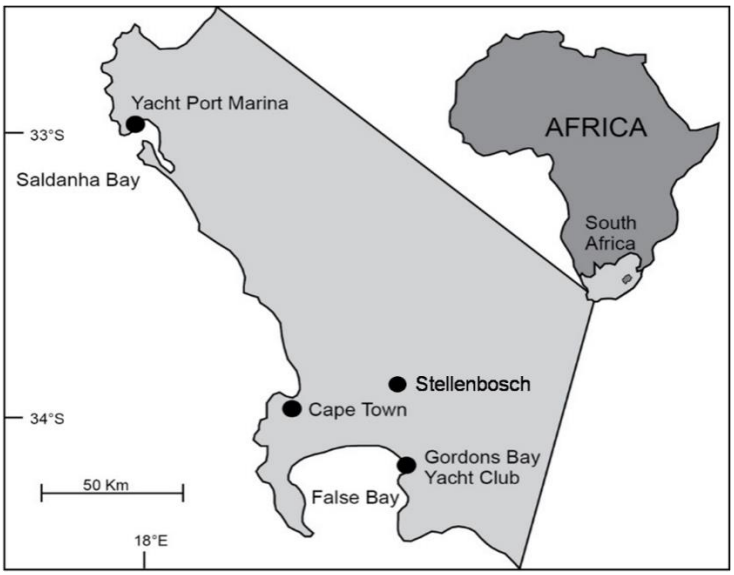

Figure 2.1: Map of locations at which specimens were collected. The mussel *Semimytilus algosus* was collected in Gordon's Bay. *Ciona robusta* and fouling assemblage plates were collected from Yacht Port Marina in Saldanha Bay.

Laboratory experiments

The effect of encapsulation on the mortality of model species and fouling assemblages was assessed in relation to two temperatures while the augmentative effect of acetic acid was considered concurrently. Thus, treatments considered the effect of temperature (two levels: 13°C and 23°C, representing the cool South African west coast and the warm east coast), encapsulation (two levels: encapsulated and non-encapsulated (i.e. encapsulation control) and acetic acid (two levels: 4% acetic acid solution and no acetic acid (i.e. acetic acid control) (Figure 2.2). All experimental and control containers (described below) contained filtered artificial seawater with a salinity of 32ppt mixed by hand at the Department of Botany and Zoology at Stellenbosch University. A single organism was placed in each container and water was added to each container, achieving a 1:3 ratio of biomass to water for all experiments. For those experiments exposed to acetic acid, a 4% acetic acid solution replaced the pure seawater. The average pH of this solution was 3.48 (± 1.21 SD). This solution was made up using the same filtered seawater as in the other treatments.

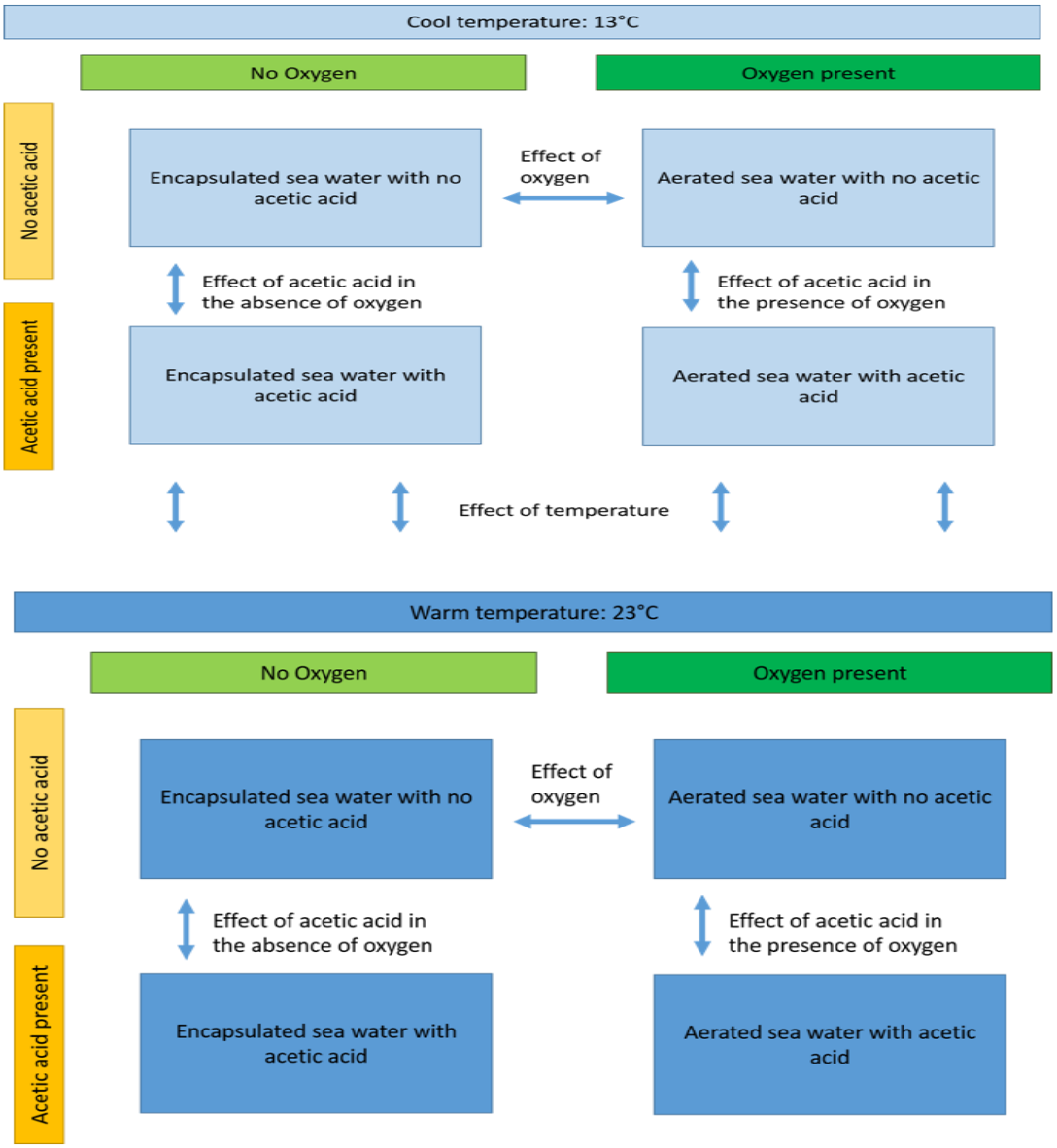

Figure 2.2: A schematic diagram illustrating the experimental design applied during the laboratory experiments.

For all experiments, controls were aerated and water was changed daily to maintain water quality. The encapsulation treatment for *Semimytilus algosus* and *Ciona robusta* consisted of 500ml (approximately 11cm x 11cm x 8cm) containers placed inside 2L airtight bags. For fouling assemblages, plates were placed into 11L plastic containers (approximately 45cm x 30cm x 15cm) with airtight lids. Prior to the experiments, a hole of 10cm x 10cm was cut in each lid and a 2L plastic access bag was secured in this space (Figure 2.3). This access bag allowed the sampling of water and the checking of mortality (described below) without disturbing the airtight seal. For experiments considering *S. algosus* and *C. robusta* six replicates were applied per treatment while five replicates were considered for fouling assemblages.

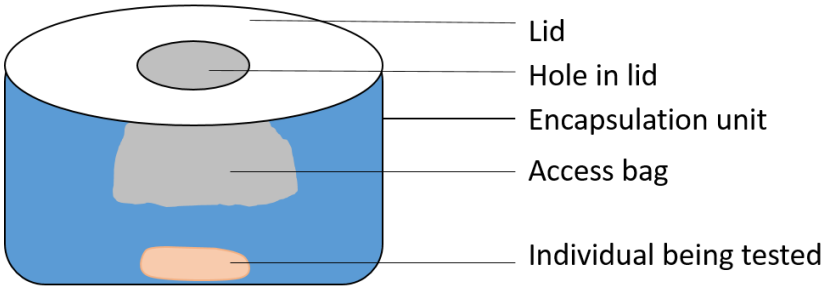

Figure 2.3: A diagrammatic representation of the experimental encapsulation units used during the laboratory experiments on fouling assemblages.

Water quality was assessed for each treatment prior to the commencement of the experiments and then monitored in each replicate every 24 hours. Parameters that were considered included pH, sulphide, ammonia and dissolved oxygen. These parameters were chosen to confirm desired treatments (i.e. effective encapsulation as reflected in low dissolved oxygen levels and the effect acetic acid as reflected in lowered pH) and to gain a measure of the effects of the treatments (e.g. increasing sulphide and ammonia concentrations). These water samples were collected using a needle and syringe with the resulting hole immediately sealed with waterproof tape. Samples were collected at the same time as checking for mortality to minimise the number of times that encapsulation bags were punctured. Using the R statistical environment (version 3.4.2), changes in these parameters were assessed by means of general mixed effects models (packages lme4 and car) after a normal distribution was confirmed, where each outcome variable (ammonia, sulphide, dissolved oxygen and pH) was assessed in relation to treatment (control aerated sea water, encapsulated sea water, aerated sea water with 4% acetic acid and encapsulated sea water with 4% acetic acid), temperature (15°C and 23°C) and time as fixed factors. Replicates were considered a random factor to account for repeated measures through time. Significance levels are indicated in the results section below.

Mortality was assessed daily, both visually and by touching biota with a needle. To reach organisms in encapsulated treatments, a 21G needle was inserted through the plastic bag and the hole then sealed using waterproof tape. For *S. algosus*, any individual that remained open when tapped vigorously was considered dead. Mortality of *C. robusta* was determined by prodding the siphons with a needle; individuals were considered dead if no movement occurred (Floerl et al. 2005). Due to the dominance of soft-bodied taxa present in the fouling communities, mortality on the plates was also determined by prodding organisms with a needle; where no movement occurred in any individuals, the community was considered dead. Treatments were terminated when 100% mortality was reached in all replicates. Similarly to the water parameters, time to mortality was statistically analysed by means of

general mixed effects models, where treatment, temperature, and ammonia and sulphide concentrations were considered fixed factors. Again, replicate was applied as a random factor to account for the repeated measures through time. Time to mortality could not be assessed for *C. robusta,* as all individuals died within 24 hours regardless of treatment. Additionally, for analyses of *S. algosus* and fouling communities, the control treatment was excluded as individuals in this treatment all survived.

2.3 Results

Treatments applied in all experiments were deemed effective with the significant decrease in pH in acetic acid treatments, the decline in dissolved oxygen in the encapsulated treatment and consistent conditions and lack of mortality in the controls. The effect of encapsulation was observed in reduced dissolved oxygen and increased ammonia and sulphide over time in both the encapsulated seawater and encapsulated acetic acid treatments. These effects were missing in both the control and acetic acid treatments. Additionally, the acetic acid and encapsulated acetic acid treatments consistently had lower pH values than their controls. These results are detailed below.

Water quality through time

Ciona robusta

a) Dissolved Oxygen

There were significant main effects of treatment (Wald test, χ_3=54.78, p<0.001), temperature (χ_1=4.59, p=0.03) and time (χ_1=35.41, p<0.001) on dissolved oxygen levels (Figure 2.4a, b). Differences among treatments were driven by significantly lower dissolved oxygen in the encapsulated treatment than all other treatments (P<0.001 in all cases; Table 2.1). An increase in temperature resulted in a significant decline in dissolved oxygen (coefficient=-0.09, t=-2.14, p=0.03), while concentrations also declined through time (coefficient=-2.03, t=-5.95, p<0.001).

b) pH

For this ascidian, significant main effects were detected for treatment (Wald test, χ_3=569.10, p<0.001) and time (χ_1=37.61, p<0.001), although no effect of temperature on pH was detected (χ_1=1.57, p>0.05) (Figure 2.4c, d). As expected both treatments to which acetic was added had significantly lower pH than the control and encapsulated treatments (p<0.01 in all cases; Table 2.1). Additionally, pH declined significantly through time (coefficient=-1.13, t=-6.13, p<0.001).

