The biology of the invasive Mediterranean fanworm, *Sabella spallanzanii*, in the context of mussel aquaculture

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Abstract

The spread of marine non-indigenous species (NIS) is increasing rapidly, largely as a result of increased global shipping, which translocates NIS via ballast water and biofouling on ship hulls and in sea chests. The worldwide management of unwanted NIS costs millions of dollars annually. The aquaculture industry is particularly impacted by NIS through increased production and processing costs. To eliminate or contain these marine NIS effectively, it is important to know about their reproductive biology, settlement preferences and growth. One such NIS for which there is limited information is the Mediterranean fanworm, *Sabella spallanzanii*. This species has spread into a number of countries, including more recently New Zealand. The research presented in this thesis investigated the reproductive biology, settlement preferences, growth and regeneration (following fragmentation) of the Mediterranean fanworm in northern New Zealand, especially in the context of Greenshell™ mussel farming.

Adult fanworm samples collected from a population in the Waitemata Harbour had an uneven sex ratio (2:1 female to male ratio) and were continuously producing eggs (gametogenesis) throughout the year with potentially several spawning events during the year. The fanworms reached maturity at a smaller body size in New Zealand, compared to fanworm populations in the Mediterranean Sea and at larger body size compared to Australia. While the presence of fanworm could be detected via eDNA and eRNA from seawater and from biofilm on settlement plates from mussel farms in the Hauraki Gulf, the signal varied markedly among samples and months, making it difficult to establish a clear temporal pattern for fanworm presence. Although, fanworm eDNA and eRNA was detected in a field situation with high turbidity and turnover, it is unknown whether the detected molecular signal originates from extracellular fanworm eDNA molecules (i.e., mucus, branchial crown fragments) or whether the signal originates from fanworm larvae. However, by visually assessing settlement of *S. spallanzanii* on different substrate material, it was confirmed that fanworm were present in the same locations where the eDNA sample was taken, even though eDNA signal was low.

Fanworm showed a clear larval settlement preferences for morphologically more complex surfaces, including fibrous mussel grow-out rope, fibre-cement plates with plastic mesh and among established biofouling such as blue mussels, tunicates and colonial bryozoans. It is likely that the morphological complex physical structures offer protection for the recently settled juveniles while at their most vulnerable, until they are able to build their protective tube to further protect themselves against potential predators and competition. A biosecurity concern has been that harvesting Greenshell™ mussels may result in the proliferation of fanworm via the release of fragmented fanworms and that these fanworm fragments could regenerate. Observations during mussel harvesting indicated that many of the fanworms remained attached to the filamentous mussel grow-out rope, even while being run through the de-clumping machine, and were returned to shore facilities attached to the rope in bulk handling bags for cleaning and drying before reuse. The size of fanworm fragments did not affect their survival or regeneration capability. Although, in general, posterior fragments had a
higher survival and were able to regenerate a new branchial crown quicker compared to anterior fragments that had higher mortality and were slower in regenerating tail segments. At sea, fanworm fragments were slower at regenerating body parts compared to those held in controlled laboratory conditions, indicating that environmental exposure (i.e., temperature, currents and bacteria) affected the speed of recovery. The experimental release of fanworm fragments during mussel harvesting did not result in their consumption by fish predators, while the use of other marine bait confirmed the presence of fish predators. Overall, the results of the research presented in this thesis provide valuable new knowledge about the biology of the Mediterranean fanworm in New Zealand conditions and will be useful for biosecurity agencies and mussel farmers to develop more effective management strategies for this species.
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Chapter 1 – General Introduction

1.1 Introduction

Marine non-indigenous species (NIS), also known as non-native, alien, exotic species, are the result of accidental or intentional transfer of marine species by human activities to locations outside their normal range (Bax et al., 2003; Colautti & MacIsaac, 2004; Molnar et al., 2008). Marine NIS are of concern because they can threaten native biodiversity, create human health risks, and become a nuisance for marine activities, usually through biofouling of marine infrastructure, especially vessels and aquaculture operations (Coutts & Dodgshun, 2007; Adams et al., 2011; Fitridge et al., 2012; Atalah et al., 2016b; Sievers et al., 2017).

Once a marine NIS becomes locally abundant or causes negative effects such as impacting native species assemblages, posing human health risks or affecting economic activities, they are frequently labelled as invasive species (Colautti & MacIsaac, 2004; Lodge et al., 2006; Bates et al., 2013; Clarke Murray et al., 2014; Champion, 2018). In New Zealand, once a NIS alters ecosystem processes, outcompetes native species, or becomes a nuisance for aquaculture and recreational vessels, they can be labelled as a pest or invasive species under the New Zealand Biosecurity Act 1993 (Hayes & Sliwa, 2003; Hewitt et al., 2004; Fitridge et al., 2012; Giangrande et al., 2020). Most NIS introductions are unsuccessful, since they are either compromised by the journey to the new environment and do not survive, or they cannot compete with the native species or the new environment is unfavourable to them becoming established (Locke et al., 2007; Giangrande et al., 2020).

Marine NIS are mostly spread through their attachment to vessel hulls, sea chests, as well as in ballast water, bilge water and with the movement of aquaculture gear and cultured organisms, including organisms for the aquarium trade (Bax et al., 2003; Hewitt et al., 2004; Molnar et al., 2008; Ramsay et al., 2008a; Floerl et al., 2009; Clarke Murray et al., 2011; Fitridge et al., 2012; Ojaveer et al., 2018). With rising global movement of vessels, and increasing transfers of aquaculture product and live aquarium species, the range and number of marine NIS spreading and establishing over greater distances is increasing (Lodge et al., 2006; Campbell et al., 2018; Ojaveer et al., 2018; Cole et al., 2019; Hayes et al., 2019; von Ammon, 2019).

Once marine NIS have founded in a new location, it is almost impossible to eradicate them and preventing further spread involves considerable labour and expense (Bax et al., 2003; Lodge et al., 2006; Locke et al., 2007; Fletcher, 2014). There are only very few examples of successful eradication of marine pests worldwide. The main factors to make these eradications successful were that the invasions were detected very early, immediate intervention was undertaken and in the case of the black-striped mussel, *Mytilopsis sallei*, in Darwin, Australia, the area could be completely isolated and all potentially infected vessels were contacted and cleaned (Bax et al., 2002). In the case of the killer algae, *Caulerpa taxifolia*, eradication in southern California was
again successful due to immediate action upon discovering a few specimens and collaboration from all parties (i.e., stakeholders, marinas, boat owners and the public) and because the few specimens could be isolated and destroyed under plastic frames and tarpaulins (Anderson, 2005).

Once the invasive species has become established and eradication is no longer possible, the only remaining options are attempting to limit the size of the invasive population in selected locations or trying to slow down further spread (Lodge et al., 2006).

How, or if, an invasive species can establish, and how severe any consequences will be on the local economy, ecosystem and biodiversity, will greatly depend on the invaded location and the state and health of the habitat and species assemblages (Bax et al., 2003; Lodge et al., 2006). Remote locations or islands, often with many endemic species, are more at risk, since the endemic species are often more vulnerable to being outcompeted by invasive species (Hewitt et al., 2004). Furthermore, environmental modifications caused by one invasive species often increases the opportunities for the subsequent arrival and establishment of other invasive species (Simberloff & Von Holle, 1999; Bax et al., 2003).

These could be reasons why New Zealand has proven to be vulnerable to the establishment of marine NIS. For 80 million years New Zealand’s biodiversity evolved largely in isolation, consequently many of New Zealand’s species are endemic and are only found amongst the unique biodiversity hotspots around the country (Morrison, 2005). The Exclusive Economic Zone of New Zealand covers an area of 4.4 million km², which is a marine area that is 14 times bigger than New Zealand’s land area (van Bunnik et al., 2007). The rich native biodiversity of New Zealand has over 80,000 species, of which 80% are marine species, of which 44% are endemic (van Bunnik et al., 2007). However, in the last 200 years, more than 300 marine NIS have established in New Zealand (Morrison, 2005; van Bunnik et al., 2007). One of the more recent NIS arrivals is the Mediterranean fanworm, Sabella spallanzanii Gmelin (Annelida: Polychaeta).

The Mediterranean fanworm was accidentally introduced to New Zealand in 2008 when it first appeared in Lyttelton Harbour and in the following year was found in Auckland’s Waitemata Harbour (Clapin & Evans, 1995; Knight-Jones & Perkins, 1998; Read et al., 2011; Fletcher, 2014). Since then it has continued to spread to major ports, marinas and more recently to mussel farms in the North Island. Despite attempts to remove it from several coastal areas, it has continued to spread (Read et al., 2011; Fletcher, 2014).

The Mediterranean fanworm appears to be a habitat generalist, most frequently growing on artificial structures, such as marina infrastructures, vessel hulls and mussel aquaculture lines but it has also been observed growing in soft sediment and intertidal areas (Fletcher, 2014; Douglas et al., 2020; Tait et al., 2020; K. James, pers. comm., 2018; B. Skelton pers. comm., 2019).

The Mediterranean fanworm causes problems for mussel farming operations in the Hauraki Gulf because the increasing abundance of fanworm growing on the farms interferes with mussel growth and increases the cost of handling and cleaning equipment as well as for harvesting the mussels (Woods et al., 2012; Forrest & Atalah, 2017). Mussel production from a farm in the Firth of Thames that is heavily infested with fanworm is typically reduced by 25 – 30% (David Blyth, pers. comm., Gold Ridge Marine Farm Ltd).
Greenshell™ mussel farms in the Hauraki Gulf make up around 30% of New Zealand’s total mussel production of around 100,000 t of mussels per year, which are collectively worth more than NZ$336 million in export revenue (Adams et al., 2011; Gribben et al., 2011; Atalah et al., 2016b; AQNZ, 2020). The mussels cultured in suspended longline culture systems in coastal areas are exposed to the establishment of a diverse range of marine biofouling organisms, including the Mediterranean fanworm, which accumulate over time on the associated mussel grow-out ropes (Adams et al., 2011; Fitridge et al., 2012; Atalah et al., 2016b). The accumulating marine biofouling increases the loading on the farm infrastructure, reduces farm productivity, while also increasing production and processing costs (Adams et al., 2011; Fitridge et al., 2012; Woods et al., 2012; Atalah et al., 2016b; Forrest & Atalah, 2017).

There are limited cost-effective options for controlling biofouling in mussel aquaculture (Fitridge et al., 2012; Woods et al., 2012; Sievers et al., 2014; Forrest & Atalah, 2017). However, harvesting and re-seeding the mussels are frequently used techniques to reduce the biofouling and the interspecific competition among the growing mussels (Fitridge et al., 2012; Forrest & Atalah, 2017). The timing of seeding mussels is confined to certain seasonal periods to avoid overgrowth from fouling organisms or other unsuitable conditions for the mussels. An improved understanding of the larval and settlement biology of biofouling organisms has the potential to lead to the development of mitigation strategies to minimize colonisation of nuisance biofoulers, such as the Mediterranean fanworm, in mussel aquaculture (Fitridge et al., 2012). Improved knowledge of the general biology of Mediterranean fanworm will also help to identify aspects of its biology that may be vulnerable to facilitating cost effective actions for managing it within the New Zealand mussel aquaculture industry.

This thesis aims to investigate the reproductive cycle, fecundity, settlement preferences, growth rate and regeneration after fragmentation of *Sabella spallanzanii* in the Hauraki Gulf, particularly in the context of Greenshell™ mussel farming. The general introduction outlines the research context and aims of the thesis and is followed by a literature review (Chapter 2), which summarizes the current knowledge of the Mediterranean fanworm. Three data Chapters (3 – 5), address the specific research aims of this thesis, each of which examine fundamental aspects of the biology of the Mediterranean fanworm in the Hauraki Gulf. Chapter 6 is the general discussion, which summarizes and discusses the key findings of each chapter in relation to the specific thesis aims and considerations for future research.

### 1.2 Specific chapter aims

Each of the chapters of this thesis presents research elements that were designed to investigate different biological aspects of *S. spallanzanii*, such as characterising the reproductive cycle and output, spawning seasonality, larval development, settlement preferences, growth, survival and recovery after fragmentation, which occurs during mussel harvesting. The overall goal was to gather insight into the specific aspects of *S. spallanzanii* in New Zealand conditions and compare these findings to previous studies that were conducted in the Mediterranean Sea, Europe and
Australia. It is intended that the results may help to improve current and future mitigation efforts for New Zealand mussel farmers and reduce the spread of this invasive species in New Zealand.

All procedures in this thesis were approved by the Ministry for Primary Industries (MPI) permission under section 52 of the Biosecurity Act 1993.

Chapter 2: Review of knowledge of biology and effects of the Mediterranean fanworm

For any new invasive species it is important to gather existing information about its biology and ecology so that its potential impact and avenues for control can begin to be determined. Furthermore, one intention of this study was to determine if the biology of the Mediterranean fanworm had adjusted to the different environmental conditions in New Zealand. Consequently, all readily available information on the biology and ecology of Mediterranean fanworm, from both New Zealand and overseas was accessed and summarised to form the basis of the literature review presented in Chapter 2.

Chapter 3: Determining the egg size, maturation and gametogenesis in relation to body size and time of year in Mediterranean fanworm

The research presented in Chapter 3 of this thesis aimed to investigate key aspects of reproduction in S. spallanzanii. The mode of reproduction in polychaetes varies widely, even among closely related species (Gambi et al., 2000). There are few reports describing reproduction in S. spallanzanii. Of significance are three specific studies, one for a Mediterranean fanworm population within its natural range in the Gulf of Taranto, in the Mediterranean Sea (Giangrande et al., 2000), and two studies from Australia, for populations near Melbourne, Australia, where it is an invasive species (Currie et al., 2000), and the other for a population near Adelaide, Australia (Lee et al., 2018).

The mode of reproduction, reproductive cycle and output have a great effect on the success and spread of an invasive species (Tovar-Hernández et al., 2011; Lee et al., 2018). Understanding the reproductive biology of S. spallanzanii in the Hauraki Gulf may provide a route to developing effective mitigation strategies for this species, including for the mussel farming industry in New Zealand (Champion, 2018).

