Managing the Unwanted Organism *Sabella spallanzanii* (Gmelin, 1791): investigating how desiccation, fragmentation and acetic acid can be used to manage this pest on aquaculture facilities.

A thesis submitted in partial fulfilment of the requirements for the degree of

Master of Science (Research)

in Biological Sciences at The University of Waikato by

Staci Louise King

THE UNIVERSITY OF
WAIKATO
Te Whare Wānanga o Waikato

2017

Abstract

Human activities have enabled non-indigenous marine species (NIMS) to cross geographic barriers outside their natural dispersal range. As a novel habitat, marine aquaculture facilities often grow NIMS, or are fouled by NIMS and facilities with poor quarantine practice during harvest and through domestic transfers of equipment and product may inadvertently act to vector NIMS. Furthermore, biofouling NIMS can cause significant economic loss to industry by reducing the quality of the farmed product and increasing production costs.

The objective of this study was to aid the aquaculture industry with how they manage the marine polychaete pest species *Sabella spallanzanii*. Specifically, in order to determine the possibility of *S. spallanzanii* being spread after harvesting, the survivorship of *S. spallanzanii* to typical mussel farming operations was examined in two experimental procedures: desiccation and fragmentation. An additional experimental procedure examined acetic acid as a potential treatment option that would effectively eliminate *S. spallanzanii* while having minimal effect on the cultured product.

Sabella spallanzanii is highly resilient to the typical re-seeding and harvest operations that occur in mussel aquaculture. Specimens survived upwards of 24 hours air exposure in desiccation treatments: a longer time frame than what would occur during re-seeding or the harvest process and subsequent landing of stock and transport to processing facilities. Long term survival and regeneration of body parts within 28 days was evident following fragmentation: a simulation of the potential disturbance caused by the harvest process. Immersion in a 5% solution of acetic acid for 1 minute killed 75% of *S. spallanzanii* with no effect on mussel survivorship. This is a promising control method that could contain *S. spallanzanii* and have minimal impact on harvest production time.

The results of these experiments will enable biosecurity and marine farm managers to make informed decisions about the management, containment and treatment of *Sabella spallanzanii* and prevent its secondary spread to new geographic areas. However, effective mitigation relies on a rapid response and a strong commitment between stakeholders to achieve a common goal.

Acknowledgements

As a “mature” student trying to raise a family, I have often compared my MSc journey to climbing a mountain when you are extremely unfit and have ‘flu. Luckily for me, I had an awesome support crew with me every step of the way, and I can’t thank them enough: a few words here feels completely inadequate.

Firstly, thanks to my supervisors; Marnie Campbell, Phil Ross and Chris Battershill. Marnie, your confidence in me and unwavering belief that I would make it to the top kept me going, as did those chunks of gold on the path every month. Phil, you put the mountain in front of me in the first place, and then made sure the temperature was comfortable while you sat in a sweat box. Chris, you gave me glimpses of all the other mountains you can see from the top, and encouraged me to dream about scaling every single one of them.

Thanks to the Coastal Marine Field Station technicians; Dave “Cakeman” Culliford, “T-Rex” Fairweather and Mark “The Guru” Paterson: three legendary superheroes whose problem solving skills can turn particularly slippery and steep slopes into an elegant staircase. Also, to Cheryl Ward for her magic tricks that made the writing process slightly less daunting, and to Kaeden Leonard for stepping in as a ‘proxy supervisor’.

To my “B” girls; Amy, Helen, Stine and Te Puea: you guys kept me sane with good conversation, food, wine, laughter and venting sessions. And then dragged me by the hair, kicking and screaming, at those times when my inner 2 year-old princess really deserved it. Stine, in particular, you’ve had my back and helped me so much from day one that I feel like we should both be listed as the authors of this thesis.

Finally, thanks to my kids Jessica and Jake who, through no choice of their own, got dragged along on this insane ride. You not only gave me the motivation to start on this journey, but pushed me to finish it. I hope I’ve proved to you that you can do anything if you just give it a go, and that cereal for dinner is quite acceptable!

Thanks must also go to Waikato Regional Council and the Ministry for Primary Industries for their financial support.

Hopefully the view from the top is awesome when this fog clears…

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Chapter 1

General Introduction

1.1 Background

Invasive species, along with climate change, overfishing, habitat damage and pollution, are commonly listed as the most major threats to global marine biodiversity (Lubchenco et al. 1991). Human activities, especially shipping, have enabled non-indigenous marine species (NIMS) to cross geographic barriers outside their natural dispersal range (Carlton & Geller 1991; Carlton 1996). While not all NIMS become invasive (i.e. spread rapidly and/or negatively impact native species or the environment), the potential for a NIMS to be invasive is possible if the environmental conditions are suitable and their usual top-down controls (e.g. natural predators, diseases) are absent (Shea & Chesson 2002). Without these controls, NIMS can quickly form dense aggregations that exclude native species, damage infrastructure, and alter ecosystems (Mack et al. 2000; Crooks 2002; Bax et al. 2003). NIMS may also facilitate the establishment of other NIMS with their propensity for positive, rather that competitive, biological interactions such as providing shelter or increasing prey species (Ricciardi 2001). Termed an "invasional meltdown" (Simberloff & Von Holle 1999), this scenario describes how the increased rate of invasion can lead the eventual collapse of an entire native community or ecosystem.

Shipping is widely regarded as the key vector responsible for introducing NIMS into new geographic regions (Carlton & Geller 1991; Ruiz et al. 1997). As an island nation reliant on maritime trade, New Zealand is particularly vulnerable to invasions in this manner (Simberloff 1995). In 1998, scientists identified 159 NIMS in NZ (Cranfield et al. 1998), however, that has recently increased to 351 (Ministry for the Environment & Statistics New Zealand 2016). Most of these species could have arrived via shipping, either in ballast water and/or as hull fouling (Ruiz et al. 1997; Cranfield et al. 1998). New Zealand takes a pro-active approach to managing biological security (referred to as 'biosecurity') (Meyerson & Reaser 2002; Hewitt & Campbell 2007) and created and implemented the *Biosecurity Act* 1993, with the purpose of providing the framework that will enable the "exclusion, eradication, and effective management of pests and unwanted organisms" (www.legislation.govt.nz). The *Biosecurity Act* 1993 combined all previous pest management legislation into one piece of legislation

that enabled principal management controls by one national body (Meyerson & Reaser 2002).

Eradication programs on established populations of NIMS are technically and financially difficult (Meyerson & Reaser 2002). Ideally, NIMS should be stopped at the border (Wotton & Hewitt 2004). However, given that this is a large undertaking (e.g., global treatment of ballast water), incursions are still likely to occur (Bax et al. 2003). Early detection and containment of potentially harmful or invasive NIMS then becomes a top management priority (Bax et al. 2001; Hewitt et al. 2004b; McKenzie et al. 2016). Once a species has entered a country post-border control comes into play. In this scenario, it is important to identify human-mediated pathways that would assist in the secondary spread of NIMS from an incursion site (Campbell & Hewitt 2013).

Along with international shipping, the aquaculture industry is also a major vector in the global geographic spread of NIMS (Welcomme 1992; Naylor et al. 2001; Molnar et al. 2008). Marine aquaculture facilities are known to facilitate the secondary dispersal of NIMS, as aquaculture often grow NIMS, or are fouled by NIMS (FAO 1997; Hewitt et al. 2006). Facilities with poor quarantine practice during harvest and through domestic transfers of equipment and product may inadvertently act to vector NIMS (Dodgshun et al. 2007). Floerl et al. (2009) has discussed the potential for subtidal aquaculture farms to act as 'stepping stones' for NIMS dispersal by providing suitable habitats between infested and pristine ecosystems. Furthermore, the damage caused by biofouling NIMS on aquaculture structures has been conservatively estimated to cost between 5-10% of production costs (Fitridge et al. 2012). Therefore, it is ecologically and economically essential for environmental and aquaculture managers to develop effective strategies to prevent the secondary spread of NIMS.

1.2 Aquaculture in New Zealand

New Zealand marine aquaculture production is dominated by three main products: New Zealand mussel, *Perna canaliculus* (marketed as Greenshell™ mussel); king salmon, *Oncorhynchus tshawytscha*; and the Pacific oyster, *Crassostrea gigas*. Marine farming utilises approximately 19,249 ha or 0.02% of

New Zealand's coastline (Aquaculture New Zealand 2012). Both the king salmon and Pacific oyster are non-indigenous species to NZ, and the NZ mussel is endemic. King salmon was deliberately introduced for farming purposes and the Pacific oyster was an accidental introduction that quickly overtook the native rock oyster (*Saccostrea glomerata*) industry due to its faster growth rate (Crimp 2007). Mussels are the most successfully farmed product in terms of export value, production, and marine space occupied (Aquaculture New Zealand 2012). The Marlborough Sounds and the Hauraki Gulf are the major mussel farming areas, growing 69% and 19%, respectively, of the 101,311 tonnes produced annually (Aquaculture New Zealand 2012).

New Zealand mussels are grown on a longline farming system (Figure 1-1). Juvenile mussels are seeded onto a continuous crop line that is suspended from a single 'backbone' line kept afloat by buoys and anchored to the sea floor at each end. After 3-6 months of growth, the mussels are stripped and reseeded onto a final production line at a density of approximately 150-200 individuals per meter. A harvestable shell size of 90-100 mm is achieved within 12-18 months. A three-hectare farm would typically consist of 9 backbone lines, each 110 m long and supported by 50-70 floats. Each float supports approximately one tonne of product (FAO 2005).

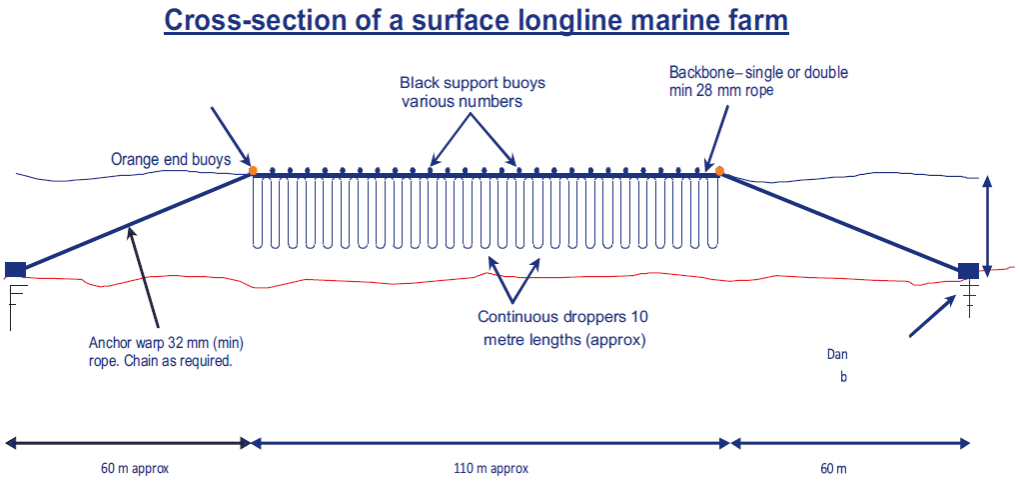

Figure 1-1 Schematic of a typical surface longline marine farming system used to grow mussels in New Zealand. (copyright: Marine Farming Association)

1.3 Case study NIM species

1.3.1 Natural history of *Sabella spallanzanii* (Gmelin, 1791)

Native to the Mediterranean Sea and European Atlantic coast, *Sabella spallanzanii* is a sessile, tube-dwelling, marine polychaete worm commonly known as the Mediterranean fanworm, European fanworm or feather duster worm (Global Invasive Species Database 2015). It can be found at depths of 0-30 m and prefers solid substrates and sheltered habitats (Currie et al. 2000) and can tolerate a temperature range of 2-29°C (Giangrande et al. 2000). The body consists of repeated segments, with a distinct thoracic region of eight segments (chaetigers) and abdominal region containing upwards of 200 chaetigers (Licciano et al. 2012). A large, spiralled branchial crown at the anterior head is extended out of the tube and used for feeding and respiration (Licciano et al. 2012). Growth occurs by adding chaetigers to the posterior end (Licciano et al. 2012). A dioecious species, large females (>300 mm body length) are highly fecund and can release >50 000 eggs during broadcast spawning events (Currie et al. 2000). Spawning occurs in autumn/winter and coincides with falling seawater temperatures and shorter days (Currie et al. 2000; Giangrande et al. 2000).