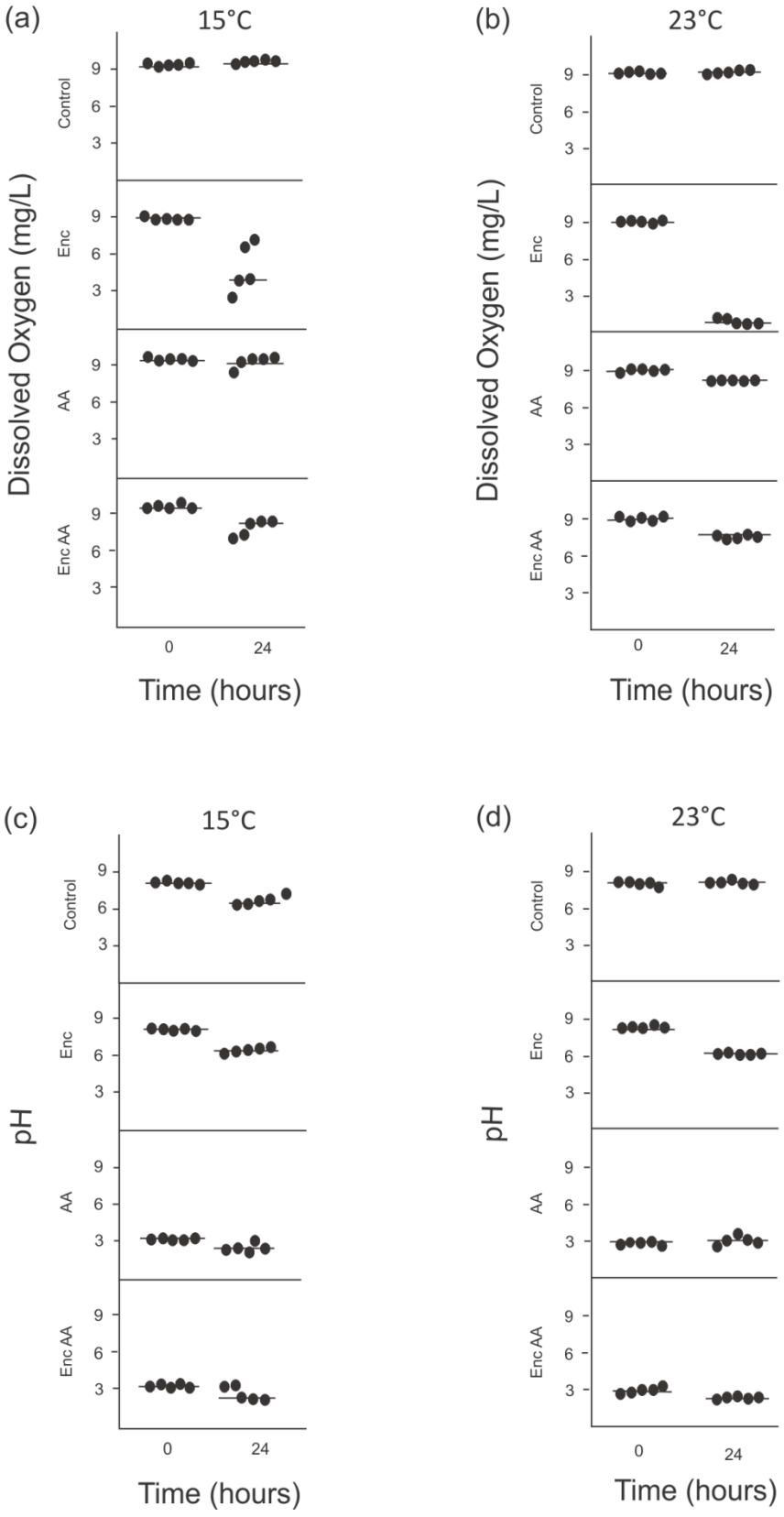

Figure 2.4: Dissolved oxygen concentration (mg/L) and pH as raw values (dots) and medians (lines) at temperatures of 15°C (a, c) and 23°C (b, d) for *Ciona robusta*. Treatments: Enc AA = encapsulated seawater with acetic acid, AA = aerated seawater with acetic acid, Enc = encapsulated seawater without acetic acid, control = aerated seawater without acetic acid.

Table 2.1: Main effect coefficient estimates and associated significance derived from the general mixed effects model considering the effect of treatment on (a) dissolved oxygen levels and (b) pH for *Ciona robusta*. Note that coefficients reflect relationships of rows to columns. ns not significant, * p<0.05. Treatments: Enc AA = encapsulated seawater with acetic acid, AA = aerated seawater with acetic acid, Enc = encapsulated seawater without acetic acid, control = aerated seawater without acetic acid.

(a) Dissolved Oxygen	Con	Enc	AA
Con			
Enc	-3.15*		
AA	0.16^{ns}	2.99*	
Enc AA	-0.79^{ns}	2.36*	-0.63^{ns}
(b) pH	Con	Enc	AA
Con			
Enc	-0.15^{ns}		
AA	-4.28*	-4.44*	
Enc AA	-4.37*	-4.53*	0.09^{ns}

c) Ammonia

Significant main effects of treatment (Wald test, χ_3=60.46, p<0.001) and time (χ_1=110.71, p<0.001) were detected, but no significant effect of temperature on ammonia was found (χ_1=2.75, p>0.05) (Figure 2.5a, b). Ammonia levels were higher in the encapsulated treatment than all other treatments (p<0.001 in all cases; Table 2.2). In addition, ammonia levels increased through time (coefficient=38.05, t=10.52, p<0.001)

d) Sulphide

There were significant main effects of treatment (Wald test, χ_3=22.70, p<0.001), temperature (χ_1=7.07, p<0.001) and time (χ_1=10.66, p<0.001) on sulphide levels (Figure 2.5c, d). The significant main effect of treatment is observed as with ammonia, where the encapsulated treatment had significantly higher sulphide levels than all other treatments (p<0.001 in all cases; Table 2.2). Sulphide levels increased with temperature (coefficient=0.32, t=2.66, p<0.05) as well as through time (coefficient=3.10, t=3.26, p<0.001).

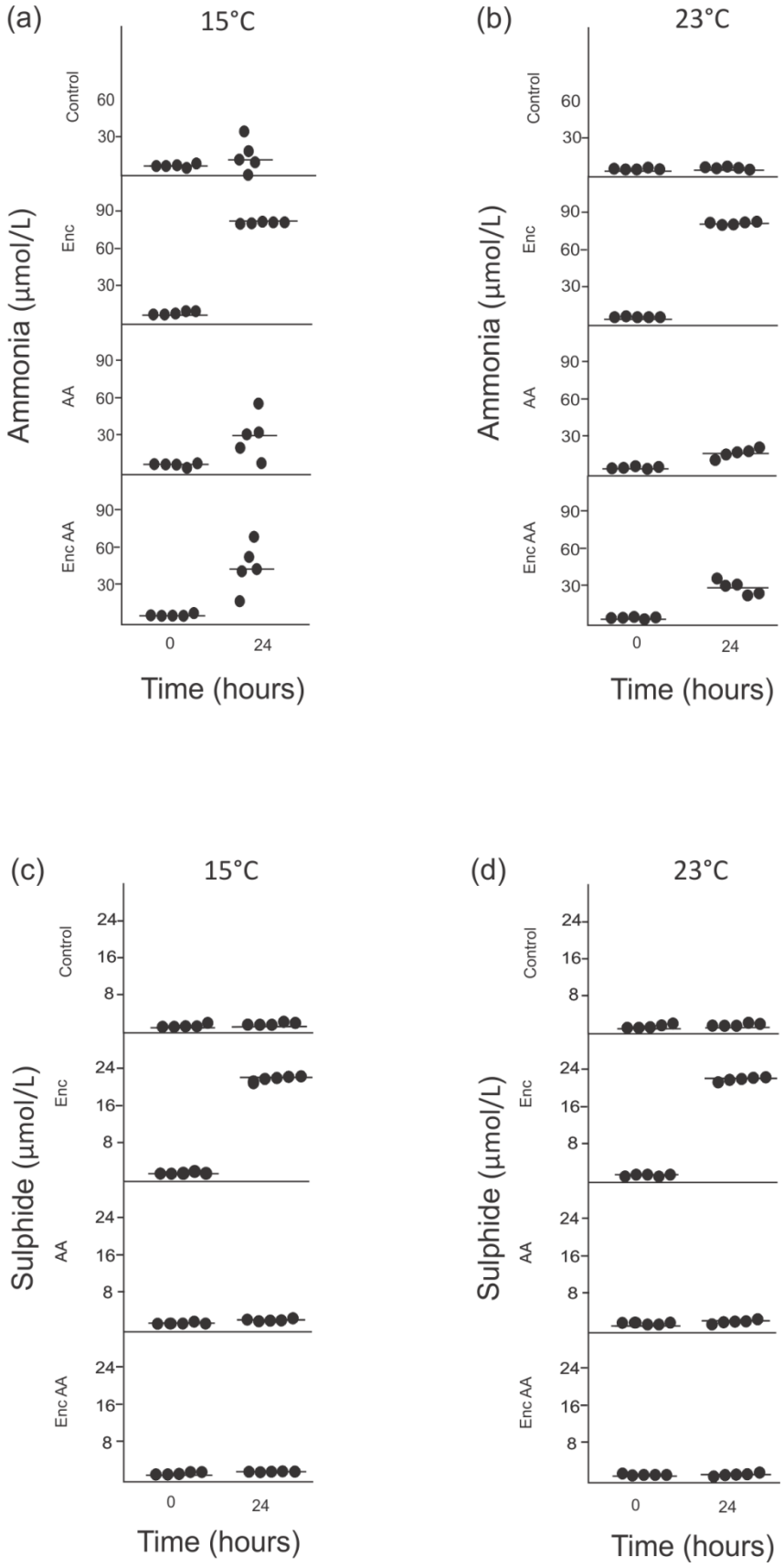

Figure 2.5: Ammonia and sulphide concentration (µmol/L) as raw values (dots) and medians (lines) at temperatures of 15°C (a, c) and 23°C (b, d) for *Ciona robusta*. Treatments: Enc AA = encapsulated seawater with acetic acid, AA = aerated seawater with acetic acid, Enc = encapsulated seawater without acetic acid, control = aerated seawater without acetic acid.

Table 2.2: Main effect coefficient estimates and associated significance derived from the general mixed effects model considering the effect of treatment on (a) ammonia and (b) sulphide levels for *Ciona robusta*. Note that coefficients reflect relationships of rows to columns. ns = not significant, * = p<0.05. Treatments: Enc AA = encapsulated seawater with acetic acid, AA = aerated seawater with acetic acid, Enc = encapsulated seawater without acetic acid, control = aerated seawater without acetic acid.

(a) Ammonia	Con	Enc	AA
Con			
Enc	38.13*		
AA	9.36^{ns}	-28.77*	
Enc AA	14.64*	-23.49*	5.28^{ns}
(b) Sulphide	Con	Enc	AA
Con			
Enc	5.50*		
AA	0.66^{ns}	-4.84*	
Enc AA	0.28^{ns}	-5.22*	0.38^{ns}

Semimytilus algosus

a) Dissolved Oxygen

There were significant main effects of treatment (Wald test, χ_3=113.75, p<0.001), temperature (χ_1 =6.74, p=0.009) and time (χ_1=49.80, p<0.001) on dissolved oxygen levels (Figure 2.6a, b). Differences among treatments were driven by significantly lower dissolved oxygen in both treatments undergoing encapsulation (p<0.001 in all cases; Table 2.3) although these did not differ from one another. As with *C. robusta*, an increase in temperature resulted in a significant decline in dissolved oxygen (coefficient=-0.05, t= -2.60, p=0.009), while concentrations also declined through time (coefficient=-0.66, t=-7.06, p<0.001).

b) pH

For this mussel, main effects of treatment (Wald test, χ_3= 1775.64, p<0.001) and time (χ_1=8.88, p=0.002) were found on pH, but no effect of temperature (χ_1 =2.50, p>0.05) were found (Figure 2.6c, d). As expected, differences in treatment occurred as a result of the addition of acetic acid to two treatments, which differed significantly from the control and encapsulated treatments (p<0.05 in both cases; Table 2.3). pH levels also increased through time (coefficient=0.02, t =1.58, p=0.002).