Therefore, the research presented in this chapter aims to investigate: 1) the pattern of gametogenesis in fanworm to determine if they differ to those previously described for Australia and the Mediterranean Sea, 2) the size at maturity for fanworm, as well as the relationship between the body size of fanworm and the size of their eggs, 3) the annual spawning cycle, and 4) the sex ratio of fanworm over a range of sizes and among seasons.
Chapter 4: The presence, settlement and growth of the Mediterranean fanworm, *Sabella spallanzanii*, among mussel farms

It is difficult to see and identify the larvae of marine invertebrates, such as Mediterranean fanworm, pre-settlement. Most new marine NIS are first observed and identified once they have already settled and grown to a size that is easily visible, by which time they may have already spawned and released larvae to establish the next generation. Detecting the larvae and early settlement stages of marine invasive species via highly sensitive digital droplet PCR (ddPCR) from environmental DNA and RNA opens up new possibilities of early and improved species specific detection. The aim of the research presented in chapter 4 was to: 1) assess effectiveness of detecting the absence or presence of the Mediterranean fanworm among mussel farms in the Hauraki Gulf using environmental DNA and RNA detection methods, 2) compare eDNA and eRNA sample detection between biofilm and seawater samples, 3) assess presence, settlement seasonality and growth of fanworm via different settlement substrates.

Chapter 5: Determine whether Mediterranean fanworm can survive and recover from fragmentation in situ in the marine environment

*Sabella spallanzanii* have been shown to have a remarkable capacity to regenerate from fragments, in experiments conducted in highly controlled laboratory conditions (Licciano et al., 2012; King, 2017). The research presented in Chapter 5 aimed to investigate whether fanworm fragments could regenerate in situ in the marine environment, to attempt to establish whether the fragmentation of fanworms during mussel harvesting could contribute to the proliferation of the species through regeneration. Unlike a laboratory setting, fanworm fragmented during mussel harvesting operations are exposed to potential predators, pathogenic bacteria and more variable and adverse environmental conditions. Specifically, this research aimed to: 1) describe the nature of the fragmentation of fanworm during mussel farming operations, 2) identify whether the scavenging fish associated with mussel harvesting operations will consume fanworm fragments, 3) determine whether fragments of fanworm can regenerate in situ if left in the marine environment and compare these results to fanworm fragments that are kept in a laboratory setting.

All procedures in this chapter were approved by the University of Auckland Animal Ethics Committee (AEC-002254).

Chapter 6: Synthesis of research results

The general discussion, which is presented in Chapter 6, discusses the collective outcomes from the preceding data chapters and summarises the improved knowledge of the biology and ecology of Mediterranean fanworm in the Hauraki Gulf. These results are discussed in terms of how they
could be useful for developing potential mitigation strategies for the species, especially for mussel
farming. In addition, future research avenues are identified that arise from this study.

Appendices 1 and 2: Details of research efforts on the spawning and fecundity measures of
the Mediterranean fanworm

Appendices 1 and 2 of this thesis provide details of additional experiments that were conducted to
attempt to spawn and raise larvae of Mediterranean fanworm in captivity and to measure fecundity
through the automated counting of eggs. These experiments failed to produce useful results but
were recorded to provide a basis for future studies to avoid repeating ineffective methodologies.
Chapter 2 – Literature review

2.1 Introduction

The Mediterranean fanworm, *Sabella spallanzanii* Gmelin (Annelida: Polychaeta), also called the giant fanworm and the European fanworm, is a filter-feeding polychaete that is native to the Mediterranean and Atlantic coasts of Europe (Clapin & Evans, 1995; Knight-Jones & Perkins, 1998; Fletcher, 2014). However, recently it has become a concern as an invasive biofouling species for both Australia and New Zealand (Bax et al., 2003; Ross et al., 2007; Read et al., 2011; Stanley & Jeffs, 2011; Fitridge et al., 2012; Fletcher, 2014; Atalah et al., 2016b).

The first confirmed occurrence of *S. spallanzanii* in the Western Pacific Region was in Australia in 1992 within Port Phillip Bay, Victoria (Clapin & Evans, 1995; Clapin, 1996; Read et al., 2011; Murray & Keable, 2013). However, archived records suggest it may be possible that the species arrived as early as 1965 in Albany, Western Australia (Murray & Keable, 2013). Genetic analysis showed that the Australian populations of *S. spallanzanii* are probably derived from one or a few introduced individuals from the Mediterranean area, most likely via ship hull-fouling or ballast water, since they have reduced genetic variability compared to the Mediterranean populations (Murray & Keable, 2013; Fletcher, 2014). Vessel hull and sea chest fouling, ballast water, and aquaculture activities are the main pathways for the transport and introduction of invasive marine species, especially to New Zealand and Australia (Coutts & Dodgshun, 2007; Adams et al., 2011; Fitridge et al., 2012; Forrest & Fletcher, 2015; Hutchings, 2018). This is because both countries are too isolated to be reached by the natural larval dispersal range of most sessile marine species (Murray & Keable, 2013).

It is most likely that southern Australia was the source of the fanworm population that established in New Zealand, since the local population also has minimal genetic divergence (Ahyong et al., 2017). Due to the spread and strong presence of *S. spallanzanii* in Australia, the fanworm was labelled as "Unwanted organism" under the New Zealand’s Biosecurity Act in 2000 (Read et al., 2011; Fletcher, 2014).

The Mediterranean fanworm was first detected in New Zealand in May 2008 and subsequently became established firstly in Port Lyttelton, and subsequently the Waitemata Harbour and then Whangarei Harbour (Read et al., 2011; Fletcher, 2014). When the first few specimen of *S. spallanzanii* were discovered in Lyttelton Harbour in 2008, substantive efforts were made to contain the spread (Read et al., 2011; Fletcher, 2014). The worms were removed by divers from the wharf piles and these areas were checked regularly for reinestation (Read et al., 2011). Periodic searches up to December 2009 discovered a continued presence of the worms in Lyttelton Harbour despite active attempts at eliminating the pest (Read et al., 2011). In May 2009 a specimen was
found outside the inner port, suggesting reproduction had occurred, and indicating a second
generation of larval settlers had been produced (Read et al., 2011).

In October 2009 several fanworm individuals were found in the Waitemata Harbour and were
removed (Read et al., 2011). Further spread of the worms around the Waitemata Harbour was
noticed by divers in 2010 and it was decided that the localised elimination programmes would stop
at both Lyttelton and Auckland, although surveillance would continue (Read et al., 2011; Fletcher,
2014).

In February 2014, a small population of the Mediterranean fanworm was discovered in Waikawa
Bay, Coromandel, Tauranga and Nelson Harbours (Fletcher, 2014). Therefore, even though efforts
have been made to remove *S. spallanzanii* at the beginning of its invasion, this species has
continued to spread within New Zealand coastal waters and now appears to be well established
over an extensive latitudinal range from Whangarei to Lyttelton (Read et al., 2011; Fletcher, 2014;
Morrisey et al., 2016).

For any new invasive species it is important to gather existing information about its biology and
ecology so that its potential impact and avenues for control can be determined. Therefore, the
purpose of this literature review is to provide a general overview of what is known so far about the
biology and effects of the Mediterranean fanworm from the most readily available literature. The
review aims to initially identify how this invasive species may affect native species, habitats and
aquaculture, as well as assessing whether it may have any uses. This initial review assists with the
design of the experiments for the research presented in this thesis, which examines aspects of the
biology and ecology of *S. spallanzanii* in the Hauraki Gulf.

### 2.2 Physical characteristics of the Mediterranean fanworm

The adult *S. spallanzanii* is a sessile, tube-dwelling worm with a prominent branchial crown of
brightly coloured bands of tentacles or filaments for filter-feeding and respiration (Read et al., 2011;
Fletcher, 2014; Giangrande et al., 2014b; Giangrande et al., 2014c) (Figure 2.1). The single fan
can be completely retracted inside the tube in response to disturbance, or shed spontaneously in
response to stress such as high temperatures, as a predator defence mechanism or in response to
physical damage (Licciano et al., 2012; Giangrande et al., 2014b). The outer firm and leathery, but
flexible tube is between 5 - 20 mm in diameter and can grow up to 800 mm in length, although in
situ the full length of the tube is often obscured by other biofouling (Figure 2.2) (O'Brien et al.,
2006; Fletcher, 2014; Giangrande et al., 2014c). Reaching up to 800 mm in length this species is
considerably larger than all the other native fanworms found in New Zealand.

The tube of *S. spallanzanii* is constructed from combining mucus, faeces and pseudofaeces and
can be buried up to 10 cm into soft sediment to anchor the worm (O'Brien et al., 2006; Ross et al.,
2007; Fletcher, 2014). The branchial crown or fan can reach 10 - 15 cm in diameter and is brightly
coloured with bands of orange, purple and white (Figure 2.1) (O’Brien et al., 2006; Ross et al., 2007; Murray & Keable, 2013; Fletcher, 2014).

Figure 2.1. Branchial crown of *Sabella spallanzanii*. Photograph by Dr Serena Cox, NIWA.

Figure 2.2. *Sabella spallanzanii* specimen with its vacated tube below.
Sabella spallanzanii can grow on hard and soft substrata but is mostly observed growing attached to artificial substrates, such as floating pontoons, wharf pilings and vessel hulls (Figure 2.3 A and B) (Holloway & Keough, 2002b; Ross et al., 2007; Read et al., 2011; Murray & Keable, 2013; Fletcher, 2014). It will also frequently attach to other benthic organisms, such as oyster or scallop shells or gravel particles, as a means of anchoring and establishing in soft sediment habitats but has also been found on subtidal rocky reef habitat (Douglas et al., 2020).

The Mediterranean fanworm appears to prefer habitats in shallow subtidal areas (1 - 30 m) that are sheltered from direct wave action (Clapin & Evans, 1995; Clapin, 1996; Currie et al., 2000; Murray & Keable, 2013). Individual fanworms can re-attach their base back to the substrate if they become detached by producing copious quantities of sticky mucus that acts as an adhesive, while their protective tube remains robust and flexible at the same time (O'Brien et al., 2006; Fletcher, 2014; Giangrande et al., 2014b).

2.3 Filtration capacity

The distinctive characteristics of S. spallanzanii are the presence of thoracic companion chaetae (bristles) and abdominal chaetae arranged in spiralling bundles, which are clearly visible under a microscope (Licciano et al., 2012; Murray & Keable, 2013; Fletcher, 2014; Giangrande et al., 2014b; Giangrande et al., 2014c). The Mediterranean fanworm is a suspension feeder, consuming a wide variety of organic matter, such as phytoplankton and zooplankton (Licciano et al., 2005; Licciano et al., 2007; Giangrande et al., 2014b; Giangrande et al., 2014c). Adult fanworms are
highly efficient at filtering particles from seawater and can process up to 12 m³ of seawater per day (Stabili et al., 2006).

The filtration capacity of *S. spallanzanii* increases with water temperature, reaching an optimum at 22 °C, with filtration rate being found to be high and constant when algal cell concentration was below 4 x 10³ cells mL⁻¹ for suspended particles ranging in size 5 – 11 µm (Clapin, 1996). At high water temperatures of 27 °C filtration rate decreased, as well as with high algal cell concentration above 10 x 10³ cells mL⁻¹ because gut capacity was exceeded (Clapin, 1996).

As a highly efficient filter feeder, it may be a serious competitor to commercially important filter feeders, such as oysters and mussels, especially where it establishes among shellfish in aquaculture structures (Fitridge et al., 2012; Fletcher, 2014; Tait & Inglis, 2016). To feed, the Mediterranean fanworm extends its branchial crown out of the tube to collect, sort and consume particles between 2 - 40 µm in diameter and most likely also below this size range (i.e., marine bacteria), which includes the major phytoplankton groups that are also of importance to bivalve filter feeders (Holloway & Keough, 2002b; Adams et al., 2011; Licciano et al., 2012; Fletcher, 2014).

Adult green-lipped mussels, *Perna canaliculus*, cannot feed efficiently on particles smaller than < 2 µm in diameter, but can ingest particles up to 620 µm (Safi & Hayden, 2010). Smaller mussels of 1 - 6 mm in shell length feed on a smaller size range of suspended particles of between 3 - 30 µm (Sim-Smith et al., 2005). In general, bivalves tend to adjust their feeding in relation to their own size and food requirements, meaning they can feed on a wide range of different sized food particles depending on their nutritional requirements (Ward & Shumway, 2004). There is a clear overlap in the particle size that both mussels and fanworms feed on. However, fanworms can extend their feeding fan beyond the reach of mussels and in dense aggregations the fans can form a canopy that could reduce the availability of suspended food particles for mussels growing beneath the canopy of fanworms (Holloway & Keough, 2002a; O'Brien et al., 2006; Ross et al., 2007; Adams et al., 2011; Fittridge et al., 2012; Atalah et al., 2016b).

In addition, to feeding on phytoplankton and zooplankton, the Mediterranean fanworm has also been reported to feed on bacteria, giving the species the potential to function as a bioindicator for detecting and monitoring microbial pollution in the marine environment as well as being used as a bioremediator by reducing organic and bacterial pollution in coastal waters (Licciano et al., 2005; Stabili et al., 2006; Licciano et al., 2007). *Sabella spallanzanii* has the potential to improve the water quality, by removing organic matter from the water and expelling it as faeces, pseudofaeces or using it for tube building (Licciano et al., 2005; Stabili et al., 2006). However, with their filter feeding activity, fanworm have the capacity to create currents that locally influence the water flow and alter sediment stability (O'Brien et al., 2006). Furthermore, a cluster of *S. spallanzanii* can provide shelter from predation for other organisms settling in between them, and the worm tubes
can also provide a substrate for algae and settlement for other sessile invertebrates, potentially affecting community structure (Pierri et al., 2019).

On the Mediterranean coast of Italy, this fanworm species reportedly has multiple natural predators, such as various fish and large invertebrates, including lobsters and cephalopods (Stabili et al., 2013). However, so far there are no known predators identified for Mediterranean fanworm in Australia or New Zealand (Stabili et al., 2013; Fletcher, 2014). It has been speculated that the elevated concentrations of dimethylarsinic acid (DMA), a relatively toxic compound, that is found in the branchial crown of *S. spallanzanii*, in addition to the leathery tube, serve as effective protection against potential predation (Fattorini et al., 2004; Fattorini & Regoli, 2004; Notti et al., 2007). The DMA in the branchial crown of *S. spallanzanii* most likely results from ingestion or breakdown of arsenic compounds from food or seawater filtration rather than being internally generated, meaning that DMA concentrations in the crown could potentially vary depending on the environment and the availability of arsenic compounds in the water column (Fattorini et al., 2004). Therefore, fanworm crowns might be more or less toxic depending where they are growing and the conditions of the water, hence, making them more or less appealing to potential predators.