1.3.2 Invasion history of *Sabella spallanzanii*

Sabella spallanzanii was first recognised as a pest species by Australian authorities after its rapid establishment, and subsequent environmental impact, throughout Port Phillip Bay in the 1990's (Currie et al. 2000). As such, in 2000 the New Zealand government listed this organism as an Unwanted Organism under the *Biosecurity Act* 1993 and established surveillance programmes around high risk areas such as ports and marinas (Inglis et al. 2006). Small numbers of individuals were discovered in Lyttelton Harbour in 2008, and then in Waitemata Harbour in 2009 (Read et al. 2011b). Eradication efforts began immediately in both harbours upon *S. spallanzanii* detection, but were abandoned in 2010 as the populations had become too widespread to eradicate and/or control effectively (Read et al. 2011b). *S. spallanzanii* is now prolific throughout Whangarei Harbour, but small incursions in Tauranga, Coromandel and Nelson are thought to have been contained (Fletcher 2014). There is evidence of *S.*

spallanzanii fouling on mussel farms in the Hauraki Gulf (T Malcolm, pers. comms.; Figure 1-2), however, the extent of the incursion has not been described.

Figure 1-2 Mussel lines infested with *Sabella spallanzanii*, near Waiheke Island, NZ. Note: many *S. spallanzanii* worms are relaxed and protruding from the tube (red arrows) and have the potential to drop back into the water (copyright: Ministry for Primary Industries, 2016)

1.3.3 Knowledge gaps

Most publications focus on the biology and environmental impacts of *S. spallanzanii,* and are from international sources (Clapin 1996; O'Brien et al. 2006; Ross et al. 2013). Regional variability in lifecycles is evident in the reporting of maximum growth length and length at sexual maturity for *S. spallanzanii* (Fletcher 2014). For example, a maximum tube length of 400 mm is often reported in the literature (Clapin & Evans 1995), however *S. spallanzanii* with a tube length of 1 m have been observed in Whangarei Harbour, NZ (I. Middleton, pers. comms.). Similarly, reports on body length at sexual maturity differ between the regions: maturity being reached at 150 mm in Europe (Giangrande et al. 2000), and at approximately 50 mm in Australia (Currie et al. 2000). Furthermore, European populations have a lifespan of approximately 5 years, but estimates in Australian populations is 2 years (Fletcher 2014). Although recent genetic testing has shown that the NZ population originated from the

Australian population (Ahyong et al. 2017), there have been no formal studies on the maximum length, length at sexual maturity, lifespan or growth rates of *S. spallanzanii* NZ populations. This is an obvious knowledge gap that needs to be filled, if efficient management of *S. spallanzanii* is to occur.

The specific impacts of *S. spallanzanii* on the NZ marine aquaculture industry have not been explored, and trials to find an effective treatment for the species are aimed at managing hull fouling. The potential for secondary spread of *S. spallanzanii* through stock and equipment transfers is high (Dodgshun et al. 2007), along with its likelihood to increase production costs as a biofouling organism (Fitridge et al. 2012). *Sabella spallanzanii* may damage infrastructure and clog machinery, causing production to slow (Currie et al. 2000). Furthermore, the high filtering capacity of *S. spallanzanii* (Stabili et al. 2006) is likely to limit food availability to the farmed product, and hence affect growth. Conversely, *S. spallanzanii* could remediate the negative impacts of marine aquaculture waste by reducing levels of particulate organic matter (POM) and harmful bacteria (Stabili et al. 2010). There is also the potential for *S. spallanzanii* to be used as a finfish food source (Giangrande et al. 2014b). These trade-offs need to be fully considered within a NZ context, noting that under the Biosecurity Act Unwanted Organisms cannot be used in any way or form.

Although *S. spallanzanii* is not currently managed in a pro-active manner in NZ, there needs to be some strategies in place that will aid aquaculture facilities infected by *S. spallanzanii,* and limit the risk of transferring this Unwanted Organism to other locations. Lessons can be learnt and insights drawn from the attempts to manage other NIMS that threaten NZ and international marine aquaculture industries. Management strategies for ascidians such as *Styela clava, Ciona intestinalis,* and *Didemnum vexillum* are applicable to *S. spallanzanii* as they are all aggressive invaders and known colonisers of aquaculture infrastructure (Clarke & Therriault 2007; Coutts & Forrest 2007; Mckenzie et al. 2017). In particular, parallels can be made with the management of *Styela clava,* a species that has severely impacted the Canadian cultured mussel industry (Thompson & MacNair 2004; Clarke & Therriault 2007).

1.4 Thesis aims and organisation

The objective of this study was to aid the aquaculture industry with how they manage this pest species. Specifically, this project aims to examine the survivorship of *Sabella spallanzanii* after being exposed to typical mussel farming operations to determine the possibility of *S. spallanzanii* being spread after harvesting. The second aim of this project was to explore a treatment option for *S. spallanzanii* that would effectively kill *S. spallanzanii* while having minimal effect on the product. To achieve these aims, the following research questions were explored in the three subsequent chapters:

Chapter 2: How resilient is *S. spallanzanii* to desiccation caused by air exposure? This chapter explores how air exposure during the mussel re-seeding or harvesting process affects the survivability of *S. spallanzanii* adults.

Chapter 3: Can *S. spallanzanii* survive and regenerate body parts following fragmentation? During the mussel harvest process attached biofouling is mechanically removed and returned to the environment. The ability of *S. spallanzanii* to survive and regenerate after simulated harvest fragmentation was explored in this descriptive chapter.

Chapter 4: Is acetic acid an effective treatment method for *S. spallanzanii* that will have little impact on mussel health? Acetic acid has been demonstrated to be an effective control agent for other NIMS that biofoul aquaculture facilities (Forrest et al. 2007; Piola et al. 2009), and to quickly treat *S. spallanzanii* in hull fouling scenarios. Treating *S. spallanzanii* in conjunction with mussels (*Perna canaliculus)* has not been investigated.

The results of these experiments will enable biosecurity and marine farm managers to make informed decisions about the treatment, management and containment of *S. spallanzanii* and prevent its secondary spread to new geographic areas. The outcomes are discussed in a synthesis chapter (Chapter 5).

Chapter 2

Resilience of *Sabella spallanzanii* to desiccation

2.1 Introduction

The domestic transfer of stock and equipment is a common practice within the New Zealand (NZ) aquaculture industry. If quarantine practices are inefficient, these practices can inadvertently extend the spatial ranges of non-indigenous marine species (NIMS) (Forrest et al. 2009). There are many pristine and high value areas (e.g. marine reserves, marine protected areas) on, or within, close proximity to the transport routes used by mussel aquaculture (Figure 2-1). Thus, there is a potential risk that these 'high value areas' could be inoculated with NIMS, resulting in loss of cultural, social, economic and ecological values (Dodgshun et al. 2007). Aquaculture transfers, along with vessel movements, have been identified as the most likely mechanisms of human-mediated spread of *Sabella spallanzanii* in NZ (Fletcher 2014).

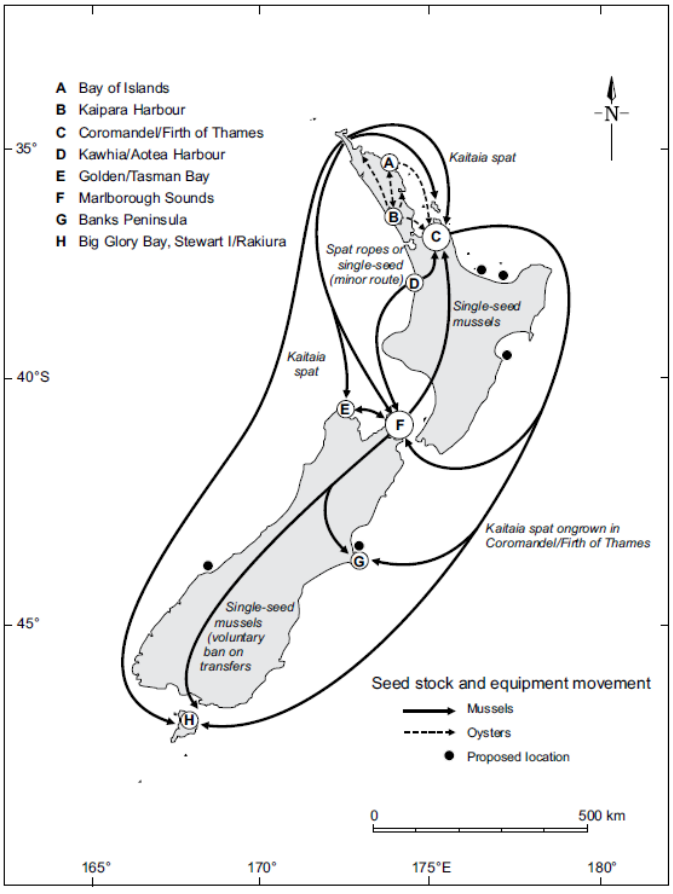

Figure 2-1 Location of existing and proposed marine farm regions in New Zealand. Larger bubble size (C and F) indicates regions with a higher concentration of farming. Arrows indicate the main vessel pathways used to move stock and equipment in the aquaculture industry (Dodgshun et al. 2007).

Juvenile mussels (referred to as 'spat') are traditionally collected from Ninety Mile Beach, Northland, and transferred to farms around NZ (Keeley et al. 2009).

Spat collection in NZ occurs over a limited spatial extent, resulting in issues of moving spat across biogeographic boundaries (Dodgshun et al. 2007) and the potential for cascade effects across the industry if wild spat collection failure occurs (Marine Farming Association 2017). This is an undesirable trait of the mussel industry in NZ that can lead to the spread of disease and NIMS if poorly managed. Spat are seeded onto lines and covered with a biodegradable, cotton-blend stocking to keep them intact after being submerged. As the spat grows, space on the lines becomes limited, and the spat lines are removed from the water (Figure 2-2) in order to re-seed the spat onto new lines at lower densities (Aquaculture New Zealand 2007).

Harvesting occurs when the mussels are 90-120 mm in length, at approximately 18 months old (FAO 2005). This process involves stripping the mussels from the lines as they are hauled onto the barge, with the stripped mussels being fed into a de-clumping machine to separate them and remove sediment and biofouling. The 'wash-water' used in the de-clumping process is discharged overboard during the harvest (Aquaculture New Zealand 2007). The mussel product is then stored in large sacks (referred to as 'fadges') to be transported to the processing factory. The mussels are able to survive for over five days out of the water (Kennedy 1976), however processing generally occurs within hours of harvest to ensure the product is of the highest quality possible (FAO 2005).

Figure 2-2 Mussel lines, heavily fouled with *Sabella spallanzanii*, awaiting re-seeding of juvenile mussels. Note: *S. spallanzanii* on the deck (circled in red), which would be washed overboard on

clean up at the end of the day. (Image provided by Rangi Walker; copyright: Waikato Regional Council).

Air exposure of mussel infrastructure (i.e. floats and lines) is used by industry as a practical method of removing biofouling after harvest (Aquaculture New Zealand 2007). However, when using air exposure as a containment method of NIMS, it is important to understand the desiccation tolerance of the target species as some species can survive from days to months of exposure (e.g., algae (Schaffelke & Deane 2005)). To date, there have been no NZ studies that have examined the resilience of *S. spallanzanii* to exposure (i.e. desiccation due to exposure to air). As a subtidal, sessile species, *S. spallanzanii* has a limited tolerance to air exposure (Currie et al. 1998). However, Fletcher (2014) has observed it fully exposed during extreme low tides in Waitemata Harbour, NZ. An ability to survive air exposure may not be unusual in this genus, although not noted in *S. spallanzanii* within the literature. For example, Murray et al (2011) have noted that the congener, *Sabella pavonia*, prefers an intertidal habitat, with an exposure to submersion ratio of 20:80.

Thus, there is the potential that *S. spallanzanii* can survive exposure to air. This, coupled with marine farming practices that may leave *S. spallanzanii* specimens on the deck of the aquaculture facility or vessels, could result in an increased potential for *S. spallanzanii* to survive air exposure and be spread. Therefore, the aim of this chapter was to examine if *S. spallanzanii* can survive exposure to air over a series of set time periods. The set time periods are based upon likely time frames that would occur in NZ during the standard operational practice of re-seeding, harvesting or movement of lines and equipment. The null hypothesis (Ho) being tested is that increasing time out of the water does not affect the survival rates of *S. spallanzanii*. In particular, desiccation treatments were used to examine changes in specimen weight, mortality and behaviour.

2.2 Methodology

All experiments for this project were conducted in a temperature controlled (18°C ± 1°C) and restricted access laboratory, modified to comply with biosecurity conditions outlined by the Ministry for Primary Industries (see Appendix A). *Sabella spallanzanii* specimens were collected from Orakei Marina,

Auckland, NZ and transported to the laboratory in Tauranga, NZ where they were kept in housing aquaria following depuration. Appendix B describes the collection methods and ensuing depuration process. Within the laboratory, husbandry of collected specimens consisted of weekly feeding, cleaning and monitoring of water quality. Further details of the husbandry regime and water quality parameters are outlined in Appendix C.