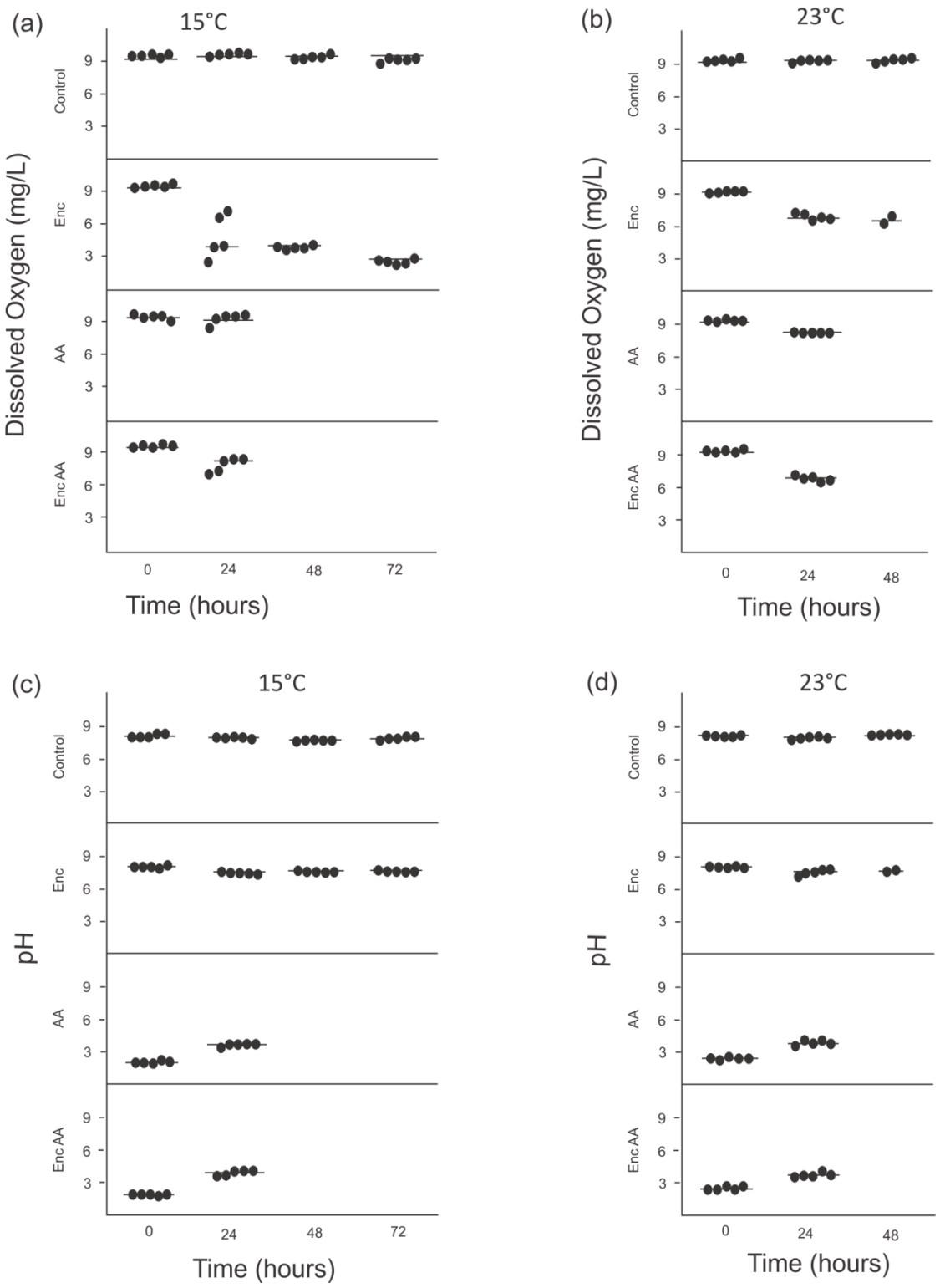

Figure 2.6: Dissolved oxygen concentration (mg/L) and pH as raw values (dots) and medians (lines) at temperatures of 15°C (a, c) and 23°C (b, d) for *Semimytilus algosus*. Treatments: Enc AA = encapsulated seawater with acetic acid, AA = aerated seawater with acetic acid, Enc = encapsulated seawater without acetic acid, control = aerated seawater without acetic acid.

Table 2.3: Main effect coefficient estimates and associated significance derived from the general mixed effects model considering the effect of treatment on (a) dissolved oxygen and (b) pH levels for *Semimytilus algosus*. Note that coefficients reflect relationships of rows to columns. ns = not significant, * = p<0.05. Treatments: Enc AA = encapsulated seawater with acetic acid, AA = aerated seawater with acetic acid, Enc = encapsulated seawater without acetic acid, control = aerated seawater without acetic acid.

(a) Dissolved Oxygen	Con	Enc	AA
Con			
Enc	-2.02*		
AA	0.82*	1.20*	
Enc AA	-1.78*	0.23ns	-0.96*
(b) pH	Con	Enc	AA
Con			
Enc	-0.31*		
AA	-4.94*	-4.63*	
Enc AA	-4.98*	-4.67*	-0.05^{ns}

c) Ammonia

Similarly to *C. robusta*, significant main effects of treatment (Wald test, χ_3=112.83, p<0.001), temperature (χ_1=7.32, p<0.001) and time (χ_1=74.07, p=0.006) were detected on ammonia levels (Figure 2.7a, b). A significant effect of treatment is explained by the control treatment having significantly lower ammonia levels than all other treatments (p<0.001 in all cases: Table 2.4). Ammonia levels for *Semimytilus algosus* increased with temperature (coefficient=1.27, t=2.71, p=0.006) and through time (coefficient=18.18, t=8.61, p<0.001).

d) Sulphide

Significant main effects of treatment (Wald test, χ_3=65.04, p<0.001), temperature (χ_1=5.99, p=0.01) and time (χ_1=21.56, p<0.001) were detected (Figure 2.7c, d). Acetic acid treatments reflected significantly higher sulphide levels than both treatments without acetic acid (p<0.001 in all cases, Table 2.4). Sulphide levels significantly increased with increasing temperature (coefficient=0.36, t=2.45, p=0.01) as well as through time (coefficient=3.05, t=4.64, p<0.001).

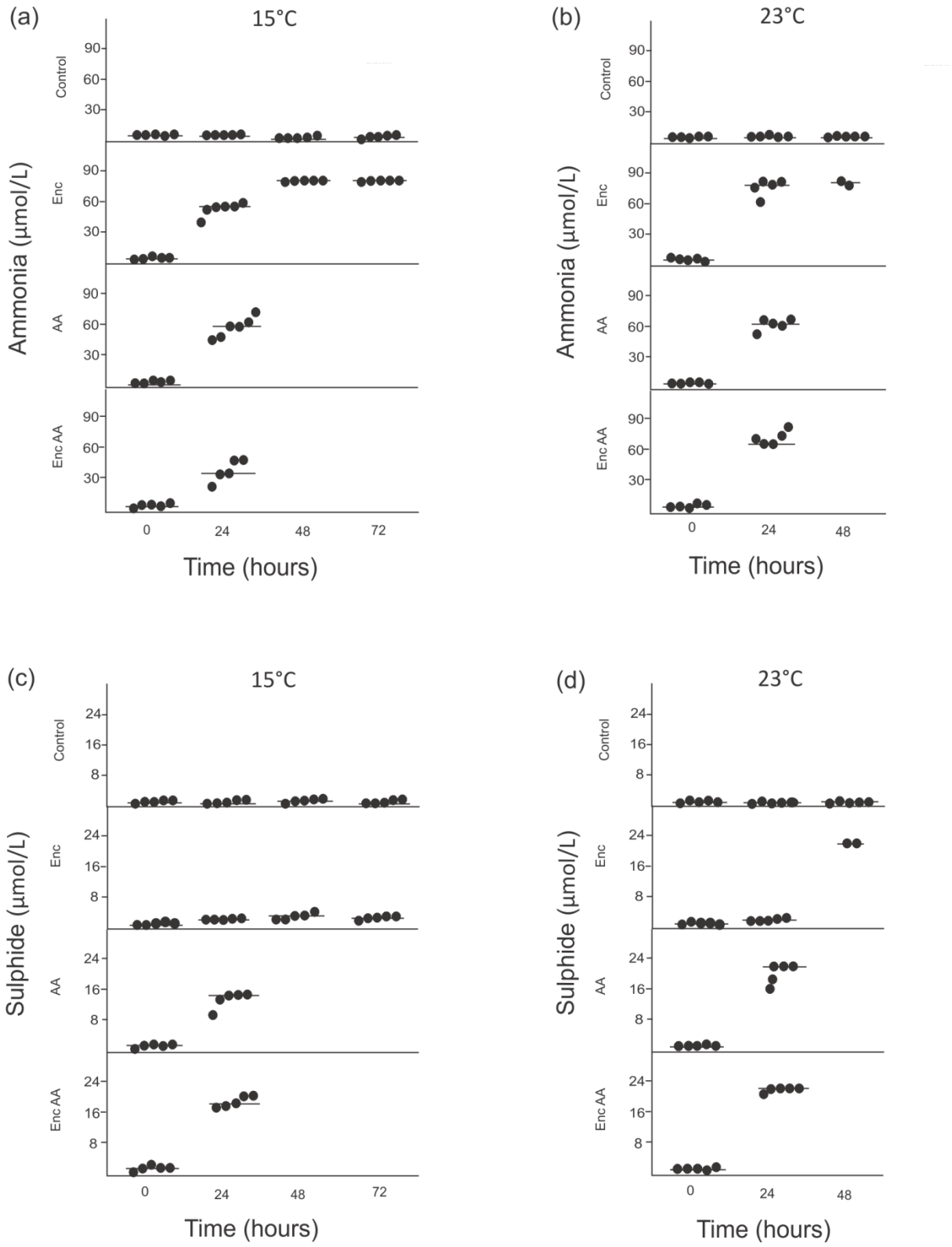

Figure 2.7: Ammonia and sulphide concentration (µmol/L) as raw values (dots) and medians (lines)at temperatures of 15°C (a, c) and 23°C (b, d) for *Semimytilus algosus*. Treatments: Enc AA = encapsulated seawater with acetic acid, AA = aerated seawater with acetic acid, Enc = encapsulated seawater without acetic acid, control = aerated seawater without acetic acid.

Table 2.4: Main effect coefficient estimates and associated significance derived from the general mixed effects model considering the effect of treatment on a) ammonia and b) sulphide levels for *Semimytilus algosus*. Note that coefficients reflect relationships of rows to columns. ns = not significant, * = p<0.05. Treatments: Enc AA = encapsulated seawater with acetic acid, AA = aerated seawater with acetic acid, Enc = encapsulated seawater without acetic acid, control = aerated seawater without acetic acid.

(a) Ammonia	Con	Enc	AA
Con			
Enc	44.63*		
AA	40.51*	-4.13^{ns}	
Enc AA	37.71*	-6.93^{ns}	2.80^{ns}
(b) Sulphide	**Con**	**Enc**	**AA**
Con			
Enc	1.91^{ns}		
AA	10.04*	8.14*	
Enc AA	11.75*	9.84*	1.71^{ns}

Fouling assemblages

a) Dissolved Oxygen

As with both *C. robusta* and *S. algosus*, there were significant main effects of both treatment (Wald test, χ_3=103.69, p<0.001) and time (χ_1=35.06, p<0.001) on dissolved oxygen levels (Figure 2.8a, b). However, unlike the *C. robusta* and *S. algosus* experiments, no main effect of temperature was detected (χ_1=1.71, p>0.05). The two encapsulated treatments had significantly lower dissolved oxygen levels than the other treatments, and also differed from one another (p<0.001 in all cases, Table 2.5). Dissolved oxygen levels decreased through time (coefficient=-1.31, t=-5.84, p<0.001).

b) pH

Similarly to dissolved oxygen levels in the fouling assemblages' experiments, main effects of treatment (Wald test, χ_3=1609.30, p<0.001) and time (χ_1=15.17, p<0.001) were detected, but not temperature (χ_1=0.49, p>0.05, Figure 2.8c, d). The treatments containing acetic acid had significantly lower pH, as expected, and the encapsulated treatment also had a lower pH than the controls (p<0.001 in all cases, Table 2.5). The pH also declined through time (coefficient=-0.24, t=-3.90, p<0.001).