### 2.4 Growth rate

There is limited data on growth rates of Mediterranean fanworm, however, differences in size at maturity and growth rates have been identified between populations in the Mediterranean Sea and in Port Phillip Bay, Australia (Currie et al., 2000; Giangrande et al., 2000). Mediterranean fanworm have been measured to grow between 8 - 10 mm per month in the Mediterranean Sea, 15 mm per month in Port Phillip Bay, Australia and up to 25 - 50 mm per month in Whangarei Harbour, New Zealand (Currie et al., 2000; Fletcher, 2014). Therefore, theoretically under optimal temperature and food supply conditions, *S. spallanzanii* could reach maturity, based on size at maturity and growth, and potentially be ready to spawn within 4 - 5 months in Australian and New Zealand conditions (Currie et al., 2000; Giangrande et al., 2000; Bybee et al., 2007; Fletcher, 2014; Giangrande et al., 2014c). However, it also might be that even though once the Mediterranean fanworm has reached a size, where they are normally mature, several months up to 1 year are needed for complete gamete development (gametogenesis) (Currie et al., 2000; Giangrande et al., 2000).

### 2.5 Reproduction in Mediterranean fanworm

The timing of spawning and fertilization in marine polychaetes is influenced by a wide variety of environmental factors, including day length or lunar phases, sea temperature and chemical cues, such as pheromones or the chemical signals from high phytoplankton concentrations, which indicate favourable conditions (Hardege et al., 1998; Watson et al., 2003; Bybee et al., 2007;
Most marine polychaetes show clear seasonal patterns of reproductive behaviour in response to environmental factors but in some species their reproductive cycle also varies inter-annually (Wilson, 1991; Rouse & Fitzhugh, 1994; Rouse, 1996; Gambi et al., 2000; Licciano et al., 2002; Bybee et al., 2006;2007; Giangrande et al., 2010). Some polychaete species reproduce sexually, some asexually and some are hermaphrodites (Rouse & Fitzhugh, 1994; Rouse, 1996; Bishop, 1998; Gambi et al., 2000; Bishop & Pemberton, 2006; Tovar-Hernández et al., 2009; Tovar-Hernández et al., 2009; Murugesan et al., 2011).

One of the first published studies on reproduction in *S. spallanzanii* concluded that they are proterandric hermaphrodites, meaning they have male sexual organs when they first mature and female organs later on in life (Giangrande & Petraroli, 1994). However, this fanworm species has been found to have a 1:1 sex ratio and spawn during winter in Taranto (Italy), the Mediterranean Sea (Gambi et al., 2000; Giangrande et al., 2000). A study of Mediterranean fanworms in Port Phillip Bay, Australia, found no evidence of hermaphroditism and confirmed that they were dioecious, i.e., had separate sexes (Currie et al., 2000). In the Mediterranean Sea, *S. spallanzanii* first reach sexual maturity at a length of 150 mm and all specimens from 150 to 200 mm were identified as being male (Currie et al., 2000; Gambi et al., 2000; Giangrande et al., 2000). However, some individuals in the population in Port Phillip Bay, Australia reached sexual maturity at a smaller size (> 50 mm), suggesting that size at sexual maturity varies by location (Currie et al., 2000; Giangrande et al., 2000; Stabili et al., 2009). Hence, there may be a difference in body size when Mediterranean fanworm reach maturity and are able to produce mature gametes for different locations.

There have only been a few studies looking at reproduction in *S. spallanzanii* and it has generally been assumed that fertilization of eggs in this species takes place inside or at the opening of the tube and that the fertilized eggs are then released, thereby increasing fertilization success (Currie et al., 2000; Giangrande et al., 2000). In the population of fanworm in the Mediterranean Sea, gametogenesis (i.e. production and ripening of gametes) takes place nearly all year round from February to December and spawning occurs once a year in February when seawater temperatures are at a seasonal low of around 11 °C (Northern Hemisphere winter) (Currie et al., 2000; Giangrande et al., 2000). However, it has been observed that reproduction in this species varies from year-to-year and sometimes does not occur at all (Giangrande et al., 2010). Larval settlement occurs after about a 2 week pelagic period, followed by metamorphosis 10 days after settlement (Giangrande et al., 2000).

It has been hypothesized that gamete fertilization in *S. spallanzanii* occurs in the worm’s tube just after the egg is released from the coelomic cavity (in situ fertilization), whilst enveloped in mucus, which may help to ensure higher fertilization success (Stabili et al., 2009). Oocytes are not viable in seawater if they are unfertilized, they just collapse and dissolve (Giangrande et al., 2000; pers. observation). However, when sperm comes into contact with seawater, it triggers their mobility. These observations suggest that *S. spallanzanii* is a ‘sperm-caster’, releasing sperm into the seawater, which are then picked up by female fanworms to fertilize their eggs (Giangrande et al., 2000).
It is hypothesized that the sperm are captured by the branchial crown of female fanworms with fertilization taking place within or near the thorax region.

Mature fanworm eggs and sperm have been observed being expelled through paired lateral coelomoducts that extend from the coelom to the thorax (Giangrande et al., 2000). Fertilized eggs have also been observed being shed at the tube opening in a mucus string (Rouse & Fitzhugh, 1994; Currie et al., 2000). Fertilized eggs appear to take a short time to develop into the first larval stage, which is a free-swimming trochophore larva (Stabili et al., 1999; Stabili et al., 2009). The planktonic larvae of *S. spallanzanii* are lecithotrophic, relying on their yolk reserves, however, more precise details of larval duration and larval development are yet to be researched (Giangrande et al., 2000).

Spawning in *S. spallanzanii* occurs with decreasing seawater temperatures between 11 - 14 °C both in the Mediterranean Sea and in Port Phillip Bay, Australia (Currie et al., 2000; Giangrande et al., 2000; Murray & Keable, 2013). In central New Zealand these seawater temperatures are reached from May to late September and therefore spawning could occur around this time (Fletcher, 2014). However, recently it has been suggested that gametogenesis in *S. spallanzanii* in New Zealand and Australia takes place all year round, similar to the pattern that has been observed in the Mediterranean Sea, however, in the Australasian populations there appears to be no distinct spawning season (Jute, 2015; Lee et al., 2018).

In the early stages of *S. spallanzanii* gametogenesis there are more coelomocytes than gametes present in the coelomic cavity, with the coelomocytes acting as energy reserves for the development of the gametes (Dales, 1961; Currie et al., 2000; Giangrande et al., 2000; Bybee et al., 2006; 2007; Jute, 2015). As the gametes mature, the number of coelomocytes typically decreases as their energy reserves are consumed by gametogenesis (Dales, 1961; Bybee et al., 2006; 2007). Coelomocytes consist of oil and glycogen, and visually appear as orange and yellowish-brown granules under a microscope (Dales, 1961). Free coelomocytes are derived from the peritoneum and only seem to occur in the coelom (Dales, 1961; Currie et al., 2000). In addition to serving as energy reserves, coelomocytes also show phagocytic activity, picking up excretory end products (Dales, 1961). In male fanworm the carotenoids, which lend the yellow colour to the coelomocytes, disappear as the coelomocytes diminish. Coelomocytes regularly disintegrate and disperse their content into the coelomic fluid. Consequently, the colour of the coelomic fluid varies due to the maturation stage and density of the gametes, i.e., when oocytes are mature, the coelomic fluid has a dark green colour, and for males the coelomic fluid is a tan brown colour (Dales, 1961). Oocytes can be differentiated from coelomocytes in that they are round cells with a single large nucleus, whereas coelomocytes are packed with oil globules and are “raspberry-shaped” (Figure 2.4; Dales, 1961; Bybee et al., 2006; 2007; Read et al., 2014). The heads of mature sperm are 2 - 3 µm in diameter and their flagellum is 8 - 12 µm in length (Figure 2.5) (Currie et al., 2000; Giangrande et al., 2000; Read et al., 2014).
Figure 2.4. Microscope picture with Eosin staining of coelomic fluid, examining cells within the coelomic cavity of a female fanworm. Eggs with the nucleus visible in the centre (A) and coelomocytes (B).

Figure 2.5. Microscope pictures of unstained coelomic fluid with sperm (A) and coelomocytes (B) in a male fanworm.

2.6 Regeneration capabilities of the Mediterranean fanworm

Regeneration capabilities vary greatly among the annelid worms (Licciano et al., 2012). Mediterranean fanworm have relatively low mortality rates in response to wounding and amputation, possessing a capacity for the rapid regeneration of a high percentage of body
fragments compared to other Sabellid species (Licciano et al., 2012; King, 2017). Furthermore, while some marine invertebrates show poor ability to regenerate whilst reproductive, in S. *spallanzanii* the regenerative capabilities did not appear to vary in relation to reproductive status (Licciano et al., 2012). Morphallaxis, which is the regeneration of specific tissue by reorganizing existing tissues, is the primary medium of regeneration in Mediterranean fanworm (Licciano et al., 2012). The wound closes by muscle contraction and tissue constriction, followed by coverage of the wound with a blastema (Müller et al., 2003; Licciano et al., 2012).

Lacerated Mediterranean fanworms regenerate easily to form abnormally short survivor individuals from the fragments, with several head and tail regenerating pairs found in the same tube after damage, indicating natural or trauma-related reproduction by fission (Read et al., 2011; King et al., 2017). However, the survival of the fanworm fragments depends on the location it has been cut, i.e., mid-body and posterior fragments show higher survivorship compared to anterior fragments (Read et al., 2011; Licciano et al., 2012; King, 2017). Therefore, damaging or cutting the fanworm during eradication or removal efforts has the potential to result in the formation of new individuals if the worms are not removed in their entirety. Likewise, when Greenshell™ mussels are harvested, from lines where both mussels and Mediterranean fanworm are present, both mussels and worms are stripped off the dropper lines and are run through a cleaning and de-clumping process, which has the potential to generate worm fragments that are then discharged back into the marine environment, where they may be able to regenerate (Woods et al., 2012).

### 2.7 Mucus of the Mediterranean fanworm

In marine invertebrates, mucus serves many different important tasks such as an external surface coating to reduce friction or hydrodynamic drag, limit water loss, aid in locomotion and feeding, aid in fertilization success and as a chemical defence against infection (Giangrande et al., 2000; Stabili et al., 2009; Stabili et al., 2011; Giangrande et al., 2014b; Giangrande et al., 2014c). Mucus composition depends on the invertebrate species and is typically a mixture of proteins, polysaccharides and water that forms a weak gel (Stabili et al., 2009; Stabili et al., 2011). Marine invertebrates especially need efficient and multiple defence mechanisms, since they are sessile or slow moving and under constant threat from microorganisms and pathogens, predators and overgrowth (Stabili et al., 2009; Stabili et al., 2011; Giangrande et al., 2014b).

Mediterranean fanworm use mucus to cement different substances together for anchoring and for tube building, i.e., by mixing faeces, pseudofaeces and sediment (Stabili et al., 2009; Stabili et al., 2011; Giangrande et al., 2014b; Giangrande et al., 2014c). *Sabella spallanzanii* secrete a large amount of mucus when being exposed to different stress conditions, i.e., manual handling and air exposure (Stabili et al., 2009; Stabili et al., 2011; S. Brand, personal observation). Furthermore, their mucus has a high lysozyme-like activity, i.e., contains enzymes which break down the cell walls of bacteria (Canicatti et al., 1992; Stabili et al., 2009; Stabili et al., 2011). This lysozyme-like
activity indicates that fanworm can defend themselves against bacterial attack, which is important since they often live in eutrophic environments such as harbours, where bacteria are particularly abundant. The antibacterial properties of the mucus may also affect the growth and presence of other colonizing species (Stabili et al., 2009; Stabili et al., 2011; Giangrande et al., 2014b; Pierri et al., 2019). During the reproductive period of S. spallanzanii the lysozyme-like activity of the mucus appears to decline markedly (Giangrande et al., 2000; Stabili et al., 2009; Stabili et al., 2011). The Mediterranean fanworms also use their mucus to envelope fertilised eggs that are released out of their tubes (Giangrande et al., 2000; Stabili et al., 2009). Therefore, the reduction of the lysozyme-like activity during the reproductive period might be a response to avoid interference with the process of fertilization and embryo development (Stabili et al., 2009).

2.8 Effect on ecosystems

Successful marine invasive species are classified as being able to affect the diversity, structure and functioning of an ecosystem and reduce its biodiversity (Giangrande et al., 2020). They often are capable of high proliferation and potential for spread, fast growth, high environmental tolerances (i.e., temperature, depth, salinity, pollution) and a lack of predators (Molnar et al., 2008). Opportunistic species can take advantage of a disturbance in an ecosystem, such as the appearance or disappearance of certain species (McCarthy et al., 2003).

Tube dwelling marine polychaetes produce complex hydrodynamic environments around them, which can reduce larval settlement near conspecific adults and may also promote the settlement of other fouling species (Callaway, 2003; Murray et al., 2011). Clustered aggregations of fanworm tubes might be advantageous for food acquisition, improving feeding efficiency as well as fertilization success (Toonen & Pawlik, 1996; Toonen & Pawlik, 2001; O’Brien et al., 2006).

As a successful invasive species, the Mediterranean fanworm, has the potential to alter indigenous marine ecosystems by altering ecosystem structure (Currie et al., 2000; Holloway & Keough, 2002a; Holloway & Keough, 2002b; O’Brien et al., 2006; Ross et al., 2007; Murray & Keable, 2013; Atalah et al., 2019; Douglas et al., 2020; Tait et al., 2020). As an opportunistic biofouling species, the Mediterranean fanworm can impact the use of human infrastructure, such as vessels and aquaculture operations. For example, the canopy-forming effect of multiple branchial crowns of many fanworms in close proximity is thought to impact the benthic habitat by altering water flow and the availability of suspended food particles (Holloway & Keough, 2002b; O’Brien et al., 2006; Ross et al., 2007). Sabella spallanzanii are highly efficient filter-feeders, they influence biodeposition and nutrient cycles (Holloway & Keough, 2002b; O’Brien et al., 2006; Ross et al., 2007). High densities of S. spallanzanii tend to form a canopy, which was found to impact the growth of the associated epifauna (Holloway & Keough 2002a). The canopy formed by clusters of S. spallanzanii individuals has been found to impact the recruitment of several epifaunal species in the vicinity, such as serpulid polychaetes, barnacles, bryozoans and sponges (Holloway & Keough
However, the development of a fanworm canopy may not always affect already established epifaunal assemblages.