2.2.1 Establishing viability in *Sabella spallanzanii* specimens

Appendix D provides the details of a pilot program that was undertaken to establish a measure of health for *S. spallanzanii* following an experimental procedure. However, the resulting reaction indexes proved to be time consuming and beyond the scope of this project. As such, specimen mortality and survival was used to quantify the survivorship of *S. spallanzanii* to a procedure. Specimens were deemed 'viable' or 'inviable' by the following process:

1. The tube was picked up from the posterior end (posterior end up, anterior end down) and the colour of any discharge noted.
    a. Clear fluid indicated a specimen was potentially viable and examined further (step 2).
    b. Brown, green or white/milky fluid suggested mortality or damage to the body, as deterioration occurred rapidly following mortality (pers. obs.) and coelomic fluid is green or white.
2. The specimen was held upright by pinching the anterior tube opening (anterior end up) and movement of the body into the narrower, and usually transparent, posterior tube end was observed.
    a. Slow, controlled abdominal movement indicated the specimen was viable.
    b. Specimens were deemed inviable if fast, uncontrolled body movement occurred and stimulus (by gently palpating the posterior) failed to cause movement.
    c. If no movement was into the posterior tube end was detected, the tube was then slowly squeezed together from the anterior opening

towards the posterior end to force the specimen into the posterior end. If gentle palpation of the abdomen failed to stimulate controlled movement of the specimen, it was deemed inviable.

3. Specimens that had ejected from their tube during the experimental treatment(s) were considered viable if chaetae movement or body retraction was observed without stimulus, or when gently prodded with a plastic pipette.

4. All specimens deemed inviable were cut from their tube (if present) and further observed under light microscopy for chaetae movement or body retraction to ensure mortality had occurred.

2.2.2 Experimental design for desiccation treatments

Sabella spallanzanii were left within their tubes during the desiccation procedure. Viable specimens were removed from the housing aquaria and suspended on a dry sample tray to emulate air exposure (as described below). Specimens were exposed to a consistent air temperature of 18°C for varying lengths of time and then re-immersed, with each specimen occupying a single aquarium. Treatment periods were: 0 minutes (control); 1 hour; 4 hours; 12 hours; 24 hours; 48 hours. Observations (biomass, survival/mortality, behaviour) of each specimen occurred immediately after the exposure treatment, and then at 24 hours and 7 days following seawater re-immersion. Four adult specimens were exposed for each time frame (i.e. total of 24).

Specimen exposure was timed to ensure that all specimens were re-immersed within a 1 hour period (i.e. 48 hr treatment specimens were removed first). Once a specimens' viability was established, the biomass (weight inclusive of tube), tube length and width (at the widest point) was recorded. Control specimens were then immediately re-immersed into individual aquaria. Treatment specimens were placed on 6 cm plastic mesh and suspended 2-3 cm above a 360 mm x 300 mm plastic tray (Figure 2-3a). The tray was lined with paper towel to prevent puddles of water occurring that could inadvertently provide hydration to specimens that ejected from their tube. All trays were placed on a rack under the

laboratory bench on the same wall as the air conditioner to control for temperature fluctuations and air movement.

At the end of the exposure periods each treated specimen was weighed and survival/mortality and behavioural observations were noted. Behavioural observations included full or partial body ejection from the tube, and visible crown movement at the anterior opening. Where a specimen had ejected from its tube, both the specimen and tube were weighed together and the tube retained with the specimen for the duration of the experiment. Specimens were then randomly placed into individual 1.25 litre aquaria, containing artificial seawater and a single air stone (Figure 2-3b), for the re-immersion portion of the experiment. Aquaria were stabilised in a 50 cm wooden quadrat to ensure they could not fall over. Following a 24 hour re-immersion period, all specimens were weighed and survival/mortality and behavioural observations were recorded. These observations were repeated seven days after re-immersion for the final experimental measurement.

Figure 2-3 Design of experimental procedure exploring the survivorship of *Sabella spallanzanii* to air exposure: a) (left) suspension of specimens for exposure period; b) (right) specimens in recovery aquaria.

On conclusion of the experimental procedure, all surviving specimens were removed from the individual aquaria and placed together in a 65 L aquarium for further observations. This aquarium was lined with a shell-grit substrate to encourage tube attachment and specimens were fed with seawater collected from Sulphur Point Marina, located adjacent to the laboratory where experiments were conducted. Following the conclusion of all experiments for this project, surviving specimens were ethically euthanized by freezing and discarded into landfill.

2.2.3 Statistical Analysis

Summary statistics were developed and visualised using Microsoft Excel. Descriptive statistics and hypothesis testing was conducted using Statistica (version 13). To meet the assumptions of ANOVA, all data were tested for normality with a Shapiro-Wilk (W) test, and for homogeneity of variance using a Levene's test. Parametric data was analysed with independent *t*-tests and one-way ANOVA, and non-parametric with Mann-Whitney (U) test and Kruskal-Wallis ANOVA. Duncan's multiple range post-hoc testing was completed on any analyses that indicated significant results. Confidence intervals were set to 95%.

2.3 Results

The median start length of specimens was 263 mm (±9.2 SE) and ranged from 162 mm to 362 mm. The median start biomass (body weight including tube) was 9.2 g (±0.5 SE) and ranged from 2.3 g to 13.9 g. Mortality occurred in 20.8% of the experimental specimens.

2.3.1 Biomass changes following air exposure

The biomass (%) of specimens, from the starting weight to immediately following exposure, differed statistically between treatments ($F_{[5,18]}$=12.97; p<0.001; Figure 2-4). All treatments, except for the 4 hr exposure, differed to the control group (Table 2-1). The 48 hr exposure However, following 24 hrs re-immersion the differences in specimen biomass were no longer evident at a statistical scale ($F_{[5,18]}$=1.1795 p=0.358; Figure 2-5). It can be inferred from this outcome that *S. spallanzanii* desiccated during exposure treatment and were then able to rehydrate when re-immersed.

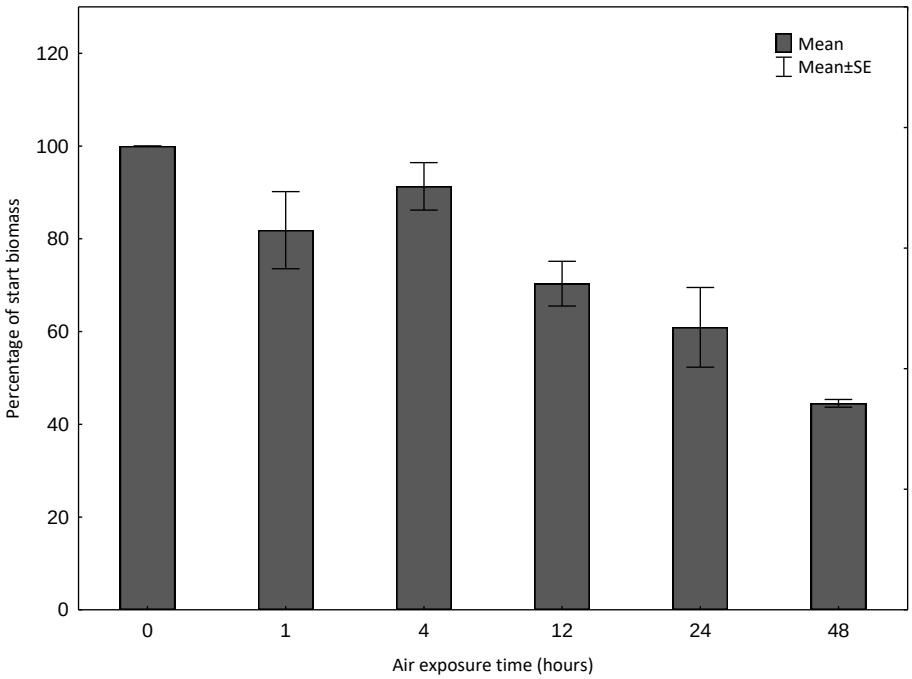

Figure 2-4 Average percentage of initial biomass of *Sabella spallanzanii* in air exposure treatments, measured immediately following treatment. n = 4 specimens per exposure time.

Table 2-1 Post-hoc results of *Sabella spallanzanii* change in biomass (%) following air exposure treatment. n.s. = not significant.

Treatment	0	1	4	12	24	48
0	-					
1	0.0449					
4	n.s.	n.s.				
12	0.0028	n.s.	0.0224			
24	0.0003	0.0226	0.0023	n.s.		
48	<0.001	0.0004	0.0001	0.0064	n.s.	-

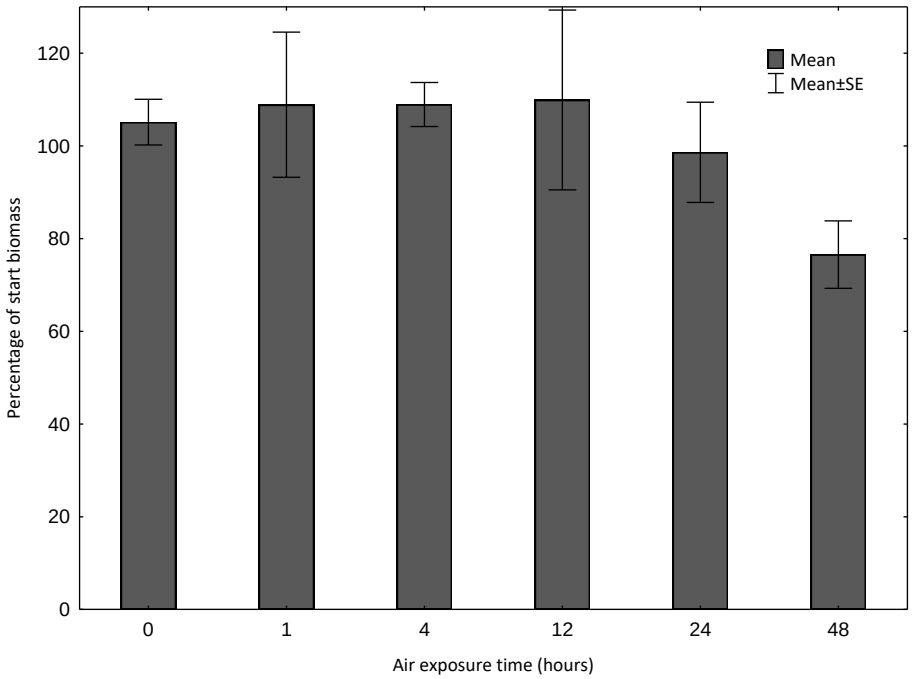

Figure 2-5 Average percentage of initial biomass of *Sabella spallanzanii* in air exposure treatments, measured 24 hours post re-immersion. n = 4 specimens per exposure time.

2.3.2 Survivorship of *Sabella spallanzanii* following desiccation

There was no significant difference (KW-$H_{[5,24]}$=5; p=0.446; Figure 2-6) in survival between treatments when measured immediately after desiccation. However, there was a significant difference in survival (KW-$H_{[5,24]}$=18.64; p=0.002; Figure 2-7) between treatments when measured 24 hours post re-immersion. The 48 hr exposure group differed to all other treatments (

Table **2-2**). All specimens in the 0-12 hr exposure treatments had 100% survival. When desiccated for 24 hours, 100% of specimens survived, but within 24 hours of re-immersion 25% had died. Specimens desiccated for 48 hours had 75% survival at the end of the treatment, but 100% mortality within 24 hours of re-immersion.

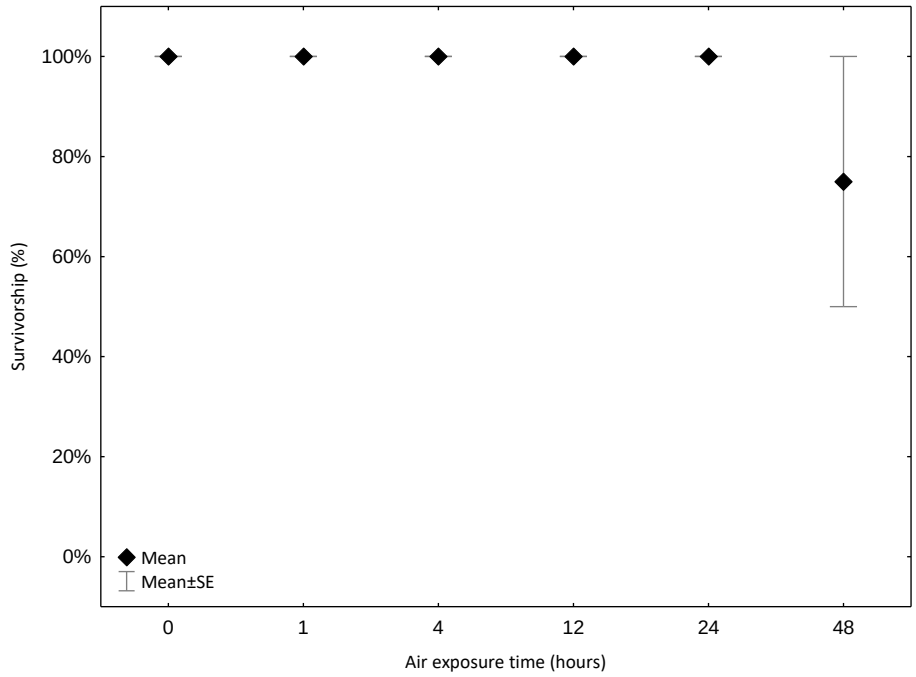

Figure 2-6 Average survival (%) of S. spallanzanii, measured immediately following desiccation treatment. n = 4 specimens per exposure time.