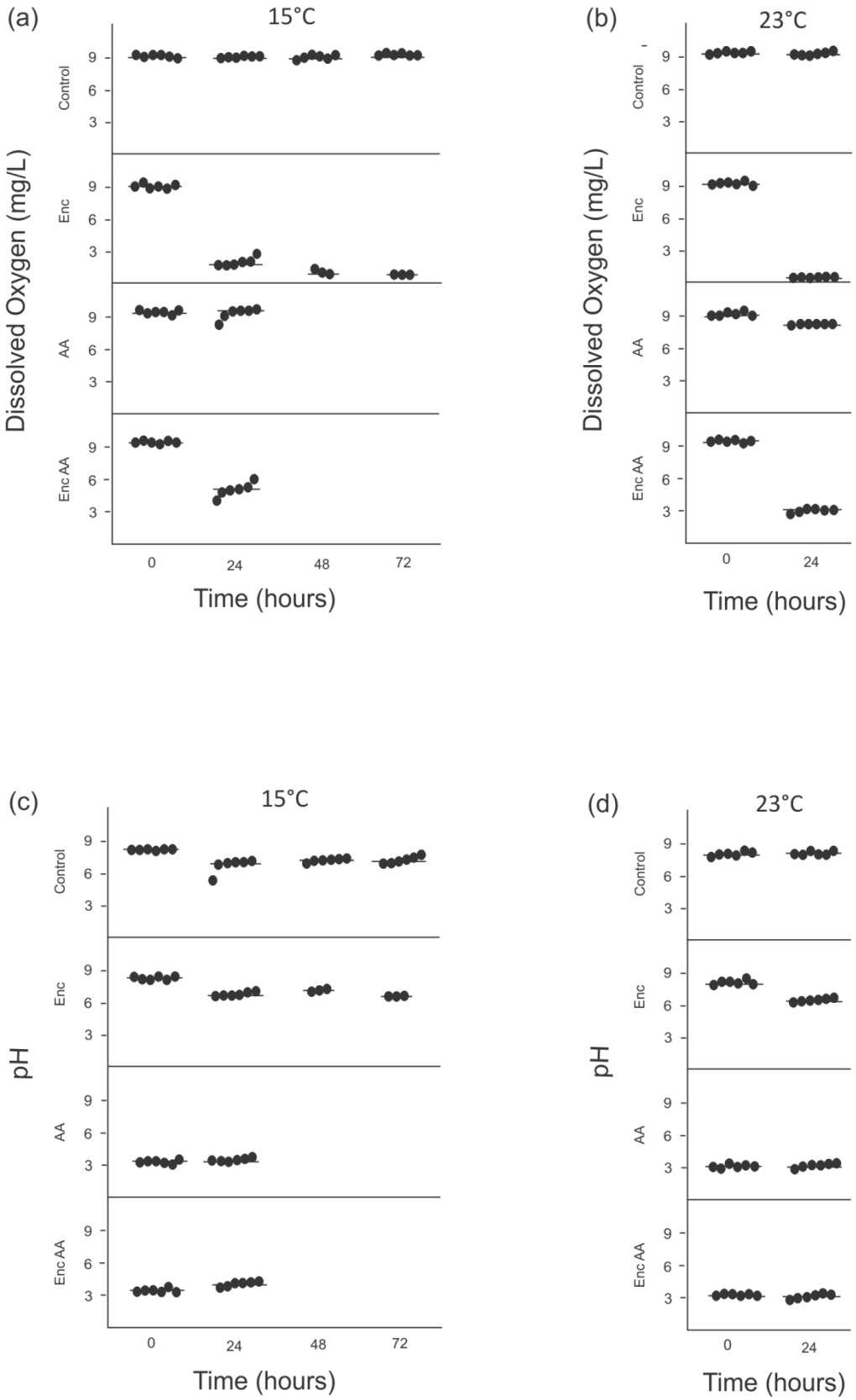

Figure 2.8: Dissolved oxygen concentration (mg/L) and pH as raw values (dots) and medians (lines)at temperatures of 15°C (a, c) and 23°C (b, d) for fouling assemblages. Treatments: Enc AA = encapsulated seawater with acetic acid, AA = aerated seawater with acetic acid, Enc = encapsulated seawater without acetic acid, control = aerated seawater without acetic acid.

Table 3: Main effect coefficient estimates and associated significance derived from the general mixed effects model considering the effect of treatment on a) dissolved oxygen and b) for fouling assemblages. Note that coefficients reflect relationships of rows to columns. ns = not significant, * = p<0.05. Treatments: Enc AA = encapsulated seawater with acetic acid, AA = aerated seawater with acetic acid, Enc = encapsulated seawater without acetic acid, control = aerated seawater without acetic acid.

(a) Dissolved Oxygen	Con	Enc	AA
Con			
Enc	-4.62*		
AA	-0.76^{ns}	3.86*	
Enc AA	-2.20^{ns}	2.42*	-1.44*
(b) pH	Con	Enc	AA
Con			
Enc	-0.57*		
AA	-4.87*	-4.30*	
Enc AA	-4.73*	-4.16*	0.15^{ns}

c) Ammonia

Again, significant main effects of treatment (Wald test, χ_3=10.41, p=0.01) and time (χ_1=86.03, p<0.001) were found on ammonia levels, and no main effect of temperature (χ_1=0.52, p>0.05, Figure 2.9a, b). The controls had significantly lower ammonia levels than the encapsulated and acetic acid treatments (p<0.001 in all cases, Table 2.6). Ammonia levels rose through time (coefficient=23.47, t=9.28, p<0.001).

d) Sulphide

Significant main effects of treatment (Wald test, χ_3=38.99, p<0.001), temperature (χ_1=7.31, p=0.006) and time (χ_1=26.54, p<0.001) were found (Figure 2.9c, d). The control treatment had significantly lower sulphide levels than all other treatments, while the encapsulated treatment had significantly higher sulphide levels than all other treatments (p<0.001 in all cases, Table 2.6). Sulphide levels also increased significantly through time (coefficient=2.88, t=5.15, p<0.001) and with increasing temperature (coefficient=0.32, t=2.7, p=0.006).

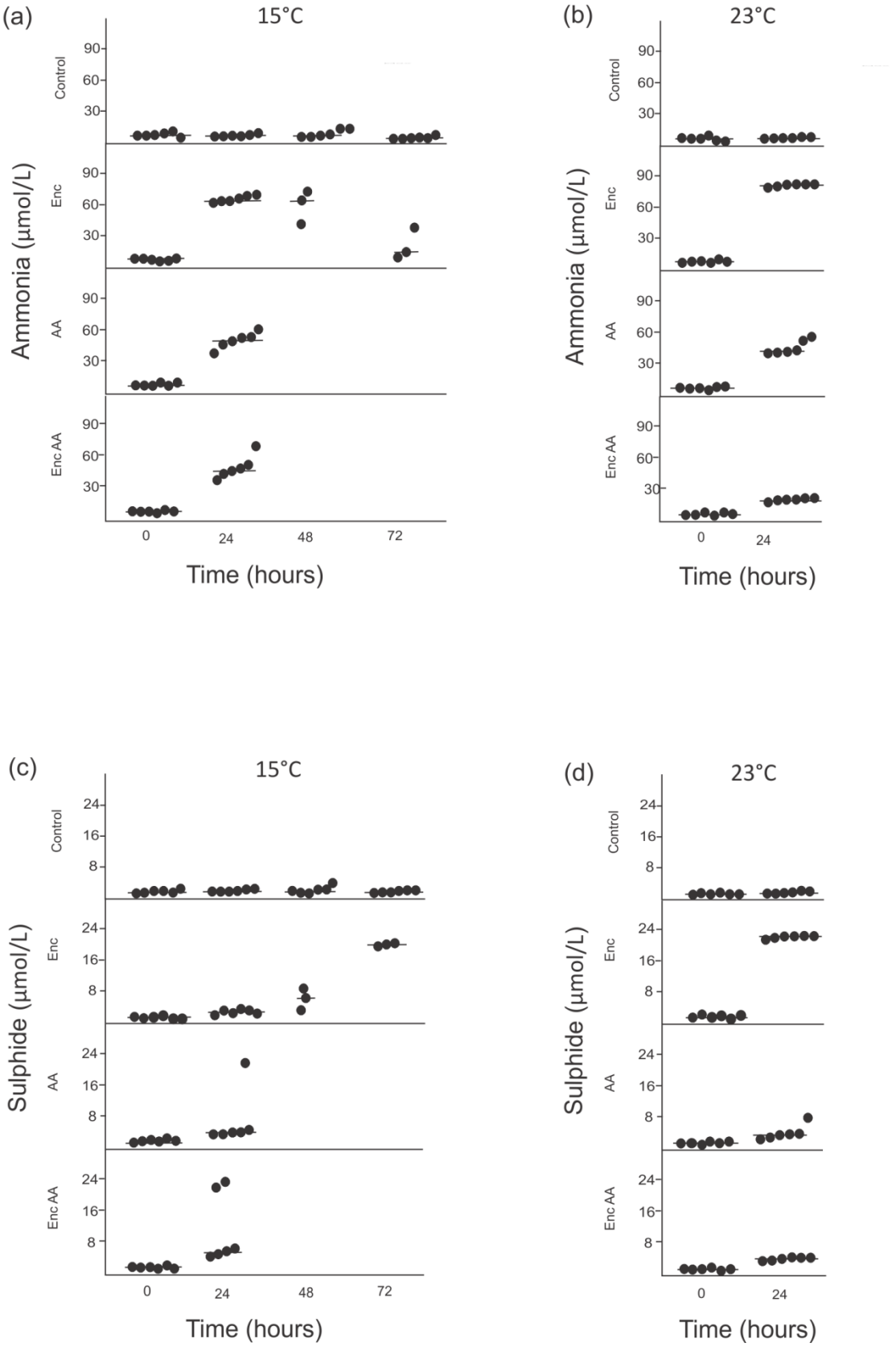

Figure 2.9: Ammonia and sulphide concentration (µmol/L) as raw values (dots) and medians (lines) at temperatures of 15°C (a, c) and 23°C (b, d) for fouling assemblages. Treatments: Enc AA = encapsulated seawater with acetic acid, AA = aerated seawater with acetic acid, Enc = encapsulated seawater without acetic acid, control = aerated seawater without acetic acid.

Table 2.6: Main effect coefficient estimates and associated significance derived from the general mixed effects model considering the effect of treatment on a) ammonia and b) sulphide levels for fouling assemblages. Note that coefficients reflect relationships of rows to columns. ns = not significant, * = p<0.05. Treatments: Enc AA = encapsulated seawater with acetic acid, AA = aerated seawater with acetic acid, Enc = encapsulated seawater without acetic acid, control = aerated seawater without acetic acid.

(a) Ammonia	Con	Enc	AA
Con			
Enc	15.62*		
AA	13.00*	-2.61^{ns}	
Enc AA	4.09^{ns}	-11.52^{ns}	-8.91^{ns}
(b) Sulphide	Con	Enc	AA
Con			
Enc	7.46*		
AA	3.75*	-3.70*	
Enc AA	3.39*	-4.07*	-0.36^{ns}

Time to Mortality

No mortality of individuals or communities was observed in control units for the entire duration of the experiments. Treatments were deemed effective as 100% mortality was recorded for organisms and communities exposed to all treatments.

Ciona robusta

All *C. robusta* individuals in treatments besides the controls died within 24 hours, regardless of temperature.

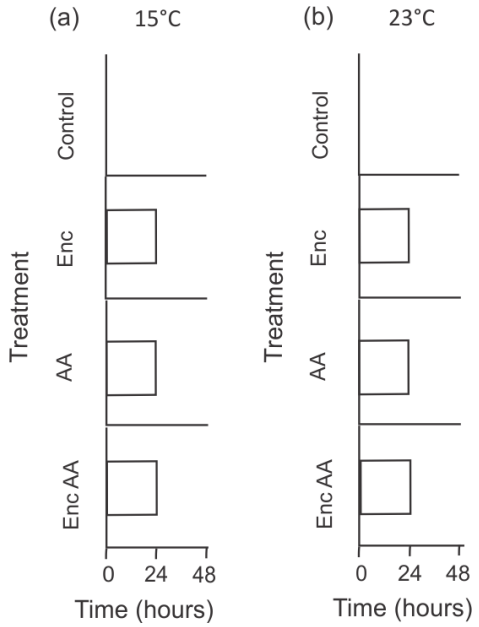

Figure 2.10: Mean time to 100% mortality of *Ciona robusta* at a) 15°C and b) 23°C. Treatments: Enc AA = encapsulated seawater with acetic acid, AA = aerated seawater with acetic acid, Enc = encapsulated seawater without acetic acid, control = aerated seawater without acetic acid. Note: No mortality occurred in the control and no variability was observed in the other treatments as all individuals died within 24hours.

Semimytilus algosus

There were significant main effects of treatment (Wald test, χ_2=308.68, p<0.001) and temperature (χ_1=52.14, p<0.001) on time to mortality, but not of ammonia (χ_1=2.18, p>0.05) or sulphide (χ_1=1.02, p>0.05). As with *C. robusta*, all individuals in the control treatment survived throughout the experiment (Figure 2.11). Individuals in the encapsulated treatment took significantly longer to die than individuals in the acetic acid (coefficient=1.44, t=15.57, p<0.001) and encapsulated acetic acid (coefficient=1.48, t=15.98, p<0.001) treatments. There was no significant difference in time to mortality between the two acetic acid treatments (coefficient=0.04, t=0.47, p=0.63). Time to mortality decreased significantly with increasing temperature (coefficient=-0.05, t=-7.22, p<0.001). Shells of individuals in the acetic acid treatments became soft and easily breakable within 24 hours, regardless of encapsulation.

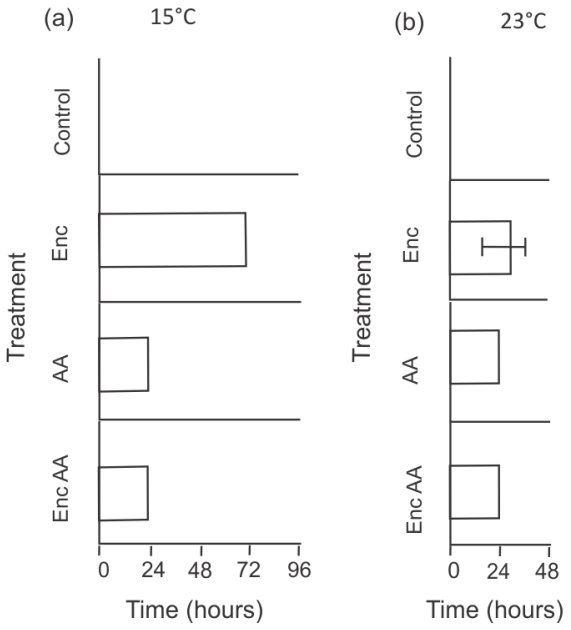

Figure 2.11: Time to 100% mortality (Mean ± SD) of *Semimytilus algosus* at a) 15°C and b) 23°C. Treatments: Enc AA = encapsulated seawater with acetic acid, AA = aerated seawater with acetic acid, Enc = encapsulated seawater without acetic acid, control = aerated seawater without acetic acid. Note: No mortality occurred in the control and where no whiskers are presented no variability was observed as all individuals died within 24hours.