For example, macrofaunal assemblages, in Port Phillip Bay, Australia were not affected by the presence of *S. spallanzanii* (Ross et al., 2007). The Mediterranean fanworm only affected the abundance of associated lumbrinerid polychaetes and gammarid amphipods. The abundance of deposit feeders, such as molluscs, did not increase with increased densities of *S. spallanzanii* (Ross et al., 2007). No effects of *S. spallanzanii* clusters were detected on infaunal colonisation in marine habitats in Port Phillip Bay, Victoria, Australia (O'Brien et al., 2006; Ross et al., 2007). However, a different experiment showed that *S. spallanzanii*’s presence increased net metabolism of epifauna in surrounding sediments and changed nutrient fluxes in the vicinity (Ross et al., 2013). *Sabella spallanzanii* halved the denitrification efficiency (the proportion of remineralised nitrogen removed from the system via denitrification) and increased the flux of ammonium eight times (Ross et al., 2013).

Similarly, a recent experiment conducted in the Rangitoto Channel, North Island, New Zealand confirmed that *S. spallanzanii* can impact solute fluxes directly, i.e., respiration and excretion, and indirectly, i.e., hydrodynamics, sediment and organism communities (Tait et al., 2020). Therefore, it is not recommended to use *S. spallanzanii* as a bioremediator of waste inputs, since high abundance of *S. spallanzanii* lead to a decrease in denitrification and reduced flux of dissolved phosphorus (Tait et al., 2020).

A second study in the Rangitoto Channel, was unable to measure any negative effects from Mediterranean fanworm on the feeding mode or motility of other associated taxa (Douglas et al., 2020). Instead, *S. spallanzanii* enhanced structural complexity and with it biodiversity (Douglas et al., 2020). The faeces of Mediterranean fanworm can provide additional food for deposit feeders, provide additional area for grazing, foraging and provide shelter for other marine organisms from predators. Consequently, the presence of Mediterranean fanworm affects the composition of a marine habitat by changing species assemblages (Douglas et al., 2020).

Even with the increased encrusting epifauna, associated with the presence of *S. spallanzanii*, there was no evidence that *S. spallanzanii* actively reduced food availability for other suspension feeders or enhanced food availability for deposit feeders through biodeposition (Douglas et al., 2020). However, in a mussel aquaculture setting, *S. spallanzanii* and mussels are in closer proximity to each other, and rely on the same food source and the ability of the fanworms’ feeding structure to reach beyond the mussels directly affects the food availability for the mussels, reducing their growth.

There appear to be differences in findings from different studies of the ecological interactions of fanworm. Some studies indicated that *S. spallanzanii* has the potential to impact nutrient cycling and species assemblage composition but how much it affects this, greatly varies among locations.
and appears to depend on the state of the local marine ecosystem and existing associated species assemblages (Holloway & Keough, 2002a; Holloway & Keough, 2002b; O'Brien et al., 2006; Ross et al., 2007; Ross et al., 2013; Giangrande et al., 2014a; Giangrande et al., 2014c; Pierri et al., 2019; Douglas et al., 2020; Tait et al., 2020). The state of the local marine ecosystem, might also directly impact the scale and the effects induced by the presence of *S. spallanzanii*, i.e., if the marine ecosystem is balanced and more tolerant against disturbances from potential invasive species (biotic resistance), or if it has already low biodiversity due to increased human disturbance such as high fishing and recreational activity, enhanced nutrient input from land runoff or sewage (Levine et al., 2004; Lodge et al., 2006; Zaiko et al., 2007; Corriero et al., 2016).

2.9 Impact on aquaculture

Marine biofouling species can directly interfere with aquaculture operations and marketability of the cultured shellfish (Fitridge et al., 2012; Sievers et al., 2014; Sievers et al., 2017). Biofouling species compete with the cultured shellfish for food and space, which typically results in reduced growth and hence, to reduced aquaculture product value (Adams et al., 2011; Fitridge et al., 2012; Sievers et al., 2014). The presence of marine biofouling in aquaculture operations can also reduce water flow, food and oxygen availability for the cultured shellfish and increase waste production (Ramsay et al., 2008a; Adams et al., 2011; Fitridge et al., 2012). Removing biofouling is costly, time intensive and often comes with production loss for aquaculture operators (Fitridge et al., 2012; Fletcher, 2014; Tait & Inglis, 2016).

In New Zealand the shellfish aquaculture infrastructure that is vulnerable to biofouling is the longline culture of the endemic Greenshell™ mussel, *Perna canaliculus*, and inter-tidal racks used for culturing the Pacific oyster, *Crassostrea gigas*, which are mostly located around Northland, the Hauraki Gulf, Tasman and Golden Bays and the Marlborough Sounds (Ren et al., 2003; Ren & Schiel, 2008; Woods et al., 2012).

Modelled simulations predict that direct impacts of the invasive species *S. spallanzanii* on the Greenshell™ mussel aquaculture sector will be around NZ $14 million over a 24 year period in New Zealand (Soliman & Inglis, 2018). Depending on the density of Mediterranean fanworm, it is estimated that they can reduce mussel biomass by 1.3 – 12.1% and that the highest annual infestation rate will be realized after 11 years, when at least 19% of all farms are infested (Soliman & Inglis, 2018). Removing *S. spallanzanii* from mussel lines to a lower density (< 100 individuals per metre of longline) could reduce the economic impact by an estimated 44%. However, the calculated impact and economic cost of fanworm fouling on Greenshell™ mussels will most likely be higher, since other marine biofoulers, such as *Styela clava*, *Mytilus galloprovincialis* and *Didemnum vexillum*, are also overgrowing the mussels and cause additional work and mussel loss for the farmers (Fletcher et al., 2013; Atalah et al., 2017; Forrest & Atalah, 2017; Soliman & Inglis, 2018).
2.9.1 Control measures against biofouling in aquaculture

Most treatment methods for controlling biofouling concentrate on vessel hull fouling (Hewitt et al., 2004). However, there is high potential for secondary distribution of invasive species through the transfer of aquaculture stock and equipment, especially since oyster and mussel seed are commonly collected in the wild and then distributed to farms located around New Zealand (Doddshun et al., 2007; Fitridge et al., 2012; Fletcher, 2014; Sievers et al., 2014).

The best approach for managing marine biofoulers in aquaculture is generally to avoid or prevent settlement of biofouling species if possible (Adams et al., 2011; Fitridge et al., 2012; Fletcher, 2014). However, prevention is difficult, because various biofouling species have different seasonality of settlement and it is often not feasible to switch farming locations or change timing of grading and re-stocking to avoid times of the year when the settlement of nuisance biofouling species is high (Sievers et al., 2014). However, knowing peak reproductive seasons of certain fouling species can be helpful in avoiding intense settlement and be helpful in developing farm stocking strategies to prevent biofouling (Sievers et al., 2014; Sievers et al., 2017).

Good biosecurity practices in aquaculture operations may also assist in reducing the spread and establishment of biofoulers among aquaculture sites, by ensuring vessels and aquaculture transfers are free of biofoulers, as well as cleaning aquaculture equipment and seed-stock prior to redeployment (Stanley & Jeffs, 2011; Fletcher, 2014; Forrest & Fletcher, 2015). Alternatively, there are several physical methods to remove biofouling from the aquaculture infrastructure and product, such as power washing, immersing fouled shellfish and infrastructure in freshwater or biocides, air-drying or heat treatment (Denny, 2008; Fitridge et al., 2012; Sievers et al., 2014). However, some marine fouling species are not affected by freshwater or biocide treatments or they need to be exposed over lengthy periods of time, which in turn can negatively affect the cultured shellfish (Fitridge et al., 2012).

Several biocides can be used to remove biofouling from vessel hulls as well as aquaculture equipment and stock. It has been shown that acetic acid is a very effective biocide for killing *S. spallanzani*, with immersion in a 1% solution for 4 minutes resulting in 100% mortality but also causing 50% mortality in the green-lipped mussel (King et al., 2017). Additionally, the use and discharge into the environment of acetic acid in large quantities after it has been used for treating biofouled shellfish can have harmful effects for the environment (Locke & Hanson, 2009). A more environmentally friendly alternative is to use freshwater instead of acetic acid. Exposing the Mediterranean fanworm to freshwater for up to 90 minutes is an effective method for killing the exposed fanworm. Furthermore, adult green-lipped mussels can tolerate immersion in freshwater for such a period with relatively high survival (A. Jeffs, unpublished data).
A novel technique as an alternative to anti-fouling paint or biocides, is the application of food grade oil to farm buoys, ropes and floats, which has been reported to reduce fouling by algae and tunicates in mussel culture by up to 90% (Bakker et al., 2011; Fitridge et al., 2012). However, the effectiveness of this method for reducing fouling by *S. spallanzanii* under New Zealand conditions is unknown. Currently, there is work on the selective breeding of Greenshell™ mussels that are resistant to biofouling, however, this option is still in its infancy and will need further development and testing (Sievers et al., 2017).

Biological controls are another approach for managing biofouling in aquaculture. Predators, such as, sea urchins, fishes, sea cucumbers and crabs have been used to control biofouling species on structures in the marine environment (Fitridge et al., 2012). However, although populations of *S. spallanzanii* in the Mediterranean Sea appear to be naturally regulated by predators, there are no known predators for Mediterranean fanworm in New Zealand or Australia (Fitridge et al., 2012; Fletcher, 2014). The lack of predation on *S. spallanzanii* could be due to the relatively high levels of dimethylarsinic acid (DMA) that is found in the branchial crowns of *S. spallanzanii*, which is relatively toxic and may deter predators (Fattorini et al., 2004; Fattorini & Regoli, 2004; Notti et al., 2007). Alternatively, it could also be that New Zealand waters lack natural predators that recognise Mediterranean fanworm as potential food item.

### 2.10 Possible uses of the Mediterranean fanworm

Filter-feeding polychaetes feed by capturing and consuming suspended particles of non-living detrital organic carbon, living bacteria, phytoplankton and zooplankton (Licciano et al., 2005; Licciano et al., 2007). Marine bacteria are commonly an important food source in many polychaetes (Licciano et al., 2005; Licciano et al., 2007). However, the role of bacteria in their diet is still largely unknown. There is some evidence that dense populations of *S. spallanzanii* may alter ambient densities of bacteria through their filter feeding activity, although this may depend on the type of bacteria involved (Licciano et al., 2005; Stabili et al., 2006; Licciano et al., 2007; Stabili et al., 2010). Mediterranean fanworm can filter, accumulate and remove bacteria and organic matter from the water column and deposit them to the seafloor as faeces and pseudofaeces, and may also integrate some of the captured material in tube construction (Giangrande et al., 2005; Licciano et al., 2005; Licciano et al., 2007; Stabili et al., 2010). The high filtering activity of *S. spallanzanii* and its ability to accumulate and concentrate bacteria from the surrounding environment may make it a useful bioremediator with the potential of being used to clear water of high levels of organic pollutants, e.g., discharges from fish farms or of human sewage effluent (Giangrande et al., 2005; Licciano et al., 2005; Stabili et al., 2006; Licciano et al., 2007).

Experimental co-culture of the Mediterranean fanworm with the Mediterranean mussel, *Mytilus galloprovincialis*, on long-lines in the Gulf of Taranto, Italy, was found to reduce the environmental impact of mussel farming and at the same time produce worm biomass that can be used as fish.
feed (Giangrande et al., 2014b; Giangrande et al., 2014c). The worms were suspended beneath the mussels to avoid potential food competition, enabling S. spallanzanii to feed on the sinking mussel waste by moving it to the sediment or removing it by incorporating into tube construction.

Some research has suggested the potential to use Mediterranean fanworm as a source of a dietary supplement for fish aquaculture or as bait for fishing (Giangrande et al., 2005; Stabili et al., 2013; Giangrande et al., 2014c). Nutrients and feed attractants are currently derived from other species of marine polychaetes and used to improve the palatability and consumption of a variety of animal feeds (Stabili et al., 2013). Mediterranean fanworm tissue has a high gross protein content of dry matter compared to other marine invertebrates. It has a good ratio of omega-6:omega-3 fatty acids that would make it attractive as a dietary supplement in artificial fish diets for the use in the culture of carnivorous fish (Giangrande et al., 2005; Murugesan et al., 2011; Stabili et al., 2013; Giangrande et al., 2014c).

Many other fanworm species, such as Sabella pavonina and Sabellastarte spectabilis, are often used in the aquarium trade as ornamental species (Bybee et al., 2006; Murray et al., 2013). However, rearing ornamental species in captivity is often difficult, hence their regenerative capacity could be used in a way of multiplying specimens more easily and quickly (Murray et al., 2011; Murray et al., 2013). The regenerative capacity of some fanworm species is relatively high. For example, S. pavonina took only 10 days for reconstructing their tubes and they were able to rebuilt body parts within 4 weeks of the experimental dismemberment of worms (Murray et al., 2011). In comparison, S. spallanzanii also has great regeneration capacities, rebuilding a branchial crown within 9–11 days and thoracic and abdominal chaetigers between 15–25 days, although the development of new tubes was never observed in the laboratory (Licciano et al., 2012).

2.11 Discussion

The limited research that has been undertaken on the Mediterranean fanworm has mostly been done on populations in the Mediterranean Sea (Italy) or Australia (Melbourne and Adelaide), which provide some useful background and comparative data for New Zealand populations (Currie et al., 2000; Giangrande et al., 2000; O'Brien et al., 2006; Ross et al., 2007; Read et al., 2011; Murray & Keable, 2013).

From the data available and the Mediterranean fanworm’s invasive history, the species appears to be a habitat generalist, with the potential to invade native ecosystems and artificial structures in sheltered coastal areas, especially structures in harbours and structures used in the aquaculture of shellfish, and affect water flux, nitrogen cycle and species assemblages (Holloway & Keough, 2002a; Holloway & Keough, 2002b; O'Brien et al., 2006; Ross et al., 2007; Ross et al., 2013; Giangrande et al., 2014a; Giangrande et al., 2014c; Pierri et al., 2019; Douglas et al., 2020; Tait et al., 2020). The biological knowledge of this species suggests, it has some flexibility in its tolerances
and responses to a variety of environmental conditions. However, the Mediterranean fanworm appears to prefer hard and artificial substrates over soft sediment, and occurs mainly in shallow waters, to a depth of up to 30 m, preferring areas with some water current but not high wave exposure (Clapin & Evans, 1995; Clapin, 1996; Knight-Jones & Perkins, 1998; Giangrande et al., 2010; Read et al., 2011; Murray & Keable, 2013; Fletcher, 2014).