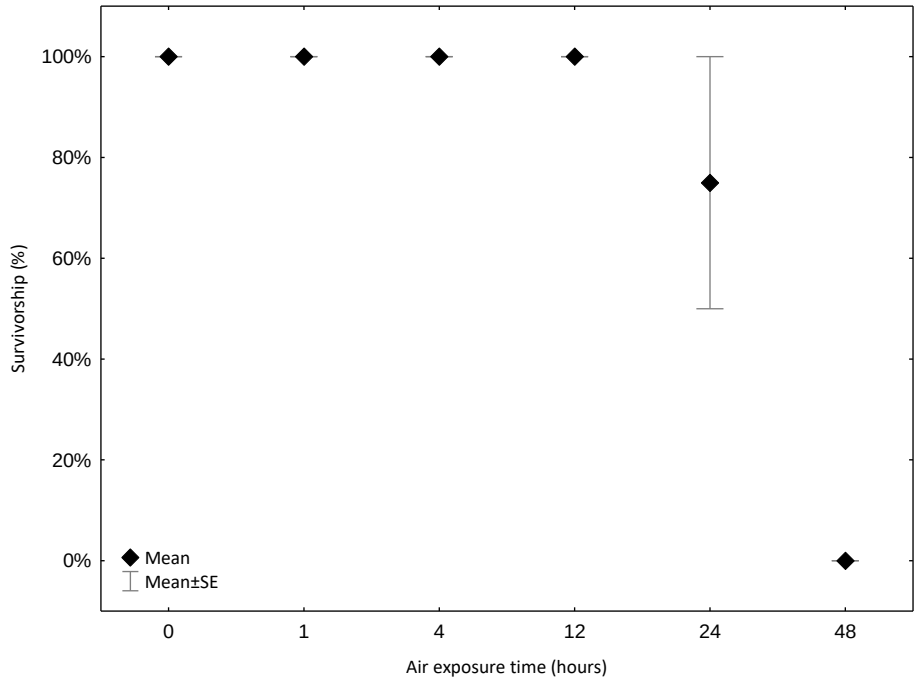

Figure 2-7 Average survival (%) of *Sabella spallanzanii*, measured 24 hours post re-immersion following desiccation treatment. n = 4 specimens per exposure time.

Table 2-2 Post-hoc results of final survivorship of *Sabella spallanzanii* to air exposure treatments. n.s. = not significant.

Treatment	0	1	4	12	24	48
0	-					
1	n.s.					
4	n.s.	n.s.				
12	n.s.	n.s.	n.s.			
24	n.s.	n.s.	n.s.	n.s.		
48	<0.001	<0.001	0.0001	0.0001	0.0002	-

There was a statistically significant difference ($t_{[22]} = -5.81$, $p < 0.001$; Figure 2-8) in the average biomass change (%) (measured immediately after exposure) of specimens grouped by final survivorship (measured 24 hours post re-immersion). All specimens that survived the experimental procedure lost no more than 37.1% of their biomass during desiccation. Those specimens that died during the experiment lost between 53% and 63.8% of their biomass during desiccation.

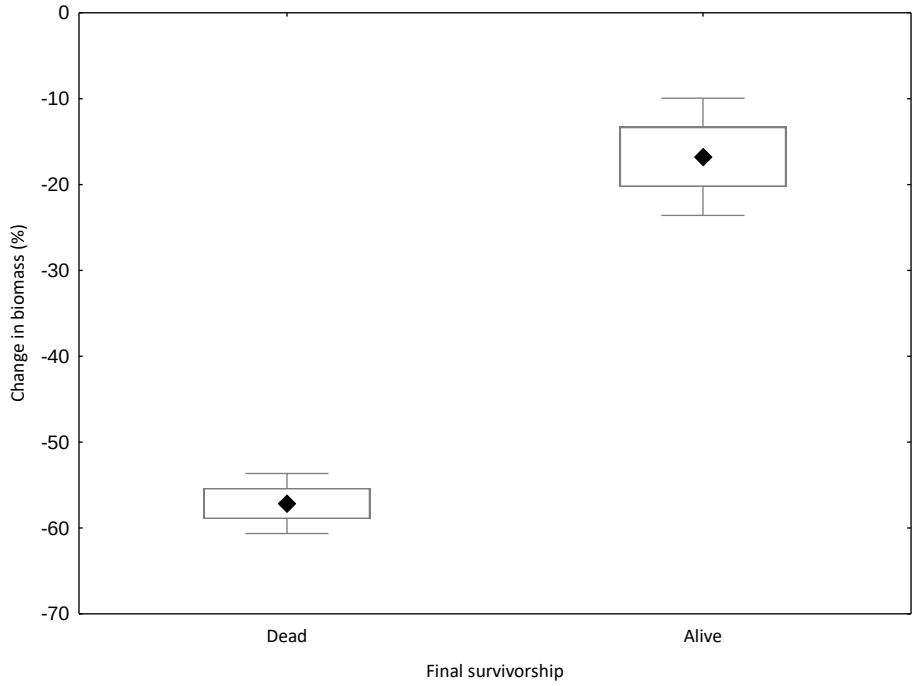

Figure 2-8 Average biomass change (%) in *Sabella spallanzanii* following air exposure treatment and grouped by final survivorship as measured post 24 hours re-immersion. n = 5 dead and 19 alive. Error bars indicate SE and box indicates quartile range.

2.3.3 Post experiment behavioural observations

All specimens that had survived the 24 hour post recovery period were still alive after 7 days when they were subsequently moved to a single aquarium for further recovery observations. After 6 weeks in recovery all specimens remained alive, however, none showed signs of re-attachment or tube regrowth.

2.4 Discussion

This experimental procedure clearly demonstrated that *S. spallanzanii* survival is affected by exposure to air, and hence the null hypothesis is refuted. When compared to the control specimens, all treatment specimens showed a clear pattern of desiccation due to air exposure, and rehydration following re-immersion. The first signs of mortality occurred in specimens exposed for 24 hrs, and significant rates of mortality occurred in the 48 hr exposure treatments. However, most specimens survived the initial exposure period but mortality occurred within 24 hrs of re-immersion. Final mortality rates were influenced by the percentage of body mass lost during the exposure component of the experimental procedure. The outcome of this experiment has clear biosecurity and aquaculture facility management implications, which are discussed further below.

These results indicate that exposing *S. spallanzanii* to air for at least 48 hours (at 18°C) during typical harvest practice would ensure that specimens were not viable. However, *S. spallanzanii* specimens that are re-seeded with the mussels, or detach during processing, and re-enter the seawater environment within a 48 hour period would potentially still be viable, posing a risk of re-infestation. In typical NZ mussel farming practice, re-seeding would take no more than 12 hours, and harvested adult mussels would arrive at the processing plant within 24 hours of being removed from the water. Mussel barge decks are also washed down at the end of each day (<12 hours) and could be a significant dispersal vector, as deck washing often occurs away from the farm after the stock has been landed, or where the barge is moored. Of note, is that in all of these situations, the air exposure time (i.e., desiccation duration) is below the threshold that causes *S. spallanzanii* mortality, as indicated in this research. Hence, *S. spallanzanii* would remain viable. This clearly demonstrates that appropriate containment or treatment methods need to be developed and implemented to reduce the likelihood that NIMS (specifically *S. spallanzanii*) are spread further within the NZ aquaculture context.

A clear indication of the ability of *S. spallanzanii* to be transferred between localised regions is evident at the Sugarloaf Wharf, Coromandel Peninsula, which

is currently infested with *S. spallanzanii* (T. Malcolm, pers. comm., Waikato Regional Council). The Coromandel Peninsula has a high concentration of mussel farms, with the Sugarloaf Wharf closely associated with these facilities and the landing of mussel stock.

Longer distance transfer of *S. spallanzanii* via aquaculture equipment and vessel movement may be reduced due to the low connectivity between regions. For example, Forrest and Blakemore (2002) have illustrated that mussel farm equipment and service vessels are infrequently moved between regions and hence this pathway may not be of concern at a national scale. Furthermore, the inter-regional transfer of mussel spat may help reduce the secondary spread of *S. spallanzanii* as the NZ mussel industry, inclusive of spat movement, is controlled by a voluntary code of practice aimed to reduce the environmental impact of the industry (Aquaculture New Zealand 2007). A key objective of the code addresses the risk of NIMS spread associated with farming activities and recommends specific actions (e.g. reporting of NIMS, discharge of biofouling) to mitigate this (Aquaculture New Zealand 2007).

What is unknown is the efficacy of the voluntary code of practice. For example, how much uptake or buy-in of the code occurs, what are the likely incentives to ensure that the voluntary code is implemented, and what, if any, penalties exist for breaching the code? Incentives and voluntary codes of practice are not always effective when dealing with issues related to environmental protection. For example, recent research in Canada demonstrates that aquaculture facilities often create anthropogenic debris (D'Anna & Murray 2015). Yet lease holders admit that the anthropogenic debris they create may not always be removed from the water or vicinity of the aquaculture leasehold (especially by small lease holders). This is due to poor economic margins that have resulted in insufficient funds to spare for cleaning the marine environment of the litter they created (D'Anna & Murray 2015), despite the illegal nature of littering in the region. Furthermore, leaseholders often admit that they will not undertake cleaning of anthropogenic litter that they have created unless they are directed to do so (D'Anna & Murray 2015). Thus, devolving biosecurity undertakings (such as the

code of practice), much like littering, may not be effective if devolved to industry that will be directly affected by the code.

Eighty percent of the specimens that didn't survive the experiment were still alive after the initial exposure period, but died during the 24 hour re-immersion period. This could potentially have been enough time to spawn as mature gametes are ejected from the tube opening on mucus strings in broadcast spawning events (Currie et al. 2000). Nash and Keegan (2004) were able to induce spawning in the Sabellid, *Bispira volutacornis*, by 'physical manipulation of the central abdomen (using a blunt instrument)'. Similarly, disturbance events, such as increased wave action, have also been shown to facilitate spawning in the Sabellariid, *Phragmatopoma californica* (McCarthy et al. 2003). This suggests that disturbance caused during the harvest and de-clumping process may be enough to stimulate *S. spallanzanii* spawning upon the barge deck and during re-immersion.

The significant relationship between mortality and biomass loss due to desiccation would benefit from further exploration. I note that the experimental conditions in the laboratory strictly limited *S. spallanzanii* specimens' contact with any moisture during the air exposure component of the experiment. However, this scenario would be unlikely to occur in actual practice. Contact with equipment or other organisms, plus atmospheric humidity, would vary the available moisture and affect the rate of desiccation, and hence the biomass loss and mortality, for each specimen. For example, water retention would be high in rope as the numerous strands tightly twisted together give it a larger surface area. Furthermore, *S. spallanzanii* remaining on the barge deck throughout the day would be exposed to spray and splashing from the harvest and de-clumping process, which would negate much of the dehydration process.

As with most graduate studies there are drawbacks and obvious flaws that become apparent after experiments have been undertaken. Within this Chapter, an obvious limitation of the experimental design was the type of mesh used to suspend the worms above the trays. Unfortunately, the specimens were able to fit through the large mesh weave, with crown extension and gravity potentially adding to the desiccation and tube ejection rates observed. A smaller mesh size

that specimens could not fit through, but still allowed moisture to drain away, would have been more efficient and address this problem.

2.5 Conclusions

The results of this chapter clearly indicate that *S. spallanzanii* can survive exposure to air for periods of up to 24 hours if followed by re-immersion in seawater. Unfortunately, the duration of desiccation is higher than what would be encountered during a mussel farm re-seeding or harvest process and movement of stock to landing and processing. A health indicator for *S. spallanzanii* was created suggesting that >53% reduction in biomass via desiccation would lead to mortality of specimens. Furthermore, there is the potential that the de-clumping process may lead to 'disturbance' of worms that may result in spawning. This is further explored in Chapter 3.

Chapter 3

Survival and regeneration of *Sabella spallanzanii* following fragmentation

3.1 Introduction

Biofouling in marine aquaculture can significantly increase production costs (discussed further in Chapter 4) and negatively impact on the farmed product (Fitridge et al. 2012). Hence, the control of biofouling is an important issue facing farm managers. Non-indigenous marine species (NIMS) are common in the fouling community as they have a tendency to colonise artificial structures (Tyrrell & Byers 2007). Therefore, the processes used by the industry to control biofouling must consider the presence of NIMS, and ensure that potentially harmful organisms are not given the opportunity to re-infest or invade the environment.