Fouling assemblages

Similarly to *S. algosus*, there were significant main effects of both treatment (Wald test, χ_2=48.19, p<0.001) and temperature (χ_1=60.95, p<0.001), but not of ammonia (χ_1=0.05, p>0.05) and sulphide (χ_1=2.73, p>0.05) on the fouling assemblage time to mortality. All organisms in the control treatments survived throughout the experiments (Figure 2.12), indicating that this treatment was a successful representation of a normal state. Organisms in both the acetic acid (coefficient=1.13, t=6.25, p<0.001) and encapsulated acetic acid (coefficient=1.09, t=6.28, p<0.001) treatments died significantly faster than those in the encapsulated treatment, and there was no significant difference between the two acetic acid treatments (coefficient=0.05, t=0.3, p>0.05). As with *S. algosus*, time to mortality decreased significantly as temperature increased (coefficient=-0.11, t=-7.81, p<0.001).

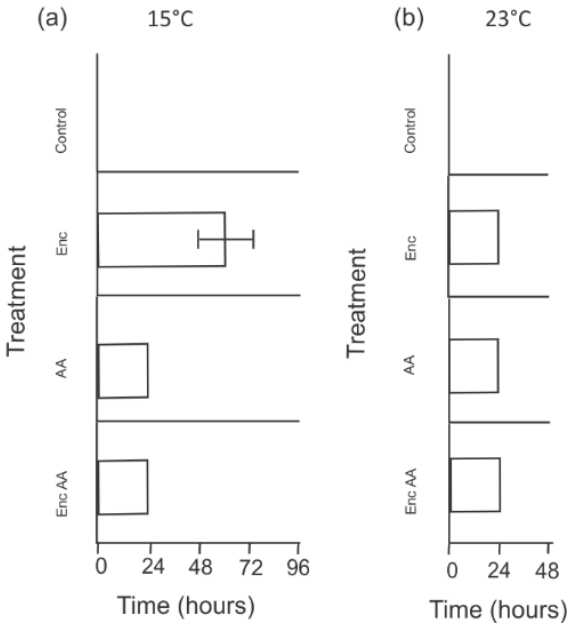

Figure 2.12: Time to 100% mortality (Mean ± SD) of fouling assemblages at a) 15°C and b) 23°C. Treatments: Enc AA = encapsulated seawater with acetic acid, AA = aerated seawater with acetic acid, Enc = encapsulated seawater without acetic acid, control = aerated seawater without acetic acid. Note: No mortality occurred in the control and where no whiskers are presented no variability was observed as all individuals died within 24hours.

2.4 Discussion

Hull fouling is a dominant vector contributing to the ever-increasing introduction and spread of non-native marine species (Floerl et al. 2010, Lacoursière-Roussel et al. 2012). Despite this, there is currently a lack of well-developed antifouling regulations and management of this vector, and invasions by alien fouling species continue to be problematic (Bax et al. 2003, Molnar et al. 2008, Sylvester et al. 2011). Encapsulation is a promising mechanism that may provide an answer to the problem of hull fouling on recreational vessels such as yachts, as it allows vessels to be treated and cleared of hull fouling while in the water (Coutts et al. 2010). This study used laboratory experiments to determine the susceptibility of common fouling organisms to encapsulation, and so aimed to develop recommendations for the effective use of this methodology in the field. It was found that encapsulation was efficient at killing a spectrum of fouling biota within three days under both warm and cool conditions, demonstrating that this mechanism may certainly be useful in the management of fouling on yachts along the South African coast.

This study demonstrated that encapsulation results in total mortality of a variety of taxa, including soft-bodied ascidians, hard-shelled mussels and fouling communities as a whole. The commonly occurring ascidian, *Ciona robusta*, was found to be more sensitive to encapsulation than the hardy mussel *Semimytilus algosus*, as all individuals died within one day in contrast to the mussels which took up to three days to die. This supports the first hypothesis that soft-bodied organisms would be more susceptible to encapsulation than hard-shelled molluscs. This finding is likely a result of the differences in morphology between these taxa. Soft-bodied biota such as ascidians and cnidarians have vulnerable soft, fleshy tissues that offer little barrier to the environment and leave them exposed directly to the marine environment (Pawlik 1993). In contrast, taxa such as mussels, have protective calcium carbonate shells (Falini et al. 1996) that offer a physical barrier between the surrounding environment and the animal. Molluscs utilise this as a defence against adverse conditions, sealing up their valves for extensive periods of time when the environment becomes unfavourable (Shumway 1977, Atalah et al. 2016).

In a previous study by Atalah et al. (2016) considering the mussel *Mytilus galloprovincialis* and the ascidian *C. robusta*, the time taken to reach mortality was the same for the ascidian but much longer for the mussels than in the current study (i.e. 18 days vs three days). It is likely that the difference in results is a reflection of the larger sized mussels (5-7cm) used by Atalah et al. (2016) compared to the 2-3cm sized mussels used in the current study. Additionally, it is possible that this difference in time is a result of different ratios of water to biomass used in their study, as this is not stated in their study.

Increasing temperature is expected to drive mortality of mussels and soft-bodied ascidians in an encapsulation system, through the increased rate of respiration and decomposition which in turn cause a decrease in dissolved oxygen and an increase in sulphides within the system (Atalah et al. 2016). This was evident in the results of this study where an increase in temperature resulted in a decreased survival time for both *S. algosus* and fouling communities. However, this was not the case for *C. robusta*, which died within 24 hours at both cool and warm temperatures, thus partially supporting the second hypothesis that an increase in temperature would reduce survival and treatment time of all taxa. While fouling communities and *S. algosus* survived for at least two days at a cooler temperature, all *C. robusta* individuals died within 24 hours regardless of temperature. As established in the first hypothesis, the vulnerable morphology of soft-bodied organisms makes them sensitive to encapsulation and results in their short survival time compared to hard-shelled biota. It is this vulnerability that precluded any effect of elevated temperature on the survival of *C. robusta*, as even at cool temperatures 100% mortality was reached within a single day.

Previous studies have demonstrated that the addition of acetic acid to the encapsulation system significantly decreases the treatment time of a variety of taxa (Forrest et al. 2007, Roche et al. 2015, Atalah et al. 2016), including both soft-bodied taxa and hard-bodied mussels. The addition of a 4% acetic acid solution to the system in these experiments reduced the treatment time significantly for both the mussels and fouling communities but not for the ascidian. Again, this apparent lack of effect on soft-bodied biota is simply a reflection of their sensitivity to adverse conditions and the fact that they had a short survival time even without the addition of acetic acid. In contrast, the survival time of both the hard, calcareous taxa and the fouling communities was reduced to less than 24 hours when acetic acid was present, regardless of encapsulation. For fouling communities this may be explained by the dominance of soft-bodied taxa whose membranous fleshy bodies would make them susceptible to chemical additives. The death of the mussels within 24 hours when placed in acetic acid reflects the breakdown of the bivalves' carbonate-based shells. These shells consist of an inner calcified shell and an outer proteinaceous layer (Breesan et al. 2014) which is broken down when exposed to acetic acid, resulting in the shells becoming soft and easily broken. When considering responses to ocean acidification, numerous experiments have demonstrated a reduction of growth, shell length and shell thickness in bivalves when exposed to reduced pH (Bamber 1990, Berge et al. 2006, Gaylord et al. 2011, Bressan et al. 2014). However, the pH created by the addition of acetic acid in these experiments was far lower than those used in experiments predicting ocean acidification, and an even stronger effect on mollusc shells was seen, with shells crumbling within 24 hours.

Peters et al. (2017a) sampled yachts from four South African marinas, and established that fouling communities on these vessels tend to be dominated by ascidians, reflecting the communities tested in this study. Notably, 45% of the species recorded on these yachts were present in the fouling communities in the present study (See appendix 1), suggesting that the communities considered in this study offered a good reflection of communities that occur on yachts in South Africa.

Given the positive results from this study, the use of encapsulation to manage and reduce hull fouling on small vessels looks like a promising venture. However, to fully consider the viability of encapsulation, its effectiveness will need to be tested in the field. Current literature suggests that two factors are likely to affect the efficacy of this approach and thus need to be considered when developing it for testing in the field: temperature and the use of additives (Atalah et al. 2016). This study confirmed the role of temperature in determining the time required to kill fouling biota through encapsulation. Notably, at 23°C all taxa besides those in control conditions died within two days, while this was extended to three days at 15°C. Despite this statistical differentiation in treatment time, the difference between treating a yacht for two or three days introduces little difference in terms of operational cost while having a standard approach is likely to facilitate the implementation if encapsulation is rolled out at a national level. As such, it is recommended that field trials apply a treatment time of three days regardless of temperature. This will add value in the form of a single ‘protocol’ which is applicable across a range of temperatures. Having one standard approach to the use of this technique in the field is therefore useful in countries such as South Africa where contrasting coasts present such a range of conditions. The use of a single approach to encapsulation in the field not only allows its use in different conditions, but also makes the implementation of this technique easier from a management perspective, especially in developing countries such as South Africa. When considering the use of a 4% acetic acid solution as an additive for the encapsulation system, this study found that irrespective of temperature, or the fouling biota in question, mortality was reached within one day. This highlights the potential value of acetic acid in encapsulation systems. However, this benefit comes at a cost, as the water containing acetic acid needs to be pumped out of the encapsulation berth after the treatment period and disposed of appropriately. Not only does this elevate the monetary cost involved in this approach, but there are currently no regulations for the disposal of large amounts of marine waste containing acetic acid in South African harbours, making the use of this additive risky. Roche et al. (2015) tested chemical addition to an encapsulation berth in the field and demonstrated the release of these chemicals into the surrounding water. The potential effects of this discharge in surrounding waters are concerning and therefore chemical additions should be avoided where possible.

It is therefore concluded that with proper testing in the field, encapsulation of fouled foreign yachts upon entry into South African harbours may be an important tool in reducing the introduction and spread of marine alien species. It is expected that an encapsulation period of three days, regardless of sea temperature differences along our coastline, will be sufficient to kill fouling organisms on the hull of the yachts and therefore decrease the risk of introductions. Chapter 3 of this thesis, will test those recommendations in the field and develop recommendations for the use of this procedure in harbours around South Africa.

Chapter 3: *In situ* encapsulation of yachts

Abstract

In-water encapsulation of the hulls vessels to kill fouling organisms is a promising technique for managing the introduction and spread of marine alien species. However, it requires scientific investigation as it has not yet been rigorously tested in the field. This chapter therefore aimed to test the results from Chapter 2 in the field and determine a timeframe for encapsulating yachts. In the field, four yachts with a fouling cover of 30-50% were encapsulated before a storm destroyed the encapsulation berth. As too few yachts were encapsulated for sound statistical analyses, pontoons below marina walkways were then encapsulated as proxies for yachts. This provided an opportunity to consider the effect of fouling cover on the time required to kill fouling biota though encapsulation and pontoons with high (80-100%) and low (30-50%) cover were considered. The mean number of days required to reach total mortality on yachts was 4.25 (± 0.5 SD), i.e. longer than the expected three days suggested by laboratory experiments. This is likely a result of a lower biomass to water ratio inside the encapsulation system in the field, and is a reminder that conclusions drawn from laboratory experiments cannot always be directly extrapolated to the field. Fouling on pontoons with both a high and low cover reached total mortality faster than yachts (3.7 (± 0.48 SD) days and 3.8 (± 0.42 SD) days respectively).This likely results from pontoons having a higher biomass to water ratio than the yachts once encapsulated. Notably, fouling cover had no effect on time to mortality on pontoons. Based on these field results it is suggested that the use of encapsulation could offer an effective method for reducing the biosecurity risk associated with yachts. However, to ensure efficacy, an encapsulation period of five days is recommended. In addition, mortality should be confirmed before the system is removed from the yacht.

3.1 Introduction

The relocation of an organism into a new region directly from its native range is called a primary introduction (Hewitt et al. 2009). In addition to these primary introductions, secondary spread from the point of introduction and the potential subsequent establishment in these new areas are important apprehensions in invasion biology. The secondary spread of invasive organisms may be a result of natural dispersion, for example through adult migration, or larval dispersion, or through human-mediated dispersal (Hewitt et al. 2009). While little can be done to address natural dispersion once organisms have established in new regions, human-mediated spread of organisms is preventable to a large extent. This prevention of human-mediated introductions and spread of alien species is one of the most effective ways of mitigating the impacts of biological invasions (Sylvester et al. 2011).