It will be necessary to learn more about the reproduction, larval development, feeding behaviour and general environmental tolerances of this species in New Zealand conditions given the existing lack of local data. Some initial research in New Zealand has examined larval settlement behaviour, reproduction, environmental tolerances, regeneration after fragmentation and early detection via environmental DNA and RNA sampling (Jute, 2015; Tait & Inglis, 2016; Zaiko et al., 2016; King, 2017; Wood et al., 2017; von Ammon et al., 2019). Some very recent studies showed that S. spallanzanii can impact native species assemblages in soft sediment habitats compared to those on hard substrate, specifically affecting nitrogen fluxes and bio-deposition, and providing additional structural complexity to the habitat that leads to local biodiversity enhancement (Ross et al., 2013; Douglas et al., 2020; Tait et al., 2020).

The investigation of the presence of gametes in S. spallanzanii over a duration of 6 months from individuals collected from various marinas around Auckland showed that gametogenesis varied amongst the different locations and even within the marinas, with gametes present from July to December (Jute, 2015). However, the 6 month sampling period was insufficient to make a clear statement about annual pattern of gametogenesis in this species. Therefore, a survey period of at least 1 year is recommended.

*Sabella spallanzanii* is one of the largest sabellid worms and can grow up to 800 mm long, reaching maturity at a size between 50 - 100 mm (Giangrande & Petraroli, 1994; Currie et al., 2000; Fletcher, 2014; Giangrande et al., 2014c). Both growth rate and maturity appear to be highly site specific and dependant on temperature and food availability (Currie et al., 2000; Giangrande et al., 2000; Fletcher, 2014; Giangrande et al., 2014c). This needs to be further investigated, since it is unknown how fast the Mediterranean fanworm grows under New Zealand conditions. Growth, longevity and maturity of Mediterranean fanworm under different environmental conditions also need to be determined, with special emphasis on their responses and limitations of environmental conditions, such as seawater temperature and food availability.

The ability of Mediterranean fanworm to recover from fragmentation is also worthy of further investigation in New Zealand conditions, as aquaculture harvesting has the potential to result in the fragmentation of Mediterranean fanworm, and if readily regenerates then it could contribute to the further spread and increase of the population (Licciano et al., 2012; Fletcher, 2014; King et al., 2017; King, 2017). It has been shown that the Mediterranean fanworm is able to regrow certain body parts from fragments in controlled optimal laboratory conditions in New Zealand (King et al., 2017). However, it is unknown if the worm would be able to regrow body parts in adverse or difficult
conditions in the natural marine environment, i.e., when exposed to bacteria, temperature variation and potential predation.

Regular surveys on harbour structures and especially vessels that are moored long-term are encouraged to detect the presence of fouling fanworms early and for them to be removed where possible. Furthermore, mussel and oyster farmers should be encouraged to clean equipment between sites and especially while moving stock and equipment between locations to further limit the spread of the Mediterranean fanworm to locations where it has not yet established (Coutts & Dodgshun, 2007; Adams et al., 2011; Fitridge et al., 2012; Sievers et al., 2014; Forrest & Fletcher, 2015; Atalah et al., 2016b; Atalah et al., 2017; Campbell et al., 2017; Sievers et al., 2017).

Additionally, it could be highly effective to involve the general public (e.g., commercial and recreational fishers, recreational scuba divers and private boat owners), to report any sightings of Mediterranean fanworm outside its known range as early detection may provide opportunities to contain further spread. Boat owners should also be encouraged to maintain clean boat hulls and to clean sea chests regularly with freshwater or bleach (Fitridge et al., 2012; Sievers et al., 2014; Forrest & Fletcher, 2015; Sievers et al., 2017). Immersion in 0.5% solution of chlorine bleach for 2 minutes proved effective in the removal of the colonial ascidian *Didemnum vexillum* from Greenshell™ mussels, *Perna canaliculus*, and left the mussels relatively unaffected (Denny, 2008). However, not every anti-fouling treatment has the same efficiency on all biofouling species. For example, freshwater immersion proves very effective against *Undiaria pinnatifida* and *S. spallanzanii* without affecting mussel health but does not prove 100% effective against *D. vexillum* (Denny, 2008). Therefore, some of these antifouling treatments are useful in a marina setting but are less useful in aquaculture treatments.

In the Mediterranean Sea, where *S. spallanzanii* is a native species another worm species invaded its niche *Branchiomma luctuosum* (Annelida, Sabellidae) (Mastrototaro et al., 2015). At first, a decrease in the abundance of the native *S. spallanzanii* was observed and at certain sites it almost disappeared completely and was substituted by *B. luctuosum*. However, nowadays the two species subsequently appeared to have reached an equilibrium, indicating that the two species might be ecologically equivalent and share mutual redundancy when co-occurring (Mastrototaro et al., 2015). The decrease and later recovery of *S. spallanzanii* in the Gulf of Taranto, Mediterranean has been observed over the course of 15 years, which shows that long term studies are necessary to observe and assess how native species react and evolve with the introduction of invasive species (Giangrande et al., 2014c; Mastrototaro et al., 2015). It is often hard to estimate consequences or the final outcome of the coexistence between native and invasive species, since even closely related species vary considerably in their life cycles and tolerances and are often highly adaptable (Holloway & Keough, 2002b). Furthermore, the marine environment is constantly changing and adapting and marine species are competing for food and space, which together plays a role in structuring epifaunal assemblages that develop after the larval settlement of organisms (Holloway & Keough, 2002a; Holloway & Keough, 2002b).
To date Mediterranean fanworm have been found in many parts of New Zealand, including Lyttelton Harbour, Waitemata Harbour, Whangarei Harbour, Port Fitzroy, Coromandel Harbour, Nelson Harbour, Tauranga Harbour, Waikawa Bay, Opua, Gisborne, Wellington, Nelson, Picton, Timaru, Dunedin, as well as mussel farms near Waiheke Island and Coromandel (Woods et al., 2015; 2020). This indicates that so far the Mediterranean fanworm has mainly been found in and near harbours, marinas and aquaculture areas which all have plenty of artificial structures available for attachment and establishment of fanworm. Fanworm populations have also been observed in soft sediment habitats in the Rangitoto Channel, near Auckland (Douglas et al., 2020; Tait et al., 2020).

If Mediterranean fanworms are observed in locations outside their existing reported range, they should be removed completely by gently gripping their holdfast and detaching them, no harsh or rapid pulling as this could induce spontaneous gamete release as a stress response or it could tear the worm in half (Fletcher, 2014). The worms should then be disposed as biosecure waste and go into a landfill. Alternatively, worms can be immersed in 5% acetic acid for 2 - 4 minutes, 10% bleach or 70% ethanol for 5 minutes to kill them and then they can be disposed of as waste (Fletcher, 2014; King et al., 2017).
Chapter 3 - Investigating reproduction in the invasive species *Sabella spallanzanii* in New Zealand

3.1 Introduction

The spread of marine non-indigenous species (NIS) has increased with the rapid growth in commercial and recreational shipping movements, and the widespread movement of marine equipment, and aquarium species (Bax et al., 2003; Floerl & Inglis, 2005; Darling et al., 2017; Ojaveer et al., 2018). Non-indigenous species can travel considerable distances to new locations that they would not normally reach by riding on the hulls of transiting ships or in their ballast water (Bax et al., 2003; Zaiko et al., 2018). Although only a few of these introduced species establish in new locations, it has been shown that with climate change many more species are likely to expand their range through accidental human introductions (Lodge et al., 2006; Hellmann et al., 2008). Some marine NIS can impact indigenous biodiversity and ecosystem functioning, as well as creating problems for human activities, such as aquaculture and marine recreation, which is when they are often labelled as invasive species (Bax et al., 2003; Colautti & MacIsaac, 2004; Floerl & Inglis, 2005; Zaiko et al., 2018).

Usually it is extremely challenging and costly, if not completely impossible, to eliminate a marine invasive species once they have become fully established in a new location (Pimentel et al., 2005; Fletcher, 2014; Pochon et al., 2017; Soliman & Inglis, 2018; Tait et al., 2020). Even if complete elimination is no longer a possibility, mitigation strategies are often still needed for invasive pest species, especially if they are biofouling species and capable of causing more severe impacts on local biodiversity and economically important activities, such as aquaculture (Holloway & Keough, 2002b; Clarke Murray et al., 2011; Soliman & Inglis, 2018).

The Mediterranean fanworm, *Sabella spallanzanii* (Gmelin, 1971), (hereafter referred to as fanworm) is a filter-feeding polychaete that is native to the Mediterranean and Atlantic coasts of Europe but is a notoriously invasive species both in Australia and New Zealand, and it has also been recorded in parts of Brazil and Indonesia (Clapin & Evans, 1995; Knight-Jones & Perkins, 1998; Currie & Parry, 1999; Currie et al., 2000; Fletcher, 2014).

There is an indication that the biology of the fanworm is quite adaptable to different environments, demonstrating that it has a high degree of plasticity in the range of habitats it can invade, being capable of establishing on soft sediment seafloor habitats, as well as on hard rock substrates and artificial plastic or cement surfaces (Holloway & Keough, 2002b; Giangrande et al., 2005; O'Brien et al., 2006; Davidson et al., 2011; Ross et al., 2013; Lee et al., 2018; Atalah et al., 2019; Douglas et al., 2020; Giangrande et al., 2020; Tait et al., 2020). This plasticity appears to be facilitated by its wide ranging tolerance of water temperature, water quality and depth, combined with a high capacity for filter feeding (Clapin, 1996; Holloway & Keough, 2002b; Giangrande et al., 2005; Licciano et al., 2005; O'Brien et al., 2006; Giangrande et al., 2010; Davidson et al., 2011; Murray & Keable, 2013; Giangrande et al., 2014c; Lee et al., 2018).
The fanworm is a biofouler on aquaculture infrastructure, especially mussel aquaculture lines and floats, since it is a habitat generalist and directly competes with mussels for food and space, whilst its external tube can also provide additional substrate for other biofouling organisms to settle and establish (Sievers et al., 2013; Fletcher, 2014; Atalah et al., 2016b; Tait & Inglis, 2016; Atalah et al., 2019). Besides slowing the growth of mussels, fouling organisms also impact production and harvest by increasing the total biomass loading on the farm infrastructure, whilst also increasing the time and costs for harvesting and processing of the mussels (Adams et al., 2011; Fitridge et al., 2012; Fletcher, 2014; Sievers et al., 2017). The fanworm is estimated to cost the mussel farming industry in New Zealand up to NZ$14 million over a 24 year period (Soliman & Inglis, 2018). Reducing the amount of fanworm settling on the mussel ropes could also reduce the economic impact of this biofouler by up to 44% (Soliman & Inglis, 2018). However, to develop effective mitigation strategies for this prolific biofouler, it is important to know more about its general biology and ecology in situ. There is limited knowledge of the reproductive biology of fanworm in New Zealand conditions, which from previous studies is known to vary in its response to establishing in new environments. For example, comparisons between native populations of fanworm in the Mediterranean to invasive populations in Australia found marked differences in their reproductive seasons, size at maturity and reproductive output (Giangrande & Petraroli, 1994; Clapin & Evans, 1995; Currie et al., 2000; Giangrande et al., 2000; Read et al., 2011; Lee et al., 2018). Improved knowledge of fanworm reproduction in New Zealand waters may provide valuable insights that could lead to improved management of this pest species. For example, co-ordinating the timing of mussel aquaculture seeding activities could provide an effective means to avoid periods of high settlement of fanworm larvae so as to prevent them establishing in seeded mussel lines.

3.1.1 Reproduction and sex ratio

It was initially assumed that the fanworm is a protandric hermaphrodite and a broadcast spawner (Giangrande & Petraroli, 1994). However, it has now been established, that this species has separate sexes (i.e., dioecious) with no distinct ovaries or testes, with the gametes being produced freely in the coelomic cavity, where they are retained freely in the coelomic fluid together with smaller coelomocytes (nutrient storage cells) that contribute to ripening of the gametes (Dales, 1961; Currie et al., 2000; Giangrande et al., 2000; Read et al., 2014; Lee et al., 2018). The development of gametes within the coelomic cavity is a common feature among polychaetes (Dales, 1961; Gambi et al., 2000; Jollivet et al., 2000). The fanworm is thought to be a ‘sperm-caster’, where the sperm is released into the surrounding seawater but the eggs are retained inside the fanworm, where they are thought to be fertilized either internally or within the fanworm’s external protective tube (Currie et al., 2000; Giangrande et al., 2000; Stabili et al., 2009; Fletcher, 2014; Read et al., 2014). The fertilized eggs are then released in long mucus strings into the water column (Dean et al., 1987; Currie et al., 2000; Bishop & Pemberton, 2006; Stabili et al., 2009). The larvae of the fanworm are lecithotrophic, as are the larvae of most sabellids, with a pelagic phase of usually more than two weeks (McEuen et al.,
1983; Rouse & Fitzhugh, 1994; Currie et al., 2000; Giangrande et al., 2000). It was observed that when unfertilized eggs are experimentally released into seawater, they collapse and degrade and are no longer viable (Giangrande et al., 2000; Stabili et al., 2009; see Appendix 1). Therefore, it is inferred that upon fertilization, a protective membrane develops, which in addition to the mucus string coating, provides another protective coating for the fertilized eggs to develop before later emerging as swimming larvae (Currie et al., 2000; Giangrande et al., 2000). Previous studies have observed a 1:1 sex ratio in 250 fanworms with body lengths of >5 cm that were sampled in Port Phillip Bay, Australia (Currie et al., 2000). In contrast, in the Mediterranean Sea there was a sex ratio of 1:1, when measuring fanworms of 25 to 30 cm (Giangrande & Petraroli, 1994). However, in that particular study, when all sizes of fanworm (5 – 30 cm) were considered, 80% of the sampled population was classified as male, skewing the overall sex ratio from 1:1 (Giangrande & Petraroli, 1994). A subsequent and more detailed study on the same fanworm population in the Mediterranean Sea reported a 1:1 sex ratio (Giangrande et al., 2000). However, it was not stated how many fanworms were examined, or their size range (Giangrande et al., 2000). It is unknown what sex ratio is present among populations of fanworms in New Zealand waters.