Chemical antifoulants that could be used to help control NIMS biofouling can adversely affect growth and survival of the farmed product. Hence, the shellfish industry relies heavily on the physical removal of biofouling (Fitridge et al. 2012). In New Zealand mussel aquaculture, this is generally done onsite during harvesting using a de-clumping machine. When mussel lines are removed from the water, they are fed through a metal ring or plates to strip the mussels off the rope (Figure 3-1a). The mussels are then fed through the de-clumping machine (Figure 3-1b) before being sorted into large sacks to await transportation to the processing plant. De-clumping machines vary, but in NZ they typically utilise a revolving drum and jets of seawater to clean and separate the mussels. Both the wash-water and associated material removed from the mussels is discharged overboard whilst harvesting is under way (Aquaculture New Zealand 2007).

Figure 3-1 Examples of machinery on mussel barges used to a) (left) strip mussels from lines and b) (right) separate mussels and remove fouling (Quality Equipment Group (QEG) 2017)

The de-clumping process is optimised to reduce damage to the mussels, however, damage to the attached biofouling is not well understood for all biofouling species. Fragments of *Undaria pinnatifida* (an introduced kelp in New Zealand) can survive the de-clumping process and retain viable spores (Forrest & Blakemore 2006). Reproductive products of the solitary ascidian *Styela clava* surviving the de-clumping process have been attributed to secondary spread of the species in Canada (Locke et al. 2009b). Additionally, due to asexual reproduction in the invasive colonial ascidian *Didemnum vexillum*, fragmentation has contributed to its rapid spread (Coutts & Forrest 2007). The tube of *S. spallanzanii* is one of the strongest of the Sabellidae and can withstand over 700 g of weight before tearing (Giangrande et al. 2014a). This suggests that S*. spallanzanii* may be robust to the de-clumping process.

It is well documented within the literature that annelids possess physiological mechanisms that enable them to replace lost or damaged body parts (Hyman 1940; Bely 2006; Murray et al. 2013). Regeneration of the tail/posterior segments is universal in annelids, however, the regeneration of a head/anterior segments is common but not ubiquitous (Zoran 2010). Among the Sabellidae the regenerative potential is highly variable, with some species able to regenerate whole individuals from mid-sections, and others lacking the ability altogether (Licciano et al. 2012). Studies on the native European populations of *S. spallanzanii* indicate a high survival potential in response to wounds and an ability to quickly regenerate lost body parts both anteriorly and posteriorly (Licciano et al. 2012). Regeneration of the head, crown and tail has been observed in Australian populations of *S. spallanzanii* (Clapin & Evans 1995). However, there have been no studies examining regeneration of *S. spallanzanii* in New Zealand (NZ) populations.

The ability of *S. spallanzanii* to replace lost or damaged body parts suggests that it has the potential to recover from damage caused by the de-clumping process used during the mussel harvest. As waste is immediately washed overboard, viable *S. spallanzanii* could then re-colonise existing infrastructure or disperse (via rafting on the currents) to new geographic habitats. Hence, the aim of this chapter was to examine the survivorship of *S. spallanzanii* to a simulated de-

clumping event. The recovery of specimens subjected to fragmentation that may occur was examined over a temporal range. A secondary aim was to observe the morphological changes and evidence of body part regeneration in *S. spallanzanii* following fragmentation.

3.2 Methodology

3.2.1 Experimental design for fragmentation procedures

A two treatment experiment was developed to determine the survivorship and regeneration of *S. spallanzanii* to injuries that would be consistent with mussel harvesting, including de-clumping. *S. spallanzanii*, whilst in their tubes, were laterally dissected into two or three fragments or left undamaged (control). Fragments were then re-immersed into individual treatment aquaria and survivorship (specified below) was recorded every four days for twenty-eight days (a lunar cycle). On conclusion of the observational period, each fragment was removed from the tube (if not previously self-ejected), photographed under light microscopy and evidence of body part regeneration (i.e., branchial crown, posterior segments) recorded. Each treatment and control sample size consisted of four specimens (n=12).

Immediately prior to the start of the experiment, specimens were removed from the housing aquaria and deemed viable following the process outlined in Chapter 2. Specimens were then weighed (weight inclusive of tube), and the tube width and length recorded. With no further action, control specimens were randomly placed into individual 1.25 L aquaria of artificial seawater and an air stone (Figure ?? Chapter 2). Treatment specimens were fragmented using the following process and each fragment placed into individual aquaria:

1. The anterior opening of the tube was squeezed closed and slowly palpated towards the posterior end until the body could be felt.
2. The tube above the body was cut off approximately 15 mm above where the specimen was felt.
3. Using a scalpel, the specimen was then cut laterally in half (2 fragments; anterior (A), posterior (P)) or into three (3 fragments; anterior (A), posterior (P), mid-section (M)).

Cutting specimens created 20 fragments in total, and an additional four control specimens (that were not fragmented).

At each 4 day observational period, survivorship (alive or dead) of each fragment and control was recorded. Establishing survivorship in controls and fragments that had ejected from the tube was unchanged from the process outlined in Chapter 2. Survivorship in fragments was established by squeezing shut the tube opening and gently palpating down the tube until the body emerged from the opposite opening. Each observation took no more than 60 seconds to reduce the chance of desiccation stress to a specimen. After specimens were re-immersed, a 30% water exchange was conducted, visible waste was removed, and 0.25 ml liquid feed was given to each fragment and control.

Following the observations on day 28, surviving fragments were removed from the tube (if present) and photographed under light microscopy. All specimens and fragments were ethically euthanized by freezing.

3.2.2 Statistical Analysis

Descriptive statistics were used to examined the results. The number of specimens available for each experiment was limited and unfortunately this number was too small to undertake statistical analyses. Instead the data is presented descriptively and is based on binomial (alive versus dead) description of the specimens.

3.3 Results

The median start length of specimens used in this experiment was 225 mm (±10.3 SE; *n*=12) and ranged from 172-294 mm. The median biomass (weight including tube) was 4.7 g (±0.8 SE) and ranged from 2.5-10.9 g. Complete mortality occurred in one control specimen, with at least one fragment of every other specimen surviving.

3.3.1 Survivorship of *Sabella spallanzanii* to fragmentation

(*Figure 3-2*) illustrates that 75% of control specimens survived the 28 day observation period. The specimens cut into 2 fragments showed 100% survival in the posterior fragment compared to 50% survival in the anterior fragment. In the

specimens cut into 3 fragments, 100% of the mid-body and posterior fragments survived and there was no survival in the anterior fragments.

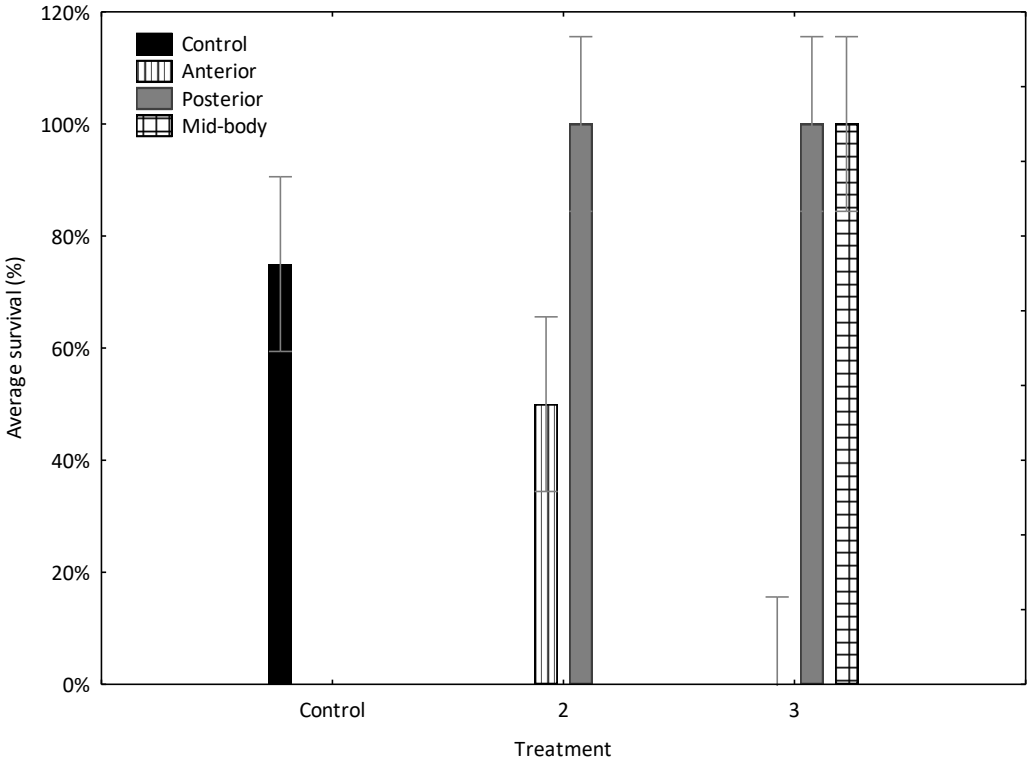

Figure 3-2 Average survivorship of *Sabella spallanzanii* to fragmentation. Measure taken 28 days after treatment n=4 specimens per treatment. Error bars indicate standard error.

3.3.2 Regeneration observations

Regeneration of body parts was evident in all surviving fragments at 28 days post-treatment. Posterior fragments (*n*=4 for each treatment) had grown full branchial crowns, anterior fragments (*n*=2, treatment 2) had added posterior chaetigers, and the mid-body (*n*=4, treatment 3) had grown both a branchial crown and added posterior chaetigers.

3.4 Discussion

Sabella spallanzanii fragments were able to survive and regenerate following the simulated de-clumping treatment. At least one fragment from each specimen survived the 28 day observational period. All posterior fragments survived both treatments, along with the mid-body fragments of specimens cut into 3. Only 50% of anterior fragments survived and these were from the specimens cut into 2. All surviving fragments had regenerated either anteriorly, posteriorly, or both in the mid-body fragments.

The regeneration process of *S. spallanzanii* has been studied in detail in its native, European range and the design of this experiment (i.e. cutting specimens into 2 and 3 fragments) was loosely based on a study by Licciano et al. (2012). The statistical analysis of the Licciano et al. (2012) study cannot be compared with this experiment due to differing sample sizes. However, the descriptive observations of fragment survival and regeneration are comparable and provide insight into the healing process that was not observed in this study. Survivorship to fragmentation was also high in the Licciano et al. with 75% of all fragments (anterior, posterior, mid-section) surviving the treatment procedure. They observed wound healing within 24 hours, and evidence of crown regrowth started at ~9 days. There was no difference in survival between the fragment type (Licciano et al 2012), in contrast to this study where anterior fragments showed higher mortality.

Of concern, is that damaged *S. spallanzanii* that do not survive the de-clumping process may still contribute viable gametes to the environment, which then have the potential to disperse to new habitats. For example, ablation is a technique used to induce spawning in *Sabellasarte spectablilis* (Bybee et al. 2006). Fertilised eggs are known to be present between the body and tube of female *S. spallanzanii* (Giangrande et al. 2000), and hence release of these eggs following damage is possible. Mucus can also contain fertilised eggs (Stabili et al. 2009), with gamete release possible following damage, as mucus is produced and released in stressful conditions (Giangrande et al. 2014a).

The precise fragmentation techniques used in this experiment may not accurately replicate the mechanical damage caused by stripping from mussel lines or the de-clumping process. High pressure water jets and the rotating drum would also be likely to damage *S. spallanzanii* by crushing, and/or tearing individuals into more fragments than created in this experiment. It is unlikely that the damage or fragmentation that occurs during harvesting would be as concise and clean as cuts from a scalpel that occurred under laboratory conditions. Therefore, more tissue damage would likely occur during harvesting conditions and hence survivorship would be different. To improve the estimate of regeneration ability of an *S. spallanzanii* individual, fragments should be collected from actual harvesting wash-water, which could then be held in aquaria for recovery to be observed. Similarly, the recovery period in laboratory conditions would not accurately reflect the dynamic environmental conditions that occur in the field, such as when a harvested specimen is washed overboard. Although water movement was provided by an air stone, this was a vertical movement and dissimilar to natural current movements. Modification of the housing aquaria to simulate currents would also allow observations to be made of *S. spallanzanii* behaviour (i.e. attachment to the substrate, regrowth of tube) following a simulated de-clumping event.

A major limiting factor of this experiment was the sample size as the data was too small for statistical analysis. Future studies of this nature would need to significantly increase the sample size in order to gain a more robust statistical analysis.

3.5 Conclusions

This observational study has shown that *Sabella spallanzanii* has a high propensity for long term survival following body damage gained in the mussel harvest and de-clumping process. Coupled with its ability to quickly regenerate body parts, it is highly likely that the standard operational practices of mussel aquaculture can aid in the secondary dispersal of S. spallanzanii. Hence, it is important to explore potential treatment options for controlling S. spallanzanii fouling in marine aquaculture.