Hull fouling refers to the growth of organisms and plants on the surfaces of vessels and infrastructure submerged in sea water (Yebra et al. 2004). Organisms commonly transported via hull fouling are sessile organisms and species living in, on and between other organisms in benthic communities (Minchin & Gollasch 2003). In addition to its importance as a vector for the introduction and transfer of non-native species, hull fouling also has adverse effects on the fuel consumption, drag and weight of vessels (Champ 2000). Although hull fouling is slowly being given more attention in the literature (Minchin & Gollasch 2003, Coutts & Taylor 2004, Coutts et al. 2010, Roche et al. 2015), and there are IMO guidelines for biofouling management, unfortunately, this vector is unregulated in most countries worldwide (Gollasch 2002).

This lack of regulation is especially true for small, recreational vessels such as yachts, as most attention is given to large commercial shipping vessels and the importance of yachts as vectors not yet been properly assessed (Floerl et al. 2005). However, it has come to light in recent studies that the relative extent of biofouling communities on yachts may often be greater than on large commercial ships (Coutts 1999). In addition to this, small recreational vessels often travel long distances and at low speeds, which along with the lack in regulations, makes them model vectors for fouling species (Minchin et al. 2006). Furthermore, the majority of recreational vessels are docked in marinas or harbours when they are not being used (Floerl & Inglis 2003a). As a result of these extended docking times, these boats are very likely to support dense fouling communities (Floerl & Inglis 2003a). One of the biggest fouling-related problems with small recreational vessels is the lack of profit-related incentives for the removal of organisms from hulls or the renewal of anti-fouling paint when compared with larger commercial vessels (Floerl & Inglis 2003a). This lack of incentive

results in more variable renewal and removal intervals (Floerl & Inglis 2003a) with some yachts, especially those that are used infrequently, being subject to irregular maintenance.

The presence of alien species in hull fouling assemblages on recreational boats has been equated to the transfer of these species (Floerl & Inglis 2005). Reflecting this, the presence of yachts has been highlighted as an important indicator of alien species numbers in South African harbours (Peters et al. 2017b). Although this does not directly link yachts as the causal agent of spread, it does highlight the important link between yachts and the invasion of marine alien species (Floerl et al. 2005, Clarke Murray et al. 2011, Ros et al. 2013). The finding by Peters et al. (2017b), coupled with the lack of regulations addressing yacht fouling in South Africa and most other regions, highlights the need to develop techniques which effectively control the spread of alien species via this vector. Although the development of anti-fouling paints looked to address and prevent hull fouling, the most effective paints, those containing tributyltin (TBT), were banned in the early 2000s (International Maritime Organisation 2010) as a result of their harmful effects on non-target organisms. One example of these harmful effects is the induction of imposex in female marine gastropods around the coast of South Africa (van Gessellen et al. 2018). It is notable that this study recorded these effects and linked them to TBT levels in 2013 and 2014, despite the compound having been banned for more than a decade. While it is clear that the ban of TBT-containing antifouling paints was necessary, this move to protect the marine environment, coupled with a lack of management of this vector, has unintentionally led to a resurgence in hull fouling and its importance as a vector for human mediated transfer of marine species (Fofonoff et al. 2003).

In-water encapsulation of vessels is a relatively new and potentially viable approach to managing fouling (Coutts et al. 2010). However, it requires scientific investigation as it has not been tested rigorously on yachts (but see Roche et al. 2015 and Atalah et al. 2016). As described previously, encapsulation is promising, as it allows organisms present on the vessel hull to be treated in situ, negating the need to take vessels out of the water for cleaning (Roche et al. 2015, Atalah et al 2016). The implications of avoiding having to dry-dock and clean vessels out of the water are that fouled vessels can be treated immediately after entering new regions, lessening the opportunity for fouling organisms to spread into the surrounding environment. Encapsulation could therefore play an important role in the development of management regulations in ports and marinas (Roche et al. 2015) that form the port of entry for international yachts. However, as highlighted previously, very few studies have tested this technique on yachts in the field (Atalah et al. 2016), resulting in the lack of a protocol with detailed field application recommendations. Studies testing an

encapsulation technique in the laboratory are far more controlled with regards to environmental conditions as well as the scale at which variables can be tested. While these controlled conditions enable sound experimental testing of variables expected to mediate the effectiveness of encapsulation, field conditions can be unpredictable and variable, resulting in uncertainty around the applicability of the methodology in the field. There is therefore a need to develop a sound protocol that is founded on findings of laboratory tests but is also rigorously tested for efficacy in the field. This will ultimately provide sufficient evidence upon which to base recommendations for the practical application of encapsulation.

Chapter aims

Considering the gaps in knowledge highlighted above, the aim of this chapter was to apply the time frame identified in the laboratory in Chapter 2 when encapsulating yachts in the field, and so test the appropriateness of this time period for ensuring successful encapsulation. Due to logistical challenges caused by a winter storm, only a limited number of yachts could be encapsulated. As a result, pontoons under marina walkways were used as proxies for yachts. Based on the results from the previous chapter, this chapter tested the *a priori* hypothesis that an encapsulation period of three days would result in total mortality of fouling organisms.

3.2 Methods and materials

Construction of encapsulation berth

An encapsulation berth measuring 9m in length, 4m in depth and 4m in width was manually constructed from sheets of 250 micron plastic. This size was chosen as >80% of yachts in South African marinas could be accommodated in a berth with these dimensions (data from Peters 2017a). Seams were bonded using LTS90B vacuum bag sealant tape and duct tape was used for reinforcement. Stainless steel eyelets were then punctured into the encapsulation berth along the top in order to secure ropes for the attachment of the berth to the vessels. Construction took place in the Department of Botany and Zoology at Stellenbosch University. Unfortunately during a rough storm that took place after the encapsulation of only four vessels, the encapsulation berth was damaged beyond repair. As too few yachts had been encapsulated to enable sound statistical analyses, the decision was made to encapsulate marina walkway pontoons. This was facilitated by the fact that the pontoons were small enough so that new encapsulation berths could be made within the timeframe of this thesis. Plastic pontoons on the underside of marina walkways were

deemed appropriate proxies for yachts as they accounted for one third of submerged surface area of yachts, reached a depth of at least 1.5m (the depth within which most fouling occurs on yachts in South African marinas (Robinson pers. obs.)), and supported the same alien fouling species as recorded on the yachts (see results section).

Pre-encapsulation sampling

Prior to encapsulation, an experienced scientific diver estimated the percentage cover of fouling on each vessel and pontoon. Additionally, the presence of alien fouling species was noted for each yacht and pontoon. Species were identified from a pre-complied target list of alien fouling species known from yachts in South African harbours (Peters et al. 2017a).The list included the sea sponge *Suberites ficus*, the triangle barnacle *Balanus trigonus*, the European crab *Carcinus maenas,* the Mediterranean mussel *Mytilus galloprovincialis*, the Chilean mussel *Semimytilus algosus* and the ascidians *Ciona robusta*, *Clavelina lepadiformis*, *Microcosmus squamiger, Botryllus schlosseri, Ascidia aspersa* and *Styela plicata*. Species on this list were selected based on their alien status in South African waters and their large, conspicuous nature, which make them easy for scientific divers to identify within fouling communities. The use of such target lists is particularly useful for rapid surveys in marina settings (Minchin et al. 2016).

In order to test the assumption that pontoons offer good proxies for yachts, alien fouling community composition was compared between yachts and pontoons with low and high levels of fouling. This was done by applying an ANOSIM in Primer 6 using the presence / absence data gained during the diver surveys.

Encapsulation process

After surveying the vessel, the diver pulled the berth up over the hull of the vessel and ropes threaded through steel eyelets were used to secure the berth onto the vessel on both sides by a person on board the yacht. As much water as possible was then pumped out to minimise the biomass to water ratio within the berth. The same process was carried out for pontoons, where ropes were attached to the planks on marina walkways.

Three yachts with a fouling cover of between 30-50% were encapsulated in Port Owen Marina on the West Coast. This level of fouling represents the class of fouling cover present on most yachts in South Africa marinas (Peters et al. 2019). As a result of an influx of freshwater into the marina due to unexpected rains up river, all further encapsulations were done at Royal Cape Yacht Club in Table Bay. Here, a fourth yacht with a fouling cover of 40% was encapsulated. All encapsulated yachts were made of fibreglass and had antifouling

coatings, but the antifouling was likely ineffective, as there was fouling present on the yachts. This layer of fouling present on the yachts forms a barrier and as a result, leaching of biocides from the antifouling coating into the encapsulation berth would be minimal. After the encapsulation of these four yachts, bad weather destroyed the berth. Following this, pontoons were used to test encapsulation in the field. This provided an opportunity to assess the effect of fouling cover on the efficacy of encapsulation. Ten pontoons with a high fouling cover (80-100%) and a further ten with a low cover (30-50%) were encapsulated. The low fouling cover was chosen as it represents typical fouling levels of yachts.

Post-encapsulation sampling

In order to determine the timeframe needed to reach total mortality of fouling biota encapsulated in the field, mortality of organisms was assessed visually through the berth every day until total mortality was reached. Organisms were considered dead if no response was observed after being prodded through the berth. Using R (version 3.4.2), the time taken to reach total mortality was analysed using a GLS model (packages nlme and MuMin). Structure (yachts, pontoons with high coverage and pontoons with low coverage), and dissolved oxygen, ammonia and sulphide levels inside of encapsulation berths on the day on which total mortality was recorded were investigated as potential predictors . The best fit model was chosen using Akaike information criterion values. Significance levels are indicated in the results section below.

Water samples were taken daily from six randomly selected positions inside and outside of the encapsulation berth. For each sample, dissolved oxygen, pH, ammonia and sulphide levels were measured. These variables were measured through time to track the depletion of oxygen and the build-up of waste products (i.e. ammonia and sulphide) through time. Water quality was assessed inside and outside of encapsulation berths for both yachts and pontoons. Dissolved oxygen, ammonia and sulphide levels on the initial day of experiments were compared to those on the day on which total mortality occurred using t-tests or Mann-Whitney tests, depending on the nature of the data. Separate analyses were conducted for samples collected inside and outside of the berths as interactions between these positions were not of interest.

3.3 Results

Community composition on yachts and pontoons

In total eight alien fouling species were recorded. These were the mussels *Mytilus galloprovincialis* and *Semimytilus algosus*, as well as the ascidians *Ciona robusta*, *Clavelina lepadiformis, Microcosmus squamiger*; *Botryllus schlosseri*, *Ascidia aspersa* and *Styela plicata*. Notably all species were recorded on yachts and pontoons and community composition did not vary between the structures (ANOSIM, R=-0.085, p=0.91, Figure 3.1). This validated the assumption that pontoons offer a good proxy for yachts.

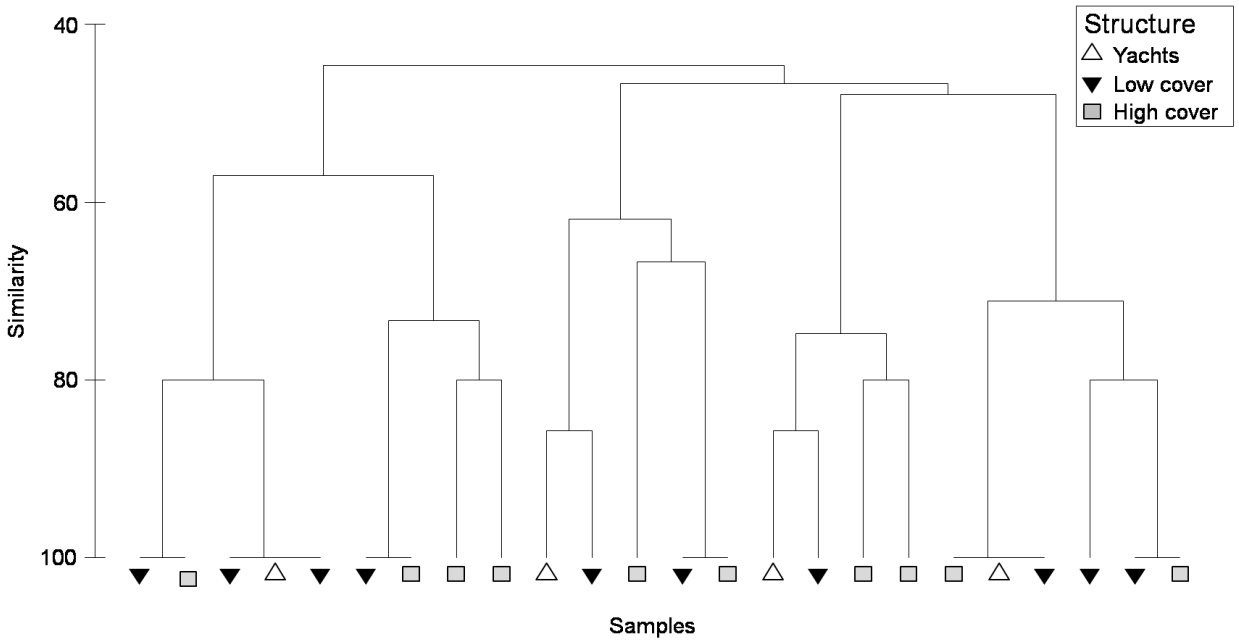

Figure 3.3: Cluster analysis on the similarity of yachts (white triangles) as well as pontoons with a high fouling cover (grey squares) and low fouling cover (black triangles). Fouling communities on the different structures do not differ significantly (ANOSIM, R=-0.085, p=0.91).