3.1.2 Gamete and fanworm maturation

A difference in the size of fanworm at sexual maturity has been observed among populations in different locations (Currie et al., 2000; Giangrande et al., 2000; Lee et al., 2018). In Port Phillip Bay, Victoria, Australia invasive fanworm reportedly produce mature gametes at a body length of about 5 cm (Currie et al., 2000), whereas populations in the Gulf St Vincent, South Australia reached reproductive maturity at a body length of 7 cm (Lee et al., 2018). Within their native range in the Mediterranean Sea, fanworms only produce ripe gametes, when they reach at least 15 cm in length, before that only smaller gametes were observed within the coelom (Giangrande et al., 2000). It has yet to be established at what size the fanworm produces mature gametes in New Zealand and whether it differs to fanworm in Australian and European waters.

The use of different classification schemes in previous studies to describe the maturation of gametes in fanworm have made comparisons among different fanworm populations more difficult. Currie et al. (2000) classified oocytes as mature by putting them broadly into two different size categories, i.e., mature (> 160 µm in diameter) or immature (< 160 µm in diameter), whereas Lee et al. (2018) modified these size categories and used three different size categories, i.e., developing oocyte (< 50 µm in diameter), early mature oocyte (50 - 100 µm in diameter) and late mature oocyte (> 100 µm in diameter). For the larger fanworms living in the Mediterranean, Giangrande et al. (2000) did not put the eggs into different size categories, however, the maximum diameter of eggs was reached at 200 - 250 µm, at which point they were assumed to be mature and ready to spawn. Furthermore, oogenesis appears to be quite variable in fanworm, where at times ova of a range of sizes (20 - 220 µm in diameter) are present simultaneously within the coelom (Read et al., 2014). The presence of some large ova amongst smaller ones, is not
indicative of a pending spawning event, since some large ova may also be retained after a spawning event and fanworm that contain mature or ripe gametes might retain them whilst awaiting more favourable conditions for spawning (Read et al., 2014).

A 6 month study undertaken in five marinas in the Waitemata Harbour, New Zealand, showed that fanworm had differing levels of gametogenic development from July to December at the different marinas and at all times there were some eggs present in the coelom of the sampled fanworm (Jute, 2015). However, there was an increasing absence of eggs measured during August and September, indicating that a potential spawning event could have taken place at this time. Jute (2015) also classified absence versus presence of eggs in fanworm based on the method of Bybee et al. (2007). This visual classification method was developed for another sabellid worm species *Sabellastarte spectabilis* and identifies different stages of gametogenesis based on the quantity of gametes and coelomocytes occupying the coelomic cavity. The gametogenic stage of fanworm has been classified into four discrete reproductive stages based on histological examination of gametes presence or absence and the amount of coelomocytes present (Bybee et al., 2007; Jute, 2015):

1. No evidence of reproductive activity in the coelom (sex cannot be determined)
2. Only coelomocytes present in the coelom (sex cannot be determined)
3. Some gametes present in the coelom (sex can be determined)
4. Coelom densely packed with gametes (sex can be determined)

In contrast to female fanworms, measuring the readiness for spawning in male fanworm is more difficult, as sperm are much smaller in size and more readily available in large numbers (Currie et al., 2000; Giangrande et al., 2000; Read et al., 2014). Additionally, the production of sperm is likely to be faster and less energetically costly compared to the production of relatively large eggs for sustaining the lecithotrophic larvae (Rouse & Fitzhugh, 1994). Therefore, gametogenesis is more readily observed and classified on the basis of the development and size of the ova than is possible for sperm.

The overall aim of this research is to better understand the reproductive biology of *S. spallanzanii*, in New Zealand, especially the timing of spawning and the pattern of gametogenesis, although depending on location there also might be differences within New Zealand fanworm populations. The results from this study allow direct comparisons with previous studies of the reproductive behaviour in both natal and invasive fanworm populations to determine whether their behaviour may have altered in response to the local conditions in New Zealand.

### 3.2 Methods

#### 3.2.1 Field and laboratory sampling

To assess reproductive biology, progress of gametogenesis, spawning seasonality and sex ratio of *S. spallanzanii*, the reproductive state of fanworms was assessed in two ways: 1) focusing exclusively on the female fanworms, and measuring development and size of eggs in relation to month and fanworm body size, 2) considering all fanworms regardless of gender, in relation to
month and fanworm body size and classifying the progress of gametogenesis according to Bybee et al. (2007) (Figure 3.1).

Accordingly, 100 fanworms were sampled randomly from the Orakei marina (36° 50" 59.8" S, 174° 48" 34.4" E) in Auckland, New Zealand on a monthly basis from April 2018 until March 2019. The fanworms are abundant throughout the surfaces of the marina pontoons at a depth from 0.5 to 1 m. The fanworm specimens were carefully removed from pontoons and piers at 0.5 m depth, placed in a bucket filled with seawater and transported back to the laboratory at the University of Auckland for dissection. Random collection ensured fanworms from a variety of sizes (5 - 30 cm in length) were collected to assess if fanworm size is related to gamete size and development. The length of each sampled fanworm was measured without their tube and without their branchial crown by laying the worm out on a ruler in a relaxed state. Fanworm length was taken to the nearest centimetre. Fanworms were then wet weighed without their tube (worm body and branchial crown) and sexed according to the colour of their coelomic fluid (green: female; tan brown: male).

![Figure 3.1. Methodology for the sampling of fanworm for one year from the Orakei marina, Auckland, New Zealand.](image)

### 3.2.2 Sex ratio

All 100 fanworm sampled each month were sexed according to the colour of their coelomic fluid (green: female; tan brown: male). To assess the colour of their coelomic fluid, a small longitudinal
incision was made along the side of the exposed fanworm and a sample of the outflowing coelomic fluid was smeared on a white tissue paper or if the colour was indeterminate, a sample was transferred to a microscope slide for further visual assessment. Where sex was not identifiable, the specimen was classified as indeterminate. Sex ratio of sampled fanworms was calculated for the entire sampling period (i.e., 1 year).

3.2.3 Fanworm and egg size

From the 100 fanworms collected every month, an initial subsample of 20 - 30 female fanworms (except for September 2018, where only 12 fanworms had any measurable eggs within them) were selected for histological analysis to assess seasonal changes in egg development and to assess fanworm size in relation to egg size and development, following the protocol of Currie et al. (2000) and Lee et al. (2018) (Figure 3.1).

A 2 - 3 cm longitudinal incision of the abdomen region of female fanworms was made and a sample of the outflowing coelomic fluid was smeared on a microscope slide. After the fluid dried on the slide, a few drops of methanol were applied to fix the cells, which were then consecutively stained by eosin, dehydrated in a graded alcohol series and mounted with resin for further analysis under the microscope. Sex and gamete development were further determined by examining the microscope slide under a Leica DMR fluorescent microscope by measuring egg size and classifying the microscope slide sample according to a method developed by Bybee et al. (2007), which considers number of eggs in relation to number of coelomocytes present. Multiple digital images were taken of the whole slide (moving the slide from left to right and downwards) to facilitate subsequent measurements of egg diameter with image analysis software (ImageJ; version 1.49) (Figure 3.1). Furthermore, gamete development in this subsample of 20 – 30 female fanworms each month, as well as fanworm body size in relation to egg development, was assessed by measuring the diameter of 100 eggs per fanworm from digital images. Male fanworms were identified and recorded and observed under the microscope but no detailed measurements of the development of spermatozoa were taken.

3.2.4 Gametogenesis stages

From the initial 100 monthly collected fanworms, a randomly selected subsample of fanworms (~50 fanworms per month) were sampled with a smear of coelomic fluid on a microscope slide and a visual assessment was made of the stage of gametogenesis based on the gametogenesis classification by Bybee et al. (2007). This assessment was used to determine if there was a seasonal component to the abundance of the different gametogenic stages and whether gametogenesis in the fanworms was synchronous (Figure 3.1). This gametogenesis assessment was used as a secondary method, in addition to the egg diameter and fanworm body length measurements, to examine gametogenesis and seasonality. Since Bybee’s et al. (2007)
classification system (stages 1 and 2) only shows coelomocytes and gender cannot be determined, coelomic fluid smears were taken from various fanworms (Figure 3.2). Fanworm body size measurements of all the fanworms that had smears taken were also recorded, to determine whether fanworms of a certain body size in a certain month fall into a specific gametogenesis stage (1 – 4 according to Bybee et al., 2007).

Figure 3.2. Histological images of the coelom of fanworm, illustrating the four stages of gametogenesis according to Bybee et al. (2007). Top left to right illustrate stages 1 and 2, bottom pictures from left to right illustrate stages 3 and 4.

3.2.5 Statistical analyses

3.2.5.1 Sex ratio

To test for differences in fanworm body length and wet weight between the three different gender categories of sampled fanworms (i.e., female, male and indeterminate), a linear regression analysis using the lm function in R and a Kruskal-Wallis test were performed (R Core Team, 2019). The Bonferroni correction for multiple testing was used for determining appropriate levels of statistical
significance. An exact binomial test was performed to determine whether the sex ratio of all sampled fanworms was 1:1 for male : female fanworms. Differences in body length and weight between male, female and indeterminate fanworms were visualized using bar charts and boxplots (ggplot2, R Core Team, 2019). Descriptive statistics are reported as mean ± standard deviation (s.d.).

3.2.5.2 Fanworm and egg size

To compare for effects of fanworm body length and months of sampling on egg size, a linear mixed effect model (random intercept model) was applied using the lme4 R package and the lmer function in R (version 3.6.0 R Core Team, 2019) (Bates et al., 2015). Model selection and best-fit model with the chosen parameters were informed by using Akaike information criterion (AIC), as well as using maximum likelihood with Satterthwaite’s method (Burnham & Anderson, 2004; Zuur et al., 2009). The final model was assessed using corresponding F statistics and restricted maximum-likelihood estimation (REML). The model used “month” and “fanworm body length” as well as their potential interaction (month : fanworm body length), as covariates and each individual fanworm (worm ID) as a random factor with the 100 measured eggs nested within individuals. January 2019 was used by the model as the intercept. Tukey’s pairwise comparison for multiple testing was used for determining appropriate levels of statistical significance.

Prior to analyses, the normality and homogeneity of variances of the data were examined using residual plots and Levene’s test. Limited departures from these assumptions were deemed acceptable given the large data set involved (Field et al., 2012). Least square means (means ± s.d.) were used for comparing the trends of differing egg sizes among the different months and fanworm body lengths, and comparing different fanworm body lengths among the months. Differences in egg diameter and fanworm body length among months were visualized using bar charts (ggplot2, R Core Team, 2019).

3.2.5.3 Gametogenesis stages

Since only coelomocytes are present in Bybee et al’s (2007) gametogenesis classification stages 1 and 2, it was not possible to determine gender, hence both female and male fanworms were included in these stages. For stages 3 and 4, gametes were present in the coelomic cavity and gender could be determined. Hence, in order to assess progress of gametogenesis, which can be inferred from egg size, the analysis focused on female gametes for stages 3 and 4 to determine if female fanworms synchronise their gametogenesis according to season or body size. To test for any association between the number of fanworms found in the different gametogenesis stages among the different months, a two-sided Fisher’s exact test was performed.

A two way analysis of variance (ANOVA) was conducted to examine the effects of gametogenesis stage and month and their interaction on fanworm body size. The data were assessed for normality and homogeneity of variance by using residual plots and Levene’s test. For statistically significant ANOVA results, differences among means were identified using pairwise comparisons with a
Bonferroni correction for multiple testing. Descriptive statistics are reported as mean ± standard deviation (s.d.).

3.3 Results

3.3.1 Sex ratio

Of a total of 1193 fanworms that were examined over the year of sampling, 661 (55.4%) were female, 324 (27.2%) were male and 208 (17.4%) were indeterminate. The sex ratio of female : male is not 1:1 (exact binomial test p < 0.001), but is around 2:1.

There is a significant difference in fanworm body length among the different gender categories (i.e., male, female and indeterminate), ($R^2 = 0.113, F_{(2, 1190)} = 76.11, p < 0.001$) with pairwise comparisons showing that both male and female fanworms had larger bodies compared to fanworms of indeterminate sex ($p < 0.001$) but there was no difference in the size of female versus male fanworms (Figure 3.3a). Sampled female fanworms ranged in length from 2 – 20 cm with a mean of 10 cm ± 3 s.d., male fanworms ranged in length of 3 – 19 cm with a mean of 10 cm ± 3 s.d. and indeterminate fanworms had a range of 2 – 19 cm with a mean of 7 cm ± 3 s.d. (Figure 3.3a). Fanworms also had a different body wet weight among the three gender categories (Kruskal-Wallis test $H_{(2)} = 119.32, p < 0.001$). Pairwise comparison showed that both male and female fanworms were heavier compared to the smaller and lighter indeterminate sex fanworms ($p < 0.001$) but there was no difference in body mass between male and female fanworms (Figure 3.3b). Female fanworms ranged in weight from 0.2 g to 9.4 g with a mean weight of 3.5 g ± 2 s.d., males ranged in weight from 0.1 g to 8.1 g with a mean weight of 3.3 g ± 1.0 s.d. and indeterminate fanworms ranged in weight from 0.1 g to 8.7 g with a mean weight of 2 g ± 2.0 s.d. (Figure 3.3b).

There was extensive variability in the measured body length and weight of indeterminate fanworms within each month contributing to large standard deviations around the means (Figure 3.3; 3.4).

From the total of 208 indeterminate fanworms, 78 were < 5 cm in body length and 130 individuals ranged in body length from 6 – 19 cm. From the 130 indeterminate fanworms that were > 5 cm, there tended to be greater numbers of indeterminate fanworms in the size range of 6 – 10 cm body length and in months May ($n = 19$), August ($n = 17$), September ($n = 17$), October ($n = 12$), November ($n = 15$) and December ($n = 17$) (Figure 3.4).
Figure 3.3. A.) Mean (± s.d.) fanworm body length, and B) mean (± s.d.) wet weight in fanworms that were female (n = 661), male (n = 324) and indeterminate (n = 208) sex. Total fanworms (N = 1193) from monthly samples taken over a one year period.
Figure 3.4. Fanworm body length (cm) of indeterminate sex (n = 208) fanworms sampled monthly over a one year period, starting April 2018. Each box plot represents the upper and lower quartiles (box ends), median (horizontal line in box), the maximum value from the data that is within 1.5 times the interquartile range over the 75th percentile (the upper whisker), the minimum value from the data that is within 1.5 times the interquartile range under the 25th percentile (the lower whisker).