Chapter 4

Response of *Sabella spallanzanii* to acetic acid: searching for a control treatment

4.1 Introduction

Biofouling in marine aquaculture facilities can cause significant economic loss by increasing production costs and reducing the quality of the farmed product (Adams et al. 2011). In an extensive review of this issue, Fitridge et al. (2012) identified five major impacts biofouling can have on shellfish aquaculture: 1) physical damage to the shell by invasive or epibiotic organisms; 2) mechanical interference to shell function that may affect feeding or increase the risk of predation; 3) reduced growth and condition due to competition for resources; 4) potential ecosystem modifications to water flow, waste build-up, oxygen levels, food availability, biodeposition and the spread of non-indigenous marine organisms (NIMS); and 5) extra weight to infrastructure, stock and equipment leading to mechanical failure/damage.

The focus of the New Zealand mussel aquaculture industry is the physical removal of biofouling at harvest time. This occurs because it is the aesthetic appeal of the species sold in the half shell that commands a premium price on the export market (Crimp 2007). As described in Chapter 3, physical removal of biofouling occurs during re-seeding and harvest processes, with the use of a de-clumping machine. However, as the biofouling is washed overboard there is the potential for the organisms (including NIMS) to be re-introduced to the environment and spread (if the fouling survives the de-clumping process). Thus, it is important to consider additional options for treating biofouling.

Finding effective methods for preventing, mitigating or treating biofouling in shellfish aquaculture is extremely difficult as the treatment needs to be effective enough that it doesn't compromise the health of the product or environment, yet efficient so as not to unduly reduce production time (Fitridge et al. 2012). The recommended natural treatments for biofouling on aquaculture infrastructure include desiccation, freshwater immersion, heat exposure and high pressure water blasting (NSPMMPI 2013). A cost-effective treatment for mussel aquaculture is a combination of freshwater immersion followed by 12 hours of air exposure (Gunthorpe 2001). However, this is not species specific and may not be suited for organisms with high desiccation tolerance (e.g. barnacles) or microscopic gametophytes (e.g. algae) (Forrest & Blakemore 2006).

The need for fast acting treatments that target NIMS has led to the investigation of eco-friendly biocides such as acetic acid, bleach, brine, chlorine, lime, and various household cleaners (Locke et al. 2009a; Piola et al. 2009; Dunmore et al. 2011; Jute & Dunphy 2016; Morrisey et al. 2016). In Canadian shellfish aquaculture, acetic acid and hydrated lime are the industry recommended treatments for the solitary ascidian *Styela clava* (Locke et al. 2009b). However, the use of hydrated lime is under review due to its potential negative environmental impact (Locke et al. 2009a). Furthermore, a study by Piola et al. (2009) found that acetic acid was a more effective treatment against fouling than hydrated lime and bleach.

Acetic acid (the active ingredient in vinegar) is regarded as an 'eco-friendly' household disinfectant and often used by commercial farmers to improve the health of stock. For example, drinking water supplemented with acetic acid reduced *Salmonella spp.* contamination in broiler chicken flocks (Le Bouquin et al. 2010) and improved the immune response of heat-stressed broiler chicks (Hassan et al. 2009). When added to the feed in shrimp aquaculture, acetic acid is also effective in controlling the pathogen, *Vibrio harveyi* (Mine & Boopathy 2011). Trials using acetic acid immersion and spray techniques to remove biofouling on mussel infrastructure have shown that it is highly successful in removing soft bodied organisms with low impact to the mussel (Forrest et al. 2007; LeBlanc et al. 2007; Piola et al. 2009). Similarly, removal of biofouling NIMS using encapsulation techniques (i.e. wrapping in plastic) is faster and more successful with the addition of acetic acid as the acid is kept in place, the acid concentration is maintained, and diffusion doesn't occur (Atalah et al. 2016).

In NZ, exploration of treatment methods to kill *Sabella spallanzanii* favour methods that have been developed for hull fouling as this is recognised as the major vector in the spread of the species (Hewitt et al. 2004a). Encapsulation trials have proven to be particularly effective treatment for boats infested with *S. spallanzanii* as it allows for a cost-effective and quick response to an incursion (Morrisey et al. 2016). Recent trials testing hypo-saline (i.e. freshwater) immersion, resulted in 100% mortality of *S. spallanzanii* in 120 min (Jute & Dunphy 2016). This led to a pilot study that involved immersing one tonne sacks

of infested *Perna canaliculus* in freshwater. Although a treatment time of 120min successfully eliminated all *S. spallanzanii* with no loss to the mussels, it was an expensive and difficult exercise (Jute & Dunphy 2016) and hence may not be a practical treatment option. There have been no trials exploring the use of acetic acid as a control treatment of *S. spallanzanii* in mussel aquaculture.

Therefore, the aim of this chapter was to explore the effectiveness of acetic acid immersion as a treatment option for *S. spallanzanii* fouling on mussel lines. A secondary aim was to establish the immersion time frame that would kill *S. spallanzanii* but have minimal impact upon the cultured mussel, *P. canaliculus.* The null hypotheses being tested are that immersion in acetic acid for varying time periods does not affect the survival of *S. spallanzanii* (H_1) or the survival of *P. canaliculus* (H_2).

4.2 Methodology

SCUBA divers removed approximately fifty *Perna canaliculus* specimens by hand from a mooring line in Pilot Bay, Tauranga (S37°38'12.2" E176°10'32.5"), with a target length of 50-100 mm. Specimens were transported immediately to the research laboratory (time from collection to laboratory was approximately 60 mins). Individual *P. canaliculus* were separated and biofouling cleaned off the shells by hand before being placed into an aerated 160 L aquarium of artificial seawater and left to depurate for 24 hours. A husbandry regime was not established as the experimental procedure began immediately after the depuration period. As described in Chapter 2, *Sabella spallanzanii* specimens were already onsite at the laboratory.

Sabella spallanzanii specimens were deemed viable (alive) or inviable (dead) using the process outlined in Chapter 2. The viability criteria for *P. canaliculus* were based upon the following observed behaviours:

- Byssal attachment to the aquaria;
- Tightly closed shells that couldn't be opened by hand; and
- A gaping shell that closed in response to light tapping with a glass pipette (Hicks & McMahon 2003; Petes et al. 2007).

Mortality was inferred if none of the above behaviours were present.

4.2.1 Experimental design for acetic acid treatments

Specimens of *S. spallanzanii* and *P. canaliculus* were fully immersed in a 5% solution of acetic acid (CH_3CO_2H; made up with artificial seawater) for varying time periods: 0 min (control), 1 min, 2 min, 4 min, 8 min, 15 min. Four individuals of each species were exposed for each time period. Following the treatment period, specimens were re-immersed into individual seawater aquaria and observations (survival/mortality, weight) were recorded at 24 hours and 48 hours post-treatment.

Before the experiment began, viable *S. spallanzanii* specimens, including the tube, were removed from the housing aquaria and measurements (weight, tube length, tube width) were recorded. *Perna canaliculus* specimens that showed byssal attachment were removed from the depuration aquarium, weighed and their length measured. The controls for both species were then immediately placed into randomly selected 1.25 L individual aquaria of artificial seawater, aerated with an airstone (**Figure 2-3**b). Experimental treatments fully submerged each specimen in the acetic acid solution for the required treatment period (1, 2, 4, 8, 15 mins) before being randomly placed into individual aquaria for post-treatment observations. Each specimen was submerged separately and the acetic acid solution changed between individuals to avoid possible dilution and to maintain independence of each treatment and specimen.

At 24 hours post-treatment, each specimens behaviour was observed to determine survival or mortality, and removed from the aquaria to be wet weighed. Upon re-immersion, visible waste in the aquaria water was removed and a 30% water exchange was conducted. Survival, or mortality, and weight was recorded again at 48 hours post-treatment. At this point the experiment was ended. All specimens used in this procedure were then euthanized ethically by freezing.

4.2.2 Statistical analysis

Microsoft Excel was used to summarise the data set and Statistica (version 13) used to test hypotheses and provide visual statistics. As the treatment results

were binomial (alive versus dead), non-parametric Kruskal-Wallis ANOVA was used to test the two hypotheses. A two-way ANOVA was used to compare the survival of both species together to each treatment time to determine if a treatment could be used to kill *S. spallanzanii* and would not affect *P. canaliculus*. Significant results were further analysed with Duncan's multiple range post-hoc tests. Confidence intervals were set to 95%.

4.3 Results

The median initial weight of *Sabella spallanzanii* specimens was 7.95 g (±0.6 SE; *n* = 24) with a minimum and maximum weight of 4.6 g and 13.5 g respectively. The median initial length of *S. spallanzanii* specimens was 240.5 mm (±7.1 SE) and ranged from 155-297 mm. *Perna canaliculus* median initial weight was 38.8 g (±1.7 SE; *n* = 24) and ranged from 20-54.3 g. The median initial length of *P. canaliculus* was 76.5 mm (±1.4 SE) and ranged from 57-86 mm. Experimental procedures resulted in the mortality of 75 % of *S. spallanzanii* specimens and 45.8 % of *P. canaliculus* specimens.

4.3.1 Survivorship of *Sabella spallanzanii* to acetic acid immersion

When measured at 24 hours after the immersion treatment, there was a statistical difference in the survivorship of *S. spallanzanii* between treatment groups ($KW\text{-}H_{[5,24]}=13.416$; $p = 0.0198$). The differences were between the control and 1 min treatment groups (100% survival) and all treatment periods over 4 mins (

Table **4-1**). All specimens in the 4 min treatment died by the 24 hour measurement period, with 75% mortality occurring in both the 8 min and 15 min treatments and 50% mortality in the 2 min treatment.

Table 4-1 Post-hoc results of *Sabella spallanzanii* survival to acetic acid treatments. Measure taken 24 hours after re-immersion. n.s. = not significant.

Time	0	1	2	4	8	15
0	-					
1	n.s.					
2	n.s.	n.s.				
4	0.003	0.003	n.s.			
8	0.018	0.016	n.s.	n.s.		
15	0.016	0.014	n.s.	n.s.	n.s.	-

At 48 hours post-treatment there was also a statistical difference in survivorship between treatment groups (KW-$H_{[5,24]}$=15.333; p = 0.0090). The control group had a 100 % survival, which differed significantly from all other treatments (**Error! Not a valid bookmark self-reference.**). Mortality was 100% in all treatments equal to or longer than 4 mins of acetic acid immersion. In the 1 min and 2 min treatments 75% of specimens died.

Table 4-2 Post-hoc results of *Sabella spallanzanii* survival to acetic acid immersion treatments, measured 48 hours post-treatment. n.s. = not significant.

Time	0	1	2	4	8	15
0	-					
1	0.0019					
2	0.0024	n.s.				
4	0.0003	n.s.	n.s.			
8	0.0003	n.s.	n.s.	n.s		
15	0.0003	n.s.	n.s.	n.s	n.s.	-

4.3.2 Survivorship of *Perna canaliculus* to acetic acid immersion

At 24 hours after treatment, there was a statistically significant difference in survivorship between *P. canaliculus* treatment groups (KW-$H_{[5,24]}$ = 12.563; p = 0.0278). The 15 min treatment differed from all other groups (

Table **4-3**), where all mussels died. In the 2-8 min treatment periods, mortality was 75%.

Table 4-3 Post-hoc result of *Perna canaliculus* survival to immersion in acetic acid. Measure taken 24 hours after re-immersion. n.s. = not significant.

Time	0	1	2	4	8	15
0	-					
1	n.s.					
2	n.s.	n.s.				
4	n.s.	n.s.	n.s.			
8	n.s.	n.s.	n.s.	n.s.		
15	0.002	0.002	0.008	0.01	0.012	-

At 48 hours after treatment, there was also a significant difference between treatments (KW-$H_{[5,24]}$= 16.245; p = 0.0062). The 8 min and 15 min treatments differed from all groups except each other, and the 1 min and 2 min treatment differed from each other (Table 4-4). All mussels died in treatments equal to and over 8 minutes. In the 2 min treatment mortality was 50%, and 25% of mussels died in the 4 min treatment. All mussels survived in the control and 1 min treatments.

Table 4-4 Post-hoc results of *Perna canaliculus* survival to immersion in acetic acid. Measure taken 48 hours post-recovery. n.s. = not significant.

Time	0	1	2	4	8	15
0	-					
1	n.s.					
2	n.s.	0.0443				
4	n.s.	n.s.	n.s.			
8	0.0006	0.0006	0.0443	0.0052		
15	0.0006	0.0005	0.0360	0.0043	n.s.	-

4.3.3 Comparison of survivorship between species

When measured at 24 hours post-treatment, there was no statistical difference when comparing overall *S. spallanzanii* and *P. canaliculus* survivorship to acetic acid treatments ($F_{[5, 36]}$=2.0526; p=0.0944; Figure 4-1). However, further analysis shows that there was a significant difference (p=0.13) between species immersed for 4 mins.