Time to mortality

The average time taken to reach total mortality on pontoons with high and low cover was 3.7 (± 0.48 SD) days and 3.8 (± 0.42 SD) days respectively, whereas for yachts this time was 4.25 (± 0.5 SD) days (Figure 3.2). All of these structures required a longer encapsulation time than the three days which was recommended based on the results in the laboratory.

The best fit GLS model was significant and included structure, ammonia, sulphide and dissolved oxygen as predictors for time to mortality. It was found that structure was the only significant main effect (Table 3.1, $p<0.001$, Fig 3.2). Yachts had a longer time to mortality than pontoons with both a high (coefficient=0.50, $t=6.28$, $p<0.001$) and low fouling cover (coefficient=0.43, $t=5.73$, $p<0.001$). Notably there was no difference in time to mortality between the high and low fouled pontoons ($p>0.05$).

Table 3.1: Statistical results from the generalised least squares model considering predictors of the time taken for fouling communities to reach total mortality.

Factor	F-value	p-value
Structure	21.34	<0.001
Dissolved oxygen	140	0.24
Ammonia	2.81	0.10
Sulphide	0.002	0.96

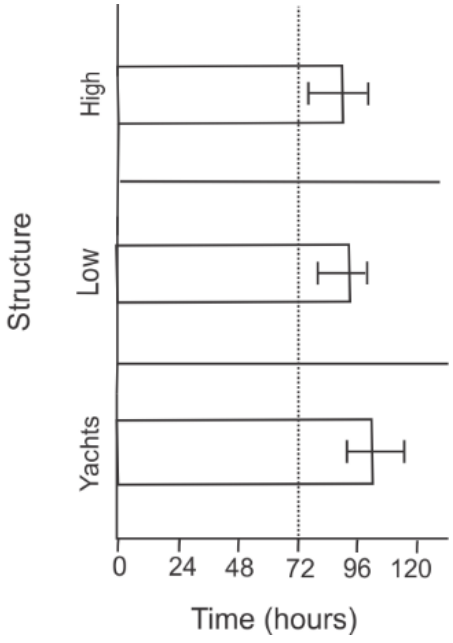

Figure 3.2: Mean ± SD time taken to reach total mortality for yachts as well as pontoons with high and low fouling cover in the field. Dotted line represents the expected effective encapsulation period of three days based on laboratory experiments.

Water quality during encapsulation

For yachts and pontoons with both a high and low fouling cover, no differences through time were detected in outside water samples surrounding the encapsulation berth (T-test, $p>0.05$ in all cases, see appendix 2).

The dissolved oxygen, ammonia and sulphide levels inside the berth, however, differed significantly between the initial and final days of encapsulation (Mann-Whitney, $p<0.001$ in all cases, see appendix 2). This trend held for yachts and all pontoons. For all three structures, the dissolved oxygen levels decreased within the encapsulation berth, both ammonia and sulphide levels rose within the system (Figure 3.3)

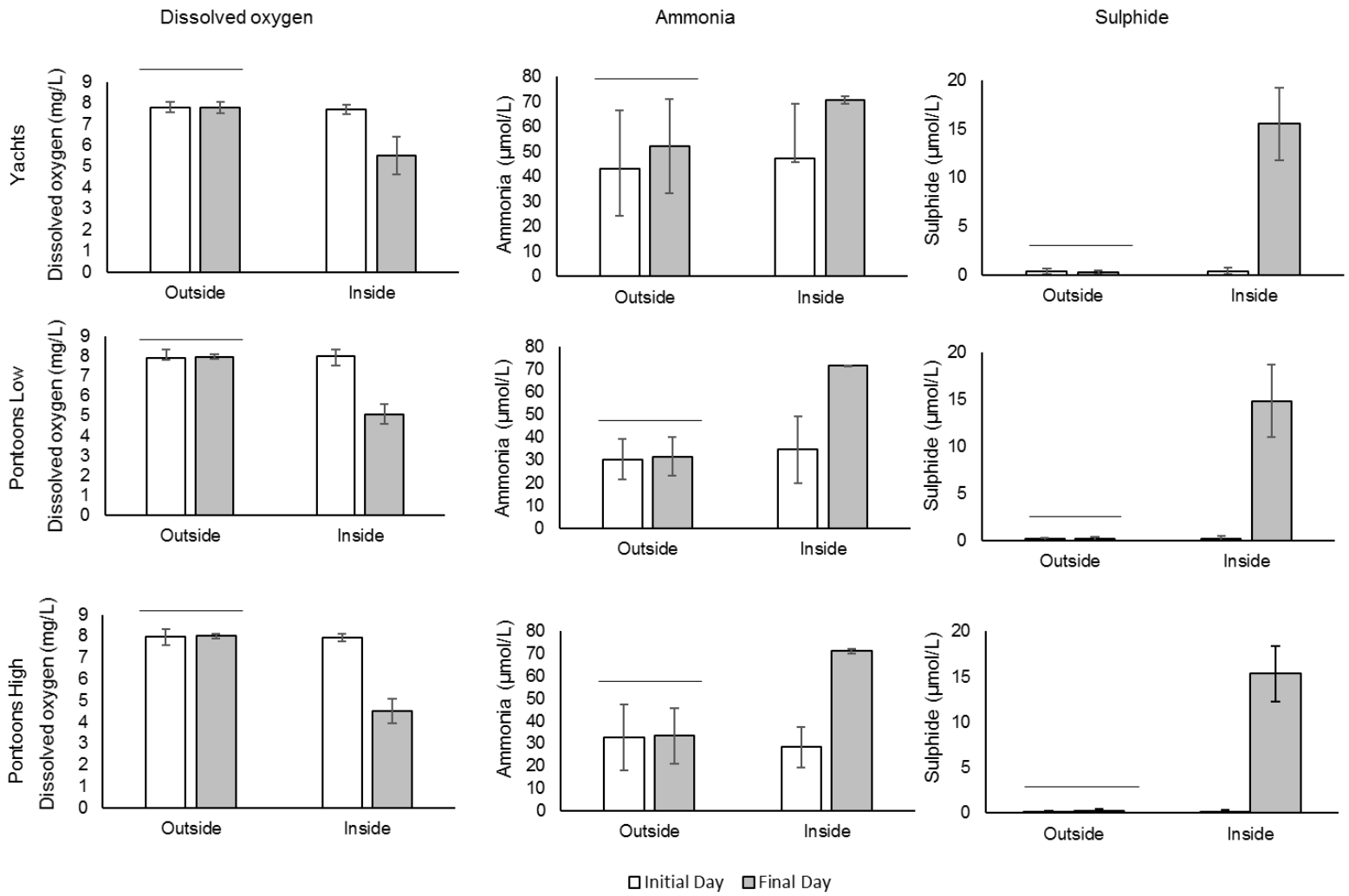

Figure 3.3: Mean ± SD levels of dissolved oxygen, ammonia and sulphide inside and outside the encapsulation berth on the first and final day of encapsulation. Line connects bars that do not differ significantly ($p<0.05$).

3.4 Discussion

Encapsulation has been highlighted as a potential management tool for hull fouling on vessels (Coutts et al. 2010, Atalah et al. 2016). This may be especially useful for the *in situ* treatment of small, recreational vessels such as yachts. However, studies testing this technique on these vessels in the field are lacking. By encapsulating yachts and pontoons in harbours, this study demonstrated the potential of this technique for treating fouling on recreational vessels while the vessels remain in the water. The most notable finding of this field study, however, was that the timeframe suggested by laboratory experiments as effective in achieving total mortality (i.e. three days) was too short. Instead, this field study found that five days of encapsulation were required to kill all fouling biota.

On average, 4.25 (± 0.5 SD) days were required for fouling communities to reach total mortality on yachts, suggesting that encapsulation systems should be left in place for at least five days to be effective. Interestingly, despite pontoons appearing to offer good proxies for yachts when testing encapsulation, communities on these structures died faster than on yachts regardless of their cover (i.e. 3.7 ± 0.48 SD days for high cover and 3.8 ± 0.42 days for low cover). The difference is likely related to the ratio of fouling biomass to water trapped within the encapsulation system. As encapsulation works by the creation of a closed system in which existing oxygen is depleted by fouling organisms and waste products build up, the lower the ratio of biomass to water, the faster mortality is likely to be achieved. The encapsulation berths made for the pontoons were cube shaped like the pontoons and thus ensured tighter fit and the easy exclusion of most of the water from the system. In contrast, structures on yachts, such as the keel and propeller, make having a tight-fitting berth difficult, especially when one berth is made for use on several yachts of different sizes. As a result, a large amount of water is unavoidably encapsulated with the yacht and the ratio of water to fouling biomass is increased, resulting in a longer encapsulation period needed to reach total mortality. This demonstrates the need to test methodologies in the context that they are likely to be applied in, as logistical constraints may only be discovered in that context. Had yachts not been encapsulated in this study and an encapsulation period been suggested based purely on laboratory results, the recommended timeframe would have been too short.

The encapsulation period required to kill communities on pontoons and yachts in this study was notably shorter than the timeframe reported by Atalah et al. (2016) for achieving mortality of communities on plates. In their study, four-year-old communities with an average cover of 84% were encapsulated on experimental blocks and showed a decrease in percentage cover within 48 hours, but only achieved total mortality after 14 days. It could be that this is a result of differences in the ratio of biomass to water in the present study compared to theirs, but the ratio used in their experiments is not stated. The communities

encapsulated on pontoons in this study were three years old (Dipenaar pers. comm.) and were dominated by similar taxa to those in Atalah et al. (2016) and included colonial ascidians and bryozoans. The timeframe of four days, which was effective in achieving mortality on the pontoons in this study, was therefore unexpectedly short but aligns with the shorter time periods noted in the laboratory findings of Chapter 2.

Pontoons with both high and low fouling cover required a maximum of four days to reach total mortality, with no statistical difference in the time to mortality between these two groups. This lack of difference indicates that the amount of fouling on the low cover treatment was still sufficient to reduce oxygen levels and increase waste product levels, creating a toxic environment within the encapsulation berth in the same period of time. This was reflected in significant declines in dissolved oxygen to similar levels in both these experimental treatments. Nonetheless, the timeframe required for encapsulation to be effective would be expected to increase with very low fouling cover or where only niche areas on vessels are fouled, as the biomass of organisms driving a decrease in dissolved oxygen and increases in waste products like ammonia and sulphide will be reduced. This in turn will result in metabolic processes within the system slowing down and encapsulation potentially not being as effective or taking much longer to be effective.

Unexpectedly, measures of dissolved oxygen, ammonia and sulphide were not identified as statistical predictors of time to mortality. This may be a result of the water measures and mortality only being recorded once daily. Taken on a finer timescale, providing better resolution, they may emerge as predictors. Nonetheless, these daily water measures demonstrate that as expected, dissolved oxygen levels declined in all encapsulation treatments while sulphide and ammonia levels rose.

As demonstrated in Chapter 2, as well as in existing literature (Atalah et al. 2016), an increase in temperature shortens the time to mortality in encapsulation systems. As such, it would be expected that an even shorter encapsulation time than the five days found in this study may be effective at achieving total mortality in warmer areas along the South African east coast. However, due to administrative constraints in South African harbours and the diving effort and support required, the implementation of one approach in all national harbours is logistically simpler than the adaptation of the approach for individual harbours or regions. In addition, the monetary and logistical cost of increasing the encapsulation time by one or two days in cooler areas is minimal. It is therefore recommended that one standard encapsulation period be implemented in all South African harbours. As these field experiments were conducted in harbours on the West coast of South Africa where temperatures are coolest (Smit et al. 2013), it is likely that the timeframe determined from these experiments would be as effective in all other harbours, even those on the warmer east coast. Based on the results of this study, an encapsulation period of five days is thus

recommended, assessing mortality of encapsulated fouling organisms before removal of the berth. This assessment is most important in cooler regions or on yachts with a lower fouling cover, as an additional day of encapsulation may be required.