3.3.2 Fanworm and egg size

A total of 22,385 eggs were measured within 225 different individual female fanworms that were sampled over 12 consecutive months (~ 20 female fanworms were sampled every month). Both of the covariate factors, fanworm body length ($F(1, 206) = 15.65, p < 0.001$) and month ($F(11, 206) = 4.94, p < 0.001$), as well as the interaction of fanworm body length and month ($F(11, 206) = 2.90, p < 0.01$) had a significant effect on egg size (Figure 3.5). Overall, it was estimated that for every 1 cm increase in fanworm body length, the mean diameter of eggs increases on average by around 4.5 µm.

The smallest mean egg diameters were measured for the months September 2018 (36 µm ± 61 s.d.) and October 2018 (97 µm ± 32 s.d.) regardless of fanworm body length. The months with the largest mean egg diameters were June 2018 (156 µm ± 36 s.d.) and July 2018 (145 µm ± 47 s.d.). Tukey’s pairwise comparison showed that the mean diameter of eggs in fanworm differed significantly between June compared to Jan – March and for June compared to August – November ($p < 0.05$) (Figure 3.5a). Mean egg diameter was also larger in July versus October ($p < 0.05$). There was extensive variability in the measured egg diameters within each month contributing to large standard deviations around the means. The measured egg diameter was most
variable in August and September 2018 at the same time as the mean egg diameter was decreasing, as mainly smaller eggs were present, while some larger eggs remained in some fanworms (Figure 3.5c).

As outlined, the mixed effect model had an interactive effect among egg size, fanworm body length and month (F(11, 206) = 2.90, p < 0.01). The sampled fanworm body sizes varied considerable among the different months (Figure 3.5c). Mean diameter of the eggs in small fanworm of 3 cm in body length (41 µm ± 68 s.d.), 4 cm (37 µm ± 46 s.d.) and 5 cm (18 µm ± 19 s.d.) were considerably smaller compared to measured egg diameters in larger fanworms, i.e. > 6 cm in body length onwards (117 µm ± 67 s.d.). The largest mean egg diameter was measured in a fanworm with a body length of > 15 cm (160 µm ± 29 s.d.). For every sampled fanworm, the measured egg diameters were highly variable, indicating that eggs of a wide range of sizes were typically present in all fanworms from a body length of 6 - 16 cm (Figure 3.5b). Fanworms of various lengths were sampled in every month and the mean fanworm length for most monthly samples was around 10 cm ± 2 s.d., except for September 2018 when the mean fanworm length was 6.6 cm ± 4 s.d., suggesting that in addition to the high variation, a greater proportion of smaller fanworms containing small eggs were sampled in September (Figure 3.5c).
Figure 3.5. A) Monthly mean egg diameter (± s.d.) measured from a total of 225 female fanworms resulting from monthly samples over a one year period from April 2018. B) Mean egg diameter (± s.d.) in a total of 225 female fanworms over a range of sizes that were sampled monthly over a one year period. C) Mean body length of fanworm (± s.d.) for monthly samples of 225 female fanworms sampled over one year, starting in April 2018.
3.3.3 Gametogenesis stages

Fisher’s exact test (p < 0.001), indicated that there is a difference in number of fanworms counted among the four gametogenesis stages and months. The main contributors to the elevated Fisher’s value for stage 1 were the months of April and May, when stage 1 was present at the highest percentages. Stage 2 was elevated in the months of August and September, whereas stage 3 was similar among all sampled months. Stage 4 was most prevalent in January, March and June, and absent or barely present in September and October (Figure 3.6).

There was a significant interaction between fanworm body size, gametogenesis stage and month (F_{(45, 575)} = 5.65, p < 0.01), where fanworm body size varied amongst the four gametogenesis stages amongst the various months (F_{(31, 575)} = 1.86, p < 0.05). Specifically fanworm body size regardless of the gametogenesis stage, differed between the following months: January and March, May, June, July, November and December (p < 0.05; Figure 3.6). There was a tendency for larger fanworms (> 9 cm) to be more likely to contain gametes and their sex can be determined (i.e., stages 3 and 4), whereas smaller worms (< 5 - 8 cm) often do not contain gametes and their sex cannot be determined (i.e., stages 1 and 2). For example, fanworm at gametogenesis stage 1 their mean body length was 6 cm ± 4 s.d., for stage 2 it was 8 cm ± 3 s.d., for stage 3 it was 9 cm ± 3 s.d. and for stage 4 it was 11 cm ± 3 s.d..

![Figure 3.6](image_url)

Figure 3.6. The proportion of four different gametogenesis stages (Bybee et al., 2007) in monthly samples of fanworms over 12 consecutive months (N = 607), with the total number of fanworms sampled each month in parentheses below the month.
3.4 Discussion

3.4.1 Sex ratio

According to Fisher’s theory, natural selection favours a 1:1 sex ratio and normally the sex ratio in a population of a species is a function of body size (Wenner, 1972). Factors that can affect the sex ratio of a species can include: restricted nutrition, activity, differential mortality, population structure and ecology (Wenner, 1972; Gosselin & Sewell, 2013; Minetti et al., 2013). Local mate competition theory suggests that natural selection favours female-biased sex ratios when matings occur within patches and brothers compete for mating (Godfray & Werren, 1996; Minetti et al., 2013). In contrast to previous studies (Currie et al., 2000; Giangrande et al., 2000) a 1:1 sex ratio was not found in S. spallanzanii in this study and there were twice as many female fanworms compared to male fanworms (Figure 3.3a; b). It is not uncommon for polychaete worms, (e.g., Spirobranchus cariniferus) to have a female biased sex ratio, however, this occurs when polychaetes are protandric hermaphrodites and the sexes have different average body sizes (Gosselin & Sewell, 2013). However, this current study shows that in Mediterranean fanworm in New Zealand, sex ratio is not a function of size. There was no difference in the body length or wet weight between female and male fanworms, meaning that fanworm body length or weight in S. spallanzanii is not indicative of their gender (Figure 3.3a). However, there was a significant difference (p < 0.001) between indeterminate gender and both female and male fanworms for both body length and weight. Smaller fanworms have no identifiable gametes with which to classify their sex and therefore are most likely immature and unable to spawn (Figure 3.3a; b).

Sex determination and sex allocation in invertebrates tends to evolve interactively through sex ratio selection (Cook, 2002). Sex determining mechanisms are primarily genetic or environmental factors or an interaction of both (i.e., temperature, food availability, photoperiod or mate availability) (Premoli et al., 1996; Cook, 2002; Minetti et al., 2013). It is possible for sex determination to vary between different populations of the same species (Godfray & Werren, 1996; Cook, 2002). Natural selection might favour certain sex-determination genes over others. Additionally, population structure and ecology can also affect sex ratio in terms of local mate competition. If one sex suffers a greater reduction in fitness through competition with siblings of the same sex, the mother is selected to bias the sex ratio towards the sex that suffers less competition (Godfray & Werren, 1996). These mechanisms could provide explanations for the biased sex ratio in fanworm, however, this would need further investigation.

Indeterminate fanworms were found throughout the year in this current study and in an Australian study (Lee et al., 2018). In the current study, some larger and heavier fanworms were found to be of indeterminate sex, ranging in body length from 6 – 19 cm, mostly in the months from May to December (Figure 3.4). It is likely that these individuals had just spawned and released their gametes and therefore their coelomic cavity was empty preventing their sex from being determined. Alternatively, these fanworms could still be in the early stages of gametogenesis and have not yet started to produce new gametes (Olive, 1970; Read et al., 2014). A previous study in
New Zealand also found an absence of gametes among all sampled fanworms in the months of August and September (Jute, 2015). Among smaller fanworms (< 5 cm) it is most likely that they are insufficiently mature to produce gametes (Olive, 1970;1992).

Previously it has been reported that fanworms in a population in the Gulf of Taranto (Italy, Mediterranean Sea) reached sexual maturity only at a body length of 15 cm and all collected specimens from 15 – 20 cm long were males but when looking at specimens from 25 – 30 cm the sex ratio was 1:1 (Giangrande & Petraroli, 1994). In contrast, a fanworm population in Port Phillip Bay (Victoria, Australia) reached sexual maturity at ~ 5 cm body length and showed a 1:1 sex ratio in all specimens > 5 cm body length (Currie et al., 2000). In this current study, none of the sampled fanworm were observed to contain both types of gametes, which would have been indicative of hermaphroditism. Although it has been hypothesised that S. spallanzanii is a protandric hermaphrodite (Giangrande & Petraroli, 1994), later studies found the species is dioecious, as only ever one type of gamete was present in each fanworm specimen at a time, and individuals are capable of repeated spawning, since eggs of a range of sizes are present within fanworms throughout the year (Read et al., 2014; Lee et al., 2018). Among the 225 sampled and closely examined fanworms in this current study, there was no evidence of hermaphroditism, confirming that S. spallanzanii is dioecious and since there was no difference in body length or weight between male and female fanworms, it shows that they are gonochoric and not protandric.

It might be that the Australian and New Zealand fanworm populations have adjusted their sex ratio due to environmental factors such as food availability, competition, temperature and pollution (Lee et al., 2018; Geffroy & Douhard, 2019). This would be an adaptive benefit for this invasive species, especially when establishing in a new area with limited mating access allowing for more reproductive flexibility and faster establishment from founder individuals (Lee et al., 2018; Geffroy & Douhard, 2019).

3.4.2 Fanworm and egg size

The presence of generally larger eggs in fanworm sampled in June and July compared to those subsequently sampled in September and October, suggests that one or several spawning events took place during August, September and October (Figure 3.5). Most often eggs of a variety of sizes were simultaneously present within individual female fanworm, ranging from the smallest egg measured (5 µm diameter) up to large eggs (220 µm diameter). However, in June and July mainly larger eggs (> 140 µm diameter) were measured in female fanworms, indicating that when the coelom is densely packed with mature gametes and less coelomocytes are present, that spawning is more likely to be imminent (Read et al., 2014). These results are consistent with a previous short term study conducted in New Zealand, that also indicated larger eggs were mainly absent in fanworms collected in August and September, despite large overall variation among egg sizes (Jute, 2015). In contrast, in fanworm populations in the Gulf of St Vincent, Australia, there was no clear difference in egg size among female fanworms sampled in the different months of the year and no indication of a spawning event for particular months (Lee et al., 2018). Therefore, it was
suggested that fanworm living in this location might be able to continuously spawn throughout the year (Lee et al., 2018).

It appears that in *S. spallanzanii* gametogenesis can take place all year round with female fanworms being at different stages of oogenesis, having different sized eggs and differing amounts of coelomocytes at different times throughout the year (Murray et al., 2011). However, these female worms might still only spawn 1 - 2 times a year, when their coelom is packed with ripe gametes and almost depleted of coelomocytes (Gambi et al., 2000; Gosselin & Sewell, 2013). However, when exactly the female fanworms reach that final stage of oogenesis may vary for each individual female, resulting in the capacity for a continuous spawning of eggs and larvae (Gambi et al., 2000; Murray et al., 2011; Gosselin & Sewell, 2013). From what has been observed throughout this study, it might be that within the seasonal temperature range at the study site (13 – 23 °C) fanworms can safely spawn all year round, potentially being able to take advantage of periods with suitable conditions for larval development, i.e., they have a high degree of flexibility in their reproductive behaviour to take advantage of suitable environmental conditions, ultimately leading to greater reproductive output and population security (Wilson, 1991; Hadfield & Strathmann, 1996; Lenz et al., 2011; Jofré Madariaga et al., 2014). In comparison to the Mediterranean, where *S. spallanzanii* do appear to have a spawning season, it is possible that the temperature range between the coldest to the warmest month (i.e., 13 – 23 °C, Auckland, New Zealand) in New Zealand and Australia is insufficient to generate a specific spawning season. Therefore, optimal conditions within the range for gamete maturation in *S. spallanzanii* are present for longer and hence provide good conditions to continuously produce and potentially spawn eggs over an extended period (Lee et al., 2018). In contrast, in the Mediterranean Sea there is a higher temperature variation (i.e., 12 – 28 °C, Gulf of Taranto, Italy), which might limit the spawning season of *S. spallanzanii* to only a few months (Giangrande et al., 2000). However, it is likely that temperature is only one factor affecting or inducing spawning and gametogenesis and other environmental conditions (i.e., phytoplankton, pollution, disturbance and competition) might also influence gametogenesis and spawning (Currie et al., 2000; Murray & Keable, 2013; Fletcher, 2014). Furthermore, there might also be variations in the reproductive behaviour among different fanworm populations.

Greater egg size in fanworm was associated with the increasing body length of female fanworm and the month of sampling, with larger eggs found in the months from April to July (Figure 3.5). While individual female fanworms of all sizes contained eggs of various sizes simultaneously, smaller female fanworms (3 - 5 cm) tended to have mainly smaller eggs (20 - 40 µm diameter) and only a few larger eggs or no larger eggs at all. In contrast, larger female fanworms that were > 6 cm in body length generally contained eggs of a wide size range but tended to be dominated by larger eggs (> 117 µm diameter; Figure 3.5). This indicates that fanworms in New Zealand with a body length of < 5 cm are not producing larger eggs in significant quantities (> 200 µm in diameter). In contrast, fanworms in the Mediterranean Sea only started producing large eggs (250 µm diameter) at a body length of 15 cm (Giangrande et al., 2000), whereas fanworm populations in Port Phillip
Bay and Gulf of St Vincent, Australia started to produce large eggs (100 – 170 µm) at a body length of 5 - 7 cm (Currie et al., 2000; Lee et al., 2018). This indicates that the size of female fanworms at sexual maturity, as well as mature egg size varies markedly amongst fanworm populations in different locations. It is common in marine invertebrates to have variations in body size and reproductive flexibility in populations in different geographic locations (Olive, 1995; Sanford & Kelly, 2011; Bates et al., 2013).