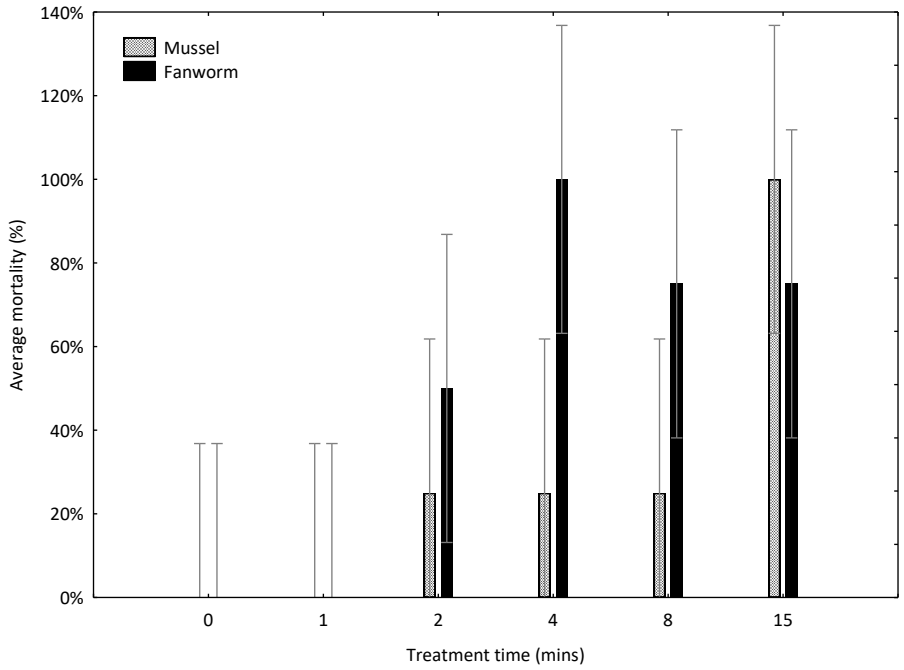

Figure 4-1 Average mortality of *Sabella spallanzanii* and *Perna canaliculus* to acetic acid immersion, measured 24 hours post recovery. *n*=4 specimens per treatment. Error bars indicate standard error.

Survivorship measured at 48 hours post-treatment indicates that there was a significant difference ($F_{[5, 36]}$=3.0000; *p*=0.0230; Figure 4-2) in survivorship of species between treatments, with the differences in the 1 minute (*p*=0.002) and 4 minute (*p*=0.003) treatments. Mortality increased with treatment time for *S. spallanzanii*, however *P. canaliculus* showed higher mortality in the 2 min treatment that the 4 min treatment.

Many individuals of both species survived the initial 24 hrs after treatment but died before the 48 hr observational periods. *Sabella spallanzanii* mortality went from 50% to 75% and *P. canaliculus* from 29% to 46% between observations.

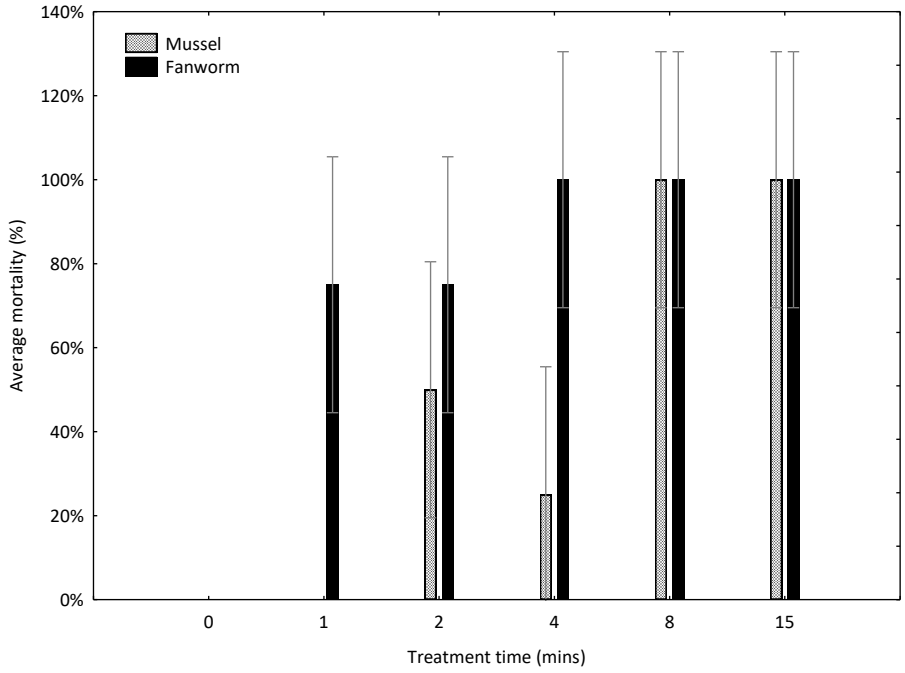

Figure 4-2 Average mortality of *Sabella spallanzanii* and *Perna canaliculus* to acetic acid immersion. Measure taken 48 hours post-treatment. n=*4* specimens per treatment. Error bars indicate standard error.

4.4 Discussion

The aim of this experiment was to explore the potential of acetic acid as a treatment option for *Sabella spallanzanii* that would be suitable for use in New Zealand mussel aquaculture. The results show that immersion in 5% acetic acid for increasing periods of time kills both *S. spallanzanii* and *Perna canaliculus*, so therefore the null hypotheses are refuted. Immersion of 1 minute in acetic acid was effective in killing 75% of *S. spallanzanii* and caused no mortality in *Perna canaliculus*. An immersion time of 4 mins killed 100% of S. spallanzanii, with a 25% mortality to mussels. However, as the 2 min immersion treatment killed 50% of mussels, this wouldn't be a recommended treatment without further testing. The short time period of chemical immersion required to have a significant impact on *S. spallanzanii* is considered a positive factor because this would have the least impact on harvest production time.

These results are comparable with the findings of similar studies, in that acetic acid immersions only require short treatment times to control fouling organisms. For example 5% solutions for 30 secs exposure killed 95% of *Ciona intestinalis* (Carver et al. 2003). Similarly, immersion in 4% solutions for 1 min eliminated many soft-bodied organisms (Forrest et al. 2007). This is supported by a study

aiming to explore treatments to eradicate tunicate fouling on mussels. Juvenile blue mussels suffered 100% mortality after immersion in a 5% solution for 5 and 10 min treatment times (Carman et al. 2016). The authors concluded from their results that acetic acid wasn't an effective treatment due to the high mortality of the farmed product, however they recognised that future studies should use shorter treatment times.

Prior to using acetic acid as a treatment during the harvest process, the methods for it application need to be refined as there are a number of variables that may negate its efficiency. For example, repeated immersions into the same solution would result in a dilution of the solution and immersion time would have to be increased accordingly (Forrest et al. 2007; LeBlanc et al. 2007). Forrest et al. (2007) also noted that mussel mortality was higher when immersion in acetic acid was followed by an air exposure period. As transport of the product overland is typical, they suggested rinsing the product before transport or treating the product after transport. Mussel mortality is also higher if acetic acid is directly applied to the flesh and unfortunately, shell gapes are common during the harvest process (LeBlanc et al. 2007). Thus, application of acetic acid during the harvest process may result in higher mortality rates than measured in this experiment.

Immersion treatments of whole mussel lines may not be logistically possible as it would require modification to the harvest process (LeBlanc et al. 2007). Piola et al. (2009) had success with a single spray of 5% acetic acid solution resulting in 65% reduction in the biofouling on experimental plates after 1 minute exposure time. A similar result could easily be achieved by spraying lines as they are removed from the water or incorporating acetic acid into the spray jets used in the de-clumping machine. However, this would result in acetic acid being discharged into the environment, which may have a negative impact as it is biocidal to non-target organisms and can affect the pH of the water (Locke et al. 2009a). Although studies have shown that acetic acid rapidly dissipates in the marine environment and would only affect a small area (Locke et al. 2009a), there is potential that mussels awaiting harvest may be impacted by any discharge.

This experimental procedure was conducted on *S. spallanzanii* whilst in the tube, which may have protected the animals body from the full effect of the treatment as it has one of the strongest tubes of the Sabellidae (Giangrande et al. 2014a). However, Read et al (2011) suggest that the tube doesn't offer protection from chemicals in the environment. As *S. spallanzanii* commonly fall or eject from the tube, future research would benefit from the comparison between treatments conducted on specimens both within and without the tube.

4.5 Conclusions

In this experimental procedure, a 1 min immersion in 5% acetic acid had no impact on mussel mortality and resulted in 75% *Sabella spallanzanii* mortality. To achieve 100% *S. spallanzanii* mortality, an immersion time of 4 mins is required, however this would result in 25-50% mussel mortality. Therefore, the results indicate that acetic acid immersion is a treatment that is effective at quickly killing *S. spallanzanii* with little or no impact on the farmed product. Hence, this treatment could be considered further by biosecurity and industry managers as a viable control option of *Sabella spallanzanii* on mussel farms (or other marine infrastructure).

Chapter 5

Thesis summary

As novel habitats, marine aquaculture facilities attract biofouling and are known to aid in the secondary dispersal of non-indigenous marine species (NIMS) (Hewitt et al. 2006). Biofouling can also cause economic loss by reducing the quality and increasing production costs (Adams et al. 2011). Listed under New Zealand's *Biosecurity Act* 1993 as an Unwanted Organism, the Mediterranean fanworm *Sabella spallanzanii* has rapidly established populations throughout NZ since its discovery in 2008 (Read et al. 2011a). There is evidence that secondary spread of this species may already be attributed to mussel aquaculture, with the discovery of an *S. spallanzanii* infestation on a wharf in Coromandel, NZ (T. Malcolm, pers. comm., Waikato Regional Council) that is closely associated with mussel aquaculture facilities. Therefore, it is both ecologically and economically important for biosecurity and aquaculture managers to develop strategies that control *S. spallanzanii* and (NIMS) fouling on mussel aquaculture.

The objectives of this thesis were to: 1) examine the survivorship of *Sabella spallanzanii* after being exposed to typical mussel farming operations to determine the potential of secondary spread after harvesting; and 2) explore a treatment option that would effectively kill *S. spallanzanii* while having minimal effect on the product. The results of these objectives are summarised below.

1) Experimental procedures showed that *Sabella spallanzanii* is highly resilient to the typical re-seeding and harvest operations that occur in mussel aquaculture. Specimens survived upwards of 24 hours air exposure in desiccation treatments: a longer time frame than what would occur during re-seeding or the harvest process and subsequent landing of stock and transport to processing facilities. Long term survival and regeneration of body parts within 28 days was evident following fragmentation: a simulation of the potential disturbance caused by the harvest process. Therefore, these processes are not effective in containing *S. spallanzanii* and may actually aid its re-infestation and help to expand its geographic range.

2) An additional experimental procedure found that *S. spallanzanii* can be quickly treated with acetic acid. Immersed in a 5% solution of acetic for 1 minute killed 75% of *S. spallanzanii* with no effect on mussel survivorship.

This is a promising control method that could contain *S. spallanzanii* and have minimal impact on harvest production time. However, as a biocidal chemical there needs to be strict controls in place on its use in order to minimise any adverse environmental impact.

These results clearly highlight the need for a management plan that will effectively contain *S. spallanzanii* to mussel aquaculture facilities and prevent any further secondary spread. Whilst a complete eradication of the species may not be feasible as it is already well established (Myers et al. 2000), these results show that treatment options are available that will contain *S. spallanzanii* and prevent further spread.

NZ aquaculture farmers, understandably, are focussed on increasing profit and tend to believe that nothing can be done to stop pest dispersal (Sim-Smith et al. 2014). However, lessons should be learnt from the management of ascidian infestations on mussel farms in Canada that have severely threatened the economic viability of the industry (Ramsay et al. 2008). Key factors in the successful response to an incursion have been due to early detection, good communication and collaboration between stakeholders, strong partnerships and rapid action(Locke et al. 2009b; McKenzie et al. 2016).

5.1 Concluding statement

The results of these experiments will enable biosecurity and marine farm managers to make informed decisions about the treatment, management and containment of *Sabella spallanzanii* and prevent its secondary spread to new geographic areas. However, successful and effective mitigation will rely on a proactive commitment between stakeholders working together to achieve a common goal.