Logistical challenges associated with the implementation of encapsulation as a management tool for hull fouling on yachts in South African harbours include the need for a competent and experienced diver and the design and production of a sturdy encapsulation berth. The diver involved in the encapsulation procedure is responsible for an initial estimation of the fouling cover on the yacht, as well as the assessment of mortality during the encapsulation period, which requires some level of experience with invasive species. Moreover, the actual encapsulation of the yachts, while relatively straightforward, requires concentration and constant communication between the diver and the person on board the yacht, as the berth needs to be lifted up by the diver and attached on either side by the person on board. Logistical constraints occurred in the making of the berth in the current study. The intention was for an encapsulation berth to be made following a similar design to Roche et al. (2016). This would have enabled the steering of the yacht into the berth, notably reducing the logistical challenges of setting up the encapsulation system. However, after extensive negotiations with commercial companies that produce inflatable boats, none were willing to become involved in the project and the berth had to be made manually at Stellenbosch University. This entailed the joining of sheets of plastic and it was these seams that later tore irreparably in a bad winter storm. These logistical challenges may have been avoided had the berth been constructed by a commercial company. For encapsulation to be viable in South Africa, the production of the berth by a commercial company will have to be secured.

In conclusion, encapsulation holds promise as a management tool to minimise the introductions and spread of marine alien species. However, the timeframe which was required in order to achieve total mortality of the fouling on yachts was notably longer than predicted by laboratory experiments, probably as a result of a lower ratio of fouling biomass to water present in the encapsulation system in the field. This provides a reminder of the differences between laboratory and field conditions and why conclusions drawn from laboratory experiments cannot always be directly applied in the field. It is concluded that to be effective on yachts in the field, encapsulation berths be left on yachts for five days at which time mortality should be confirmed before removing the berth.

Synthesis

The introduction of species to new regions can have detrimental economic and ecological impacts in recipient environments (Kumschick & Nentwig 2010, Pysek et al. 2012, Kumschick et al. 2017). The number of introductions of alien species is increasing rapidly (Kumschick et al. 2016) as a result of both direct and indirect human actions (Jeschke et al. 2014). In marine systems, the dominant pathway responsible for human-mediated introductions and spread of non-native species is shipping (Hewitt et al. 2009), with two principal vectors, ballast water and hull fouling (Sylvester et al. 2011). Antifouling paints are used to minimise hull fouling. The most effective antifouling paints contained tributyltin (TBT), however, once the harmful effects of TBT on non-target organisms were discovered, paints containing this biocide were banned (IMO 2001). Due to TBT being very effective, few studies considered alternative measures to reducing hull fouling (Minchin & Gollasch 2003). This led to a lack of commercially available effective alternative solutions once TBT was banned. As a result vessels need to routinely be removed from the water for cleaning. This process is costly for vessel owners and requires appropriate infrastructure in harbours. The lack of well-developed and effective antifouling techniques that can be applied while vessels remain in the water thus remains a challenge to authorities tasked with managing the spread of marine alien species (Piola et al. 2009). Efforts have been made to manage hull fouling internationally, such as with the existing IMO guidelines and more recently the development of Craft Risk Management Standard for Biofouling (CRMs) in New Zealand which require vessel owners to have biofouling management plans and adhere to regulations surrounding the permitted levels of fouling present on vessels. However, focus is on higher risk vessels such as commercial and research vessels. In addition, regulations such as the above and the implementation of these are lacking in South Africa.

Encapsulation, the wrapping of a structure, usually in plastic, depriving fouled organisms of light, oxygen and food (Coutts & Forrest 2007), has recently been suggested as a promising technique for killing fouling on a range of structures (Roche et al. 2015, Atalah et al. 2016). One of the reasons for its potential is that encapsulation allows vessels to be treated *in situ*, negating the need to remove them from the water (Roche et al. 2015). However, while encapsulation has been studied on a number of individual organisms under laboratory conditions, it has not yet been tested rigorously on yachts in the field. There is thus a need for a more detailed consideration of this topic to support the development of guidelines for applying this technique as a tool for managing hull fouling as a vector of marine alien species.

Chapter 2 used laboratory experiments to determine the susceptibility of a range of common alien fouling organisms to encapsulation. The main objective of this chapter was to use laboratory experiments to determine a timeframe for encapsulation of yachts in the field. The invasive mussel *Semimytilus algosus*, invasive ascidian *Ciona robusta* and fouling communities containing a variety of alien species including *Amphibalanus amphitrite*, *Jasus lalandii*, *Bugula neritina*, *Ciona robusta* and *Botryllus schlosseri* among others were encapsulated in a laboratory setting. Treatments included an aerated control, encapsulation in seawater, aerated seawater with a 4% acetic acid solution and encapsulated seawater with a 4% acetic acid solution. All controls survived throughout the experiments. All individuals and communities in acetic acid died within 24 hours regardless of encapsulation. Mussels in the encapsulation treatment took significantly longer to die than soft bodied ascidians and communities in the same treatment. This likely reflects the protection offered to mussels by their calcareous shells. However, total mortality of all communities and individuals in the encapsulation treatment was reached within three days. As such, this was the timeframe carried forward into Chapter 3 for testing encapsulation of yachts in the field.

Using the results from Chapter 2, Chapter 3 aimed to test an encapsulation timeframe of three days on yachts in the field and ultimately provide recommendations for the use of encapsulation as a management tool. An encapsulation berth measuring 9m in length, 4m in depth and 4m in width was manually constructed from sheets of 250 micron plastic at Stellenbosch University. After encapsulating four yachts, a bad winter storm damaged the berth. Pontoons were then considered as proxies for yachts. Pontoons with a low fouling cover of 30-50% and pontoons with a high fouling cover of 80-100% were encapsulated, providing an opportunity to assess the effect of fouling cover on the required time for encapsulation to be effective. The period of three days recommended by laboratory experiments was too short to achieve total mortality on yachts in the field. This highlights that methodologies require thorough testing in the context in which they will be used in order to provide accurate recommendations for their application. Yachts required an average encapsulation period of 4.25 (± 0.5 SD) days to reach total mortality, while pontoons required an average of 3.7 (± 0.48 SD) and 3.8 (± 0.42 SD) days to reach total mortality for high and low fouling cover respectively. While the required encapsulation period for yachts was significantly longer than for pontoons, there was no statistical difference between the encapsulation time required for pontoons with a high and low fouling cover. The results from this chapter suggest that it is the biomass to water ratio which is important when applying encapsulation and that this ratio is an important driver of the time taken to reach mortality. Structures on yachts such as the keel and propeller make having a tight-fitting berth challenging and it is very difficult to minimise the water enclosed in the system. This results in the encapsulation of a large amount of water with the yacht and an increased ratio of water

to fouling biomass. The cube-shape of pontoons, on the other hand, enable the use of tight-fitting berths which result a lower ratio of water to biomass, ultimately shortening the period of encapsulation required to reach total mortality.

While the use of encapsulation has potential as a tool in managing hull fouling as a vector for the introduction and spread of marine alien species, this technique is unlikely to be an appropriate tool for routine antifouling maintenance on yachts. This is because of a number of practical and logistical considerations. Firstly, although organisms die during encapsulation, they tend not to fall off the hull. While this minimises the release of dead fouling organisms into the surrounding area as the encapsulation berth is removed, this means that to be used as a routine antifouling measure, yachts would have to be encapsulated and then scraped, which requires either being taken out of the water after encapsulation to be cleaned or a diver to scrape the vessels in the water post-encapsulation. Routine cleaning and antifouling maintenance of yachts already often requires being dry docked, and encapsulation prior to scraping therefore only adds extra demands before dry docking a vessel. Secondly, using encapsulation for routine antifouling maintenance would require the construction of a large number of encapsulation berths for all harbours (i.e. 19 in the South African context) and the maintenance and upkeep of these berths. This would offer notable costs to an already underfunded biosecurity department. Lastly, besides the diving effort required to survey and encapsulate yachts, large groups of support staff would be needed in order to encapsulate and monitor vessels during the process, making routine encapsulation a labour intensive and expensive exercise. Encapsulation would, however, be useful when implemented to address fouling on foreign vessels visiting South African waters, as well as on national vessels returning home from overseas. This would be particularly useful as a way to kill any non-native organisms attached to the vessels before they have an opportunity to spread into the surrounding environment. The number of foreign recreational vessels entering South Africa annually is relatively low at 200-300 per annum (Robinson, unpublished data), making the encapsulation of each of these vessels feasible. Moreover, during current customs practices for foreign vessels arriving at ports in South Africa, yachts are often docked at the customs jetty for up to seven days while awaiting customs clearance, providing the opportunity for encapsulation that would not further infringe on the stay of the yacht. Encapsulating foreign vessels during this time would thus centralize the administration of customs and biosecurity. However, for a national encapsulation program to be effective, this application of encapsulation requires the standardized construction of an encapsulation berth. In order to provide the most durable and versatile berths possible, it would be best to involve government, academics and commercial industry in the construction and implementation of berths. The collaboration between these three parties has been suggested as a promising management approach to biofouling (Davidson et al. 2016). The construction

of a sturdy berth similar to one used by Roche et al. (2016) will ensure logistical constraints such as bad weather have a minimal effect on operation. In addition, berths will be needed at all major ports in South Africa receiving international vessels, these include Durban, Richard's Bay, Saldanha Bay, Table Bay and Hout Bay. As a result of the geographic orientation of the South African coastline, these are the only harbours which yachts use as ports of entry. To implement the use of encapsulation on foreign vessels, a skilled diver with knowledge of bioinvasions will be required in order to identify alien and invasive species present on the vessels as well as assess the mortality of these organisms. The use of a berth into which vessels can be steered will make its application easier and minimise the requirements on divers.

Adaptive management is an approach in which the uncertainty of a process or technique is reduced through time with constant reviewing of the process. This enables consistent improvement of the technique and allows decisions to be made based on outcomes from the technique being tested. As the four yachts encapsulated in this study is too low a number to provide sound statistical analyses, adaptive management of encapsulation on yachts in harbours would provide further insight and allow the optimisation of the use of encapsulation as a management tool through time.

In conclusion, it is recommended that a national program be implemented for the encapsulation of foreign vessels in South African harbours. This should take place as part of the procedures undertaken while vessels are docked at the customs jetty in major ports. The use of an encapsulation berth at each of the above-mentioned ports of entry into South African waters will ensure that all foreign yachts are encapsulated, a move that will reduce the biosecurity risk posed by these vessels. With the assistance of a competent diver, the vessels should be encapsulated for a period of five days, after which a mortality assessment should be undertaken before deciding if the yacht can be removed from the berth. With the commercial construction of durable encapsulation berths for each port and an evidence-based management approach to continual improvement of the encapsulation processes, this emerging technique has the potential to considerably reduce the biosecurity risk posed by yachts visiting South African harbours.

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Appendices

Appendix 1

A list of species present in fouling assemblages. Status following Robinson et al. (2016).

Taxa	Status in South Africa
Kingdom: Animalia	
Cirripedia	
Amphibalanus amphitrite	Native
Austromegabalanus cylindricus	Native
Isopoda	
Paridotea reticulate	Native
Decapoda	
Jasus lalandii	Native
Bryzoa	
Menipea triserata	Native
Bugula neritina	Invasive
Bugula dentate	Invasive
Echinoidea	
Parechinus algulosus	Native
Ascidiacea	
Ciona robusta	Invasive
Botryllus schlosseri	Invasive
Botryllus magnicoecus	Native
Clavelina lepadiformis	Invasive
Ascidia aspersa	Alien
Kingdom: Chromista	
Ochrophyta	
Scytosiphon simplicissimus	Native
Kingdom: Plantae	
Chlorophyta	
Ulva spp.	Native
Rhodoophyta	
Porphyra capensis	Native
Schizymenia apoda	Native
Pachymenia orbitosa	Native

Appendix 2

Appendix 2.1: Statistical results from Mann-Whitney U test and t-test comparing water measures (a) inside and (b) outside the encapsulation berth on the first and final day of experiments for pontoons with a high fouling cover.

High Pontoons		
(a) Inside		
Factor	p-value	w
Ammonia	<0.001	3600
Dissolved oxygen	<0.001	0.001
Sulphide	<0.001	3600
(b) Outside		
Factor	p-value	t-value
Ammonia	0.77	0.29
Dissolved oxygen	0.44	0.78
Sulphide	0.10	1.68

Appendix 2.2: Statistical results from Mann-Whitney U test and t-test comparing water measures (a) inside and (b) outside the encapsulation berth on the first and final day of experiments for pontoons with a low fouling cover.

Low Pontoons		
(a) Inside		
Factor	p-value	w
Ammonia	<0.001	3599
Dissolved oxygen	<0.001	0.001
Sulphide	<0.001	3600
(b) Outside		
Factor	p-value	t-value
Ammonia	0.4	0.85
Dissolved oxygen	0.32	0.99
Sulphide	0.10	1.65

Appendix 2.3: Statistical results from Mann-Whitney U test and t-test comparing water measures (a) inside and (b) outside the encapsulation berth on the first and final day of experiments for yachts

Yachts		
(a) Inside		
Factor	p-value	w
Ammonia	<0.001	498.5
Dissolved oxygen	<0.001	16.5
Sulphide	<0.001	576
(b) Outside		
Factor	p-value	t-value
Ammonia	0.15	1.48
Dissolved oxygen	0.82	-0.22
Sulphide	0.23	-1.21