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Appendices

7.1 Appendix A

Biosecure laboratory and MPI Biosecurity permissions to conduct research on *Sabella spallanzanii*

All experiments were conducted in a portable laboratory located onsite at the University of Waikato's Coastal Marine Field Station, Tauranga, New Zealand (S37°40'16.3" E176°10'0.2"). The laboratory contained power, fluorescent lighting, extractor fan and water (via an external hose attached to mains water) and was modified for this project to include an air conditioner (to provide stable air temperature) and catchment bin for waste water (to meet Biosecurity standards). Access to the laboratory was strictly limited to authorised personnel only. All equipment and water that came into contact with *Sabella spallanzanii* was sterilised with household bleach (Sodium Hypochlorite (NaCl0), 36.7 g/litre) before being removed from the laboratory. Equipment was sprayed with a 5% solution of bleach, covered with fresh water and soaked for at least 15 mins, then rinsed and air dried. Undiluted bleach was stirred into waste water at a ratio of 2:1 ml:litre (bleach:water) and, after approximately 15 mins, discarded onto a lawn area containing no direct drainage into storm water channels. All solid biological waste was stored in a freezer until the conclusion of all experimental procedures, then discarded into landfill.

Specimens were housed in four 65 litre glass aquaria that were 610mm x 300mm x 380mm in size. The aquaria were aerated with two air stones and contained no added substrate. The room temperature was maintained at 18°C, with the natural light regime from laboratory windows used whenever possible. Red lighting was used for experiments or assessments conducted at night. Aquaria were labelled with biosecurity details, the treatment occurring, and the experimental start and end dates.

Ethical approval to carry out this research was not required, as under the NZ *Animal Welfare Act* 1999, *S. spallanzanii* is not deemed to be an "animal". 'Generic Permission' to conduct bona fide research on *S. spallanzanii*, is granted by the Ministry for Primary Industries (MPI) under sections 52 and 53 of the

Biosecurity Act 1993. Permission was granted by the Biosecurity Chief Technical Officer to carry out this research, with a number of conditions being required:

1. *Sabella spallanzanii* must be held in a manner that prevents any part of the organism from being lost, mislaid, released back into the marine environment.
2. Transportation of *S. spallanzanii* is permitted between the collection are and research site, ensuring a direct route is taken.
3. During transportation, *S. spallanzanii* and the transport water must be held within secure containers to prevent escape or leakage and supervised at all times. Containers must be clearly labelled with content details AND organism status AND instructions not to release under any circumstances AND contact details of the person responsible.
4. *Sabella spallanzanii* must be held within secure containers and clearly labelled with organism status AND details on the risks posed by the organism.
5. Research of *S. spallanzanii* must be undertaken in a contained land-based facility.
6. Any equipment that comes into contact with *S. spallanzanii* must be treated after use and any water used must not be discharged directly back into the marine environment.
7. At the end of the research, all *S. spallanzanii* specimens shall be rendered non-viable or destroyed by heat, desiccation or other effective means.
8. Prior to conducting research, the Chief Technical Officer (MPI) must be notified of the intended research purpose and that all permission conditions have been met.
9. All persons involved in the research must understand and comply with the permission conditions.
10. Should accidental release of *S. spallanzanii* occur, the Chief Technical Officer (MPI) must be notified as soon as practicable and appropriate action taken immediately to retrieve, contain or control the organism.

7.2 Appendix B

Specimen collection and transportation

Sabella spallanzanii specimens were collected on 11th May, 2016 at Orakei Marina, Auckland (S36°50’58.8” E174°48’34.9). Specifically, all 150 specimens were collected from the first three finger berths of “D pier”. Specimens were removed from the wooden pontoons using a paint scraper to ensure the whole animal tube was removed intact. Epibionts (encrusting species growing on the tube), such as algae and hydroids, were removed from the *S. spallanzanii* tubes prior to placing the specimen into a sealable 50 L container of seawater. Four containers in total were used, with each holding 30 - 40 *S. spallanzanii* individuals with a target size of specimens being 150-300 mm in length. Collection took place over 1.5 hours.

Transportation of specimens to the laboratory took three hours. Sealed containers were placed into a 160 L bin to contain any leaks (if they occurred) and to secure containers within the vehicle. On arrival at the laboratory, the specimens were randomly spread between two 160 litre containers of artificial seawater that was aerated with two air stones and left to depurate for 48 hours. After the depuration period, all specimens were randomly spread across four ‘housing aquaria’ that contained fresh artificial seawater and were aerated.

7.3 Appendix C

Settlement period and husbandry regime

Specimens were left within the housing aquaria for a period of 10 weeks to acclimate. During this period a husbandry regime was implemented and specimens were monitored to note visible signs of stress (e.g., crown detachment) and recovery from collection and transportation. During the acclimation period, a number of simple behavioural patterns were observed; i) attachment to substrate; ii) natural vertical orientation; and iii) reaction to stimulus (further explained in Appendix D).

The husbandry routine consisted of weekly water quality testing (e.g., temperature, oxygen, pH, ammoniums), aquaria cleaning and removal of waste material, followed by a 30% water exchange and feeding. Water quality parameters remained consistent throughout the project (Table C7-1). The water exchange was increased to 50% if Ammonia (NH_3/NH_4), Nitrite (NO_2) or Nitrate (NO_3) readings were elevated. Specimens were fed a concentrated microalgae commercial rotifer feed (Rotigrow+) diluted with reverse osmosis water at a ratio of 1:40 (algae:seawater).

Table C7-1 Average water quality parameters of Sabella spallanzanii housing aquaria, measured weekly. n = 4 aquaria and 18 sampling periods.

	Temp (°C)	pH	Oxygen (%)	Ammonia (mg/L)	Nitrate (mg/L)	Nitrite (mg/L)
Mean	16.8	7.7	95.7	0.1	4.3	0.5
Std. Error	0.1	0.0	0.4	0.0	0.5	0.1
Minimum	15.4	7.0	84.5	0.0	0.0	0.0
Maximum	18.1	8.4	101.7	2.0	20.0	5.0

At no time during this project did any specimens attach to the aquaria. Mesh was suspended approximately 5 cm from the bottom of two housing aquaria, and specimens placed upright between the gaps to encourage attachment in a vertical orientation, but this had no influence. All specimens in the housing aquaria without mesh remained in a horizontal position.

7.4 Appendix D

Establishing a *Sabella spallanzanii* behavioural reaction index

In the field, *Sabella spallanzanii* react to stimuli by withdrawing their crown and body into their protective tube and then reappearing after a period of time (pers. obs.). This behaviour could be used as a method to determine the reaction of *S. spallanzanii* to stimuli, such as water movement, touch, shadows, and its recovery after experimental treatments. Thus, a number of non-invasive trials were conducted during the final weeks of the acclimation period in order to establish a method for quantifying the 'health' of *S. spallanzanii,* within the tube, following an experimental procedure. The crown activity of specimens was used as the response variable, which is easily observed without handling the specimen.

Over a two-week period (14 days), thirteen sampling events took place at various times over a 24 hour window to cover a range of natural light availability scenarios. A red light was used when observations occurred at night. The stimuli reaction tests consisted of the following:

- Whilst extended from the tube, the crown of a specimen was lightly tapped with a plastic pipette to result in the specimen retracting into their tube;
- The recovery time was then recorded. Recovery period consisted of the time that the retraction occurred (time start) to when full crown extension beyond the tube occurred (time end).
- At each sampling period, this stimuli trial was conducted on 3-5 specimens in each of the four housing aquaria (*n* = 12-20 specimens per trial), with a maximum recovery time being 10 mins. Average recovery times for each trial are provided in (Figure D7-1).

On average, *S. spallanzanii* took 253 ± 18 seconds to recovery from a touch stimulus. This method of quantifying the health of *S. spallanzanii* via its recovery to stimuli was rejected, however, as it relied on the crown being extended and the timing of this behaviour was too inconsistent to be used as an indicator of recovery. Crown extension also implies the specimen is feeding, and as this

would only occur in specimens that were relatively healthy it wouldn't give an indication of how affected a specimen was following an experimental treatment.

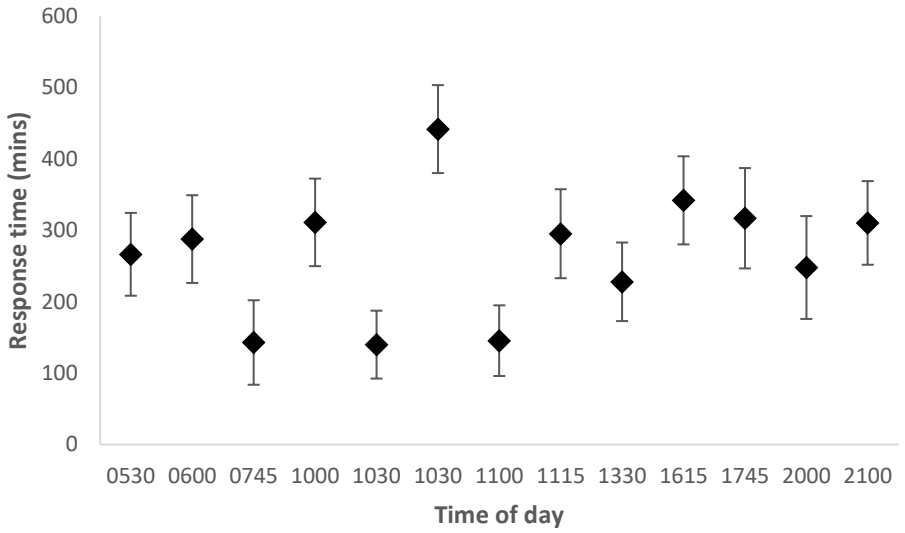

Figure D7-1 Average crown recovery time (sec) of Sabella spallanzanii to external stimulus. n=12-16 per time period. Error bars indicate standard error.

Thus, a second trial was implemented. Trial 2 used time-lapse video to quantify the time and the frequency with which an untreated specimen showed signs of 'healthy' behaviour (i.e. crown extension). This would provide a baseline indicator of 'healthy' behaviour with which to compare the same behaviour following an experimental treatment.

Unlike Trial 1, Trial 2 removed five specimens from the housing aquaria and placed them into one of twenty-five individual 1.25 L aquaria that were to be used for specimens following an experimental procedure. These aquaria were laid out into 5 rows of 5 aquaria, and a 50^2 cm aluminium quadrat (modified to hold a GoPro camera) was placed over the 25 aquaria (Figure D2). In order to test if clear video coverage could be obtained of all aquaria at once, one specimen was placed in the centre aquarium and the remaining specimens were placed in the central aquarium of each outside row. An acrylic platform suspended above the aquaria enabled a GoPro camera to record all aquaria at once for an extended time period (the GoPro was plugged into the mains power to overcome battery limitations). Time lapse video was then set to record one frame every 10 seconds continuously for 24 hours. A wall clock was placed in the video frame, to enable the exact time of frames to be recorded, and a lamp with a red bulb was

used to illuminate the frame at night. The resulting time lapse video was approximately 5 mins duration. Unfortunately, this method of determining 'healthy' behaviour was also rejected due to the fish eye effect of the GoPro camera, coupled with water movement from the air bubbles, which made it difficult to clearly see all aquaria and crown movements. Hence, reviewing the video was extremely time consuming and deemed to be beyond the scope of this project.

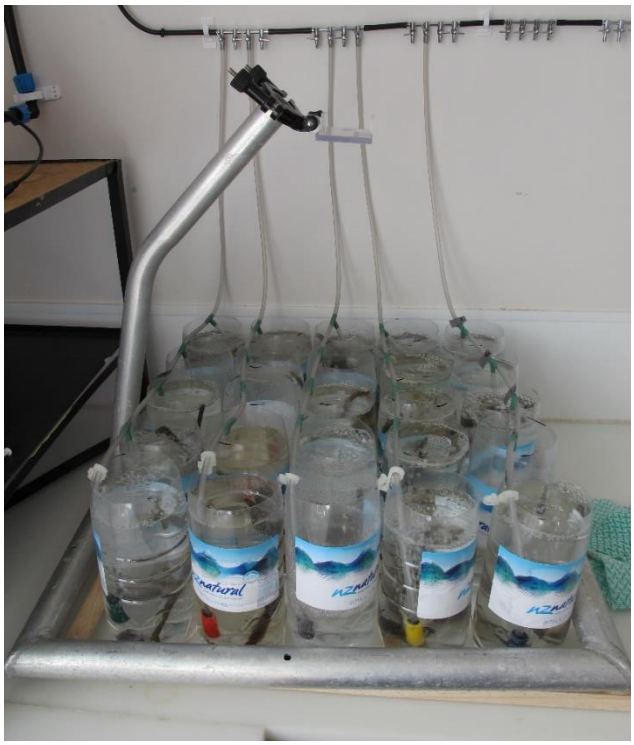

Figure D2 Design of trial to observe crown movements of Sabella spallanzanii with time lapse video.

As both trials failed to produce a suitable index that could be used to establish the health of a specimen (within the tube) following an experimental procedure, it was decided that survival or mortality would be used to quantify the reaction of a specimen to an experiment. As outlined further in Chapter 2, survival could be established using a number of observations: clear (as opposed to green, brown or cloudy) fluid discharge from the tube; movement of the body into the posterior tube end; fast retreat from the posterior tube end following gentle palpation of the body. Visible chaetae movement and body retraction was used as the survival indicator in specimens not within the tube.