3.4.3 Gametogenesis stages

The proportion of sampled fanworms at stage 4 of the gametogenesis stage classification (Bybee et al., 2007), which indicates the coelomic cavity is packed with gametes and low in coelomocyte numbers, is low from July until November, indicating that a single or multiple spawning events have occurred during that time (Figure 3.6). Furthermore, most fanworms collected during these months were at stage 1 and 2, indicating having no or very few gametes within the coelom, consistent with post-spawning recovery (Read et al., 2014). Gametogenesis stage 4 was most common among sampled fanworms in December until June, indicating that the eggs were completing their development during these warmer months (Figure 3.6). There is a high degree of overlap of the different gametogenesis stages among the months of fanworm sampling, especially for stages 3 and 4, which indicates eggs of various sizes are present to various degrees in almost every month (Figure 3.5; 3.6). Previously, stages 3 and 4 in fanworms sampled in New Zealand were mainly measured in July, November and December, with the largest egg sizes being measured in July (Jute, 2015). Gametogenesis stages 1 and 2 were more prevalent in April, May, August and September and less common in June, July, January and February. This indicates that in the months, where stages 1 and 2 were least common, and stages 3 and 4 were dominant, most fanworms had gametes within them and their sex could be identified, indicating spawning events could have taken place during these months (Jute, 2015).

Oogenesis in fanworms can take between 9 to 12 months in the Mediterranean Sea until the eggs are mature, whereas spermatogenesis is a rather rapid process, which is completed within 3 months (Giangrande et al., 2000). Similar observations were made of fanworm in Port Phillip Bay, Australia, where oogenesis, spermatogenesis and the number of coelomocytes present, followed a cycle (7 – 10 months) and resulted in roughly synchronized spawning events between June and August, based on histological examination of male and female fanworms sampled monthly (Currie et al., 2000). In contrast, no synchronicity for male and female gametogenesis or seasonality has been found in a fanworm population in the Gulf of St Vincent, Australia, although, a higher number of mature eggs (> 100 µm diameter) were found in April and May and a lower number in September, January and February (Lee et al., 2018). The results of this current study is consistent with the study from the Gulf of St Vincent, Australia, where although less ripe stage 4 eggs were found from July to November, different sized eggs (stage 3) were present in every month. Collectively these results suggest that some spawning by individuals may have taken place during this time but it is unlikely that mass synchronous spawning events were occurring. Consequently,
this greatly limits the potential for a mitigation strategy for aquaculture based on avoiding seasonal fanworm spawning events for key operational activities such as seeding or moving mussel grow-out ropes (Fitridge et al., 2012; Fitridge & Keough, 2013; Sievers et al., 2014; Bannister et al., 2019; Sievers et al., 2019). However, removing small worms (< 5 cm) before they reach maturity and start producing viable eggs, might be useful in diminishing fanworm numbers by ultimately reducing the number of viable eggs and larvae in the water column.

3.5 Conclusion

In this study no distinct spawning season for fanworm in northern New Zealand could be identified, although elevated spawning activity appeared to have occurred from July to October. The high variability in egg size and ongoing production of eggs in addition with the female skewed sex ratio is indicative that S. spallanzanii has high reproductive flexibility, allowing them to reproduce or spawn over an extended time frame with little environmental limitation. This species is highly tolerant of a wide range of environmental conditions, capable of establishing on muddy benthic environment, among mussels in suspended culture, on hard artificial substrates or in intertidal rock pools (Fletcher, 2014; pers. comm. K. James). Also, the Mediterranean fanworm tolerates a wide range of temperatures (4 – 27°C) and depths (0.5 – 30 m), making it likely that a combination of several environmental factors such as food availability, competition pressure, and pollution might affect the fanworms reproductive output and sex ratio (Clapin, 1996; Depledge & Billinghurst, 1999; Zheng et al., 2010; Fletcher, 2014). Even though there is no distinct spawning season, small fanworms (< 5 cm) are likely immature and do not produce viable eggs. Collectively, these findings provide valuable insights into the biology of the Mediterranean fanworm and indicate that seasonal mitigation strategies have limited potential and other avenues should be explored to effectively reduce fanworm establishment and spread.
Chapter 4 - The presence, settlement and growth of the Mediterranean fanworm, *Sabella spallanzanii*, among mussel farms

4.1 Introduction

The spread of marine non-indigenous species (NIS) is greatly facilitated by human mediated pathways, such as increasing international shipping traffic, aquarium trade and aquaculture activities (Floerl & Inglis, 2005; Coutts & Forrest, 2007; Coutts et al., 2010; Darling & Mahon, 2011; Darling et al., 2017; Ojaveer et al., 2018). NIS often arrive in new international locations by hitchhiking on international vessels, via the fouling on ship hulls as well as being carried in sea chests and ballast water (Bax et al., 2003; Floerl & Inglis, 2005; Floerl et al., 2009; Bates et al., 2013; Roche et al., 2015; Growcott et al., 2017; Johnston et al., 2017). Following arrival at new locations, the spread of NIS can be facilitated through the movements of domestic vessels and aquaculture gear. Whilst some NIS have no adverse effects on the local ecosystem, others (commonly referred to as invasive species) can cause biodiversity loss, impact ecosystem functions, and compromise economic activities, e.g., affect productivity in the aquaculture sector (Bax et al., 2002; Adams et al., 2011; Clarke Murray et al., 2012; Bates et al., 2013; Campbell et al., 2017; Johnston et al., 2017). One such example is the Mediterranean fanworm, *Sabella spallanzanii*, which is an invasive species of great concern in New Zealand and is of particular interest for national and regional coastal management authorities, mussel farmers and biosecurity practitioners.

Biofouling is a significant obstacle to efficient production in most shellfish aquaculture (Adams et al., 2011; Fitridge et al., 2012; Atalah et al., 2016b; Sievers et al., 2017). For example, marine biofouling of green-lipped mussels, *Perna canaliculus*, is estimated to cost the New Zealand aquaculture industry up to US $16.4 million per year (Forrest & Atalah, 2017). Biofouling species such as *S. spallanzanii*, compete with the mussels for phytoplankton and space, and cause reduced growth of the mussels and compromise the mussel’s attachment with byssal threads, leading to crop losses of 50 – 60% (Ramsay et al., 2008b; Bannister et al., 2019). These mussel crop losses are estimated to cost the New Zealand shellfish industry up to NZ $26.4 million over a 24 year period (Soliman & Inglis, 2018). There is a need to learn more about the fanworm’s dispersal ability and temporal presence in the water and whether its growth can outcompete the growth of mussels.

The Mediterranean fanworm arrived in New Zealand in 2008 most likely via ship hull fouling or ballast water, as evidenced from its life traits (i.e., ability to colonize vessel hulls and pelagic larval stage) and incursion history, i.e., first detection records associated with shipping hubs (Read et al., 2011; Fletcher, 2014). Further domestic spread of fanworm in New Zealand has most likely been
facilitated by the movement of infested vessels among coastal locations. In addition to being able to survive in sea chests and ballast water, this species also overgrows coastal infrastructure, including moorings and aquaculture facilities, which in turn become stepping stones for secondary spread via regional shipping and movement of marine equipment (Floerl & Inglis, 2005; Floerl et al., 2005; Hopkins & Forrest, 2008; Floerl et al., 2009; Campbell et al., 2017; Growcott et al., 2017). The most effective way to deal with invasive marine species, is preventing their arrival with appropriate biosecurity measures. Early detection of an incursion can provide an opportunity to take action before an invasive species becomes established and while they are still low in numbers (Ficetola et al., 2008; Ojaveer et al., 2018; Tait et al., 2018; van den Heuvel-Greve et al., 2021).

Strategies to prevent settlement of NIS on biofouling-prone surfaces and entrainment on transport vessels include application of antifouling chemicals, paint, wrapping, physical removal, air exposure, power washing, exposure to brine solution, freshwater, chlorine bleach and heat treatment (Dunphy et al., 2005; Forrest et al., 2007; Denny, 2008; Fitridge et al., 2012; Bannister et al., 2019). However, it is often difficult to effectively detect NIS early enough at low population densities and when they are present among other marine species, especially when the target organisms are relatively small and inconspicuous (Darling & Mahon, 2011; Floerl et al., 2012; Darling et al., 2017; Ojaveer et al., 2018). Computer modelling, that considers spatio-temporal patterns of propagule distribution and molecular diagnostic tools such as environmental DNA (eDNA) metabarcoding and species-specific assays can aid with this challenging task (Wood et al., 2013; Pochon et al., 2015; Zaiko et al., 2016; Atalah et al., 2017; Pande et al., 2017).

Environmental DNA (eDNA), which refers to genetic material from environmental samples, such as soil, water, biofilm and air, is used to detect species based on their genetic material found in the environment (Ficetola et al., 2008; Barnes et al., 2014; Barnes & Turner, 2016; Darling et al., 2017; Collins et al., 2018; Cristescu & Hebert, 2018; Taberlet et al., 2018). Environmental DNA tools have shown promising results for detecting species and describing the community composition, both in freshwater and the marine environment, i.e., target species detection and community profiling with droplet digital PCR (ddPCR) (Rees et al., 2014; Laroche et al., 2017; Lacoursière-Roussel et al., 2018; Taberlet et al., 2018). Thus, high-throughput sequencing (HTS) or metabarcoding is an easy and cost-effective approach to monitor biodiversity and screening for putative pests from eDNA samples (van den Heuvel-Greve et al., 2021). In contrast, species-specific detection targets the eDNA signal derived from one to a few specific target species, using ddPCR or quantitative PCR (qPCR) with specific primers and probe set (Zaiko et al., 2018). However, eDNA based detections do not distinguish between living and dead material from organisms, which may create problems because shed and extracellular DNA can persist in seawater for months (Darling & Mahon, 2011; Barnes et al., 2014; Darling et al., 2017; Pochon et al., 2017; Collins et al., 2018; LeBlanc et al., 2020; Wood et al., 2020; van den Heuvel-Greve et al., 2021). In contrast to eDNA, environmental RNA (eRNA) is produced in biologically active (living) organisms and degrades more rapidly, therefore, it tends to provide a more accurate picture of whether there are living organisms of the targeted species present in an area sampled for eRNA (Hofreiter et al., 2001; 2015; Sassoubre et al., 2016; Darling et al., 2017; Laroche et al., 2017; Pochon et al., 2017; Zaiko et al., 2018; Cristescu, 2019). However, the downside of using
eRNA is that it is far less stable and more prone to degradation than eDNA, and requires additional resources for processing (Laroche et al., 2017).

To successfully manage the effects of key marine pests, such as *S. spallanzanii*, on mussel farms while reducing production costs and shellfish losses, more information is needed on the patterns of settlement, recruitment and subsequent growth of fanworm. Therefore, effective surveillance systems need to be established, that also potentially employ novel molecular detection approaches (Ojaveer et al., 2018). Therefore, the aims of this current study were to: 1) assess the effectiveness of eDNA and eRNA for detecting the temporal presence of fanworm in the water column and arriving on settlement plates around mussel farms in the Hauraki Gulf, 2) assess fanworm recruitment and growth on different settlement substrates, both around a mussel farm and within a marina, 3) compare the effectiveness of molecular methods versus visual detection of fanworm on passive sampling devices. If the fanworm show a specific preference for a particular substrate, mitigation strategies could be used to change or treat specific surfaces to reduce larval settlement. This knowledge would be useful in developing effective management strategies for this highly invasive species and might help the aquaculture industry to reduce fanworm numbers among mussel farms.

4.2 Methods

4.2.1 Sample Collection

4.2.1.1 Seawater and biofilm sampling for eDNA and eRNA analyses

eDNA and eRNA were recovered from samples of seawater and biofilm on settlement plates and then analysed to detect the presence of fanworm at three mussel farm sites in the Hauraki Gulf, near Coromandel, New Zealand: Wilson Bay (36° 57” 06.6” S, 175° 24” 33.4” E), Esk Point (36° 48” 28.5” S, 175° 26” 21.4” E), Papakarahi Bay (36° 48” 21.1” S, 175° 27” 07.1” E) (Figure 4.1). Sampling was conducted monthly from December 2017 until January 2019, except for May, June and November, when weather conditions and vessel availability prevented sample collection.
At each site, three replicate seawater samples (12 L) were taken from 5 m depth and filtered through a 500 µm nybolt mesh filter (56 mm diameter) to remove unwanted debris and then through a sterile 80 µm nybolt mesh filter (56 mm diameter), that was used to collect representative samples of fanworm larvae, which are >80 µm size. The filtering aimed to minimize any “noise” in the eDNA/ eRNA signal from other potential sources, e.g., nucleic acids bound to particulate matter, shed cells, free-floating molecules. However, this method cannot distinguish between eDNA/eRNA from larvae versus that derived from adult fanworm, e.g., fractions of branchial crown, dead or excreted mucous. Each filter mesh with associated filtrate was then placed in a sterile 2.0 ml tube containing 1 ml of LifeGuard™ Soil Preservation Solution (Qiagen, CA, USA) using sterile tweezers. Between sampling events, tubes and filter units were decontaminated by soaking overnight in 10% bleach solution, followed by thorough rinsing with tap water and drying. Samples were delivered to the laboratory on ice within 4 hours after collection and frozen (-80 °C) immediately until further processing to recover eDNA and eRNA.

Biofilm samples came from six settlement plates deployed at each of the three mussel farm sites. The settlement plates were made from grey PVC sheet (20 × 20 × 0.4 cm) and were suspended from an anchored surface float, and attached to a weighted rope at each of the three sites, three plates were deployed at 2 m depth and three plates at 8 m depth (n = 18 per month). The plates were deployed for 1 month prior to sampling to accumulate sufficient biofilm. At each sampling event, the collected plates (n = 18) were replaced with clean plates. Additionally, HOBO Water Temp Pro v2 loggers were attached to the ropes with the biofilm plates to record seawater temperature at a frequency of every 30 minutes at 5 m depth. The seawater temperature data were read out every month, when plates were collected and the loggers were then re-deployed. After removal from the water, the accumulated biofilm material was removed in situ using individual sterile stainless steel surgical blades (Swann-Morton, Sheffield, UK) and isolated into sterile 2.0 ml tubes containing 1 ml LifeGuard™ Soil Preservation Solution (Qiagen, CA, USA). Laboratory gloves were worn throughout the sampling process and caution was taken to avoid cross-contamination between samples by replacing the blades and cleaning the sampling gear with bleach, and thoroughly rinsing with freshwater. Samples were delivered to the laboratory on ice within 4 hours after collection and frozen (-80 °C) immediately until further processing to recover eDNA and eRNA.

4.2.1.2 Passive sampling for presence, settlement and growth of fanworm

In addition to eDNA and eRNA analyses of seawater and biofilm samples, a set of different substrate materials, i.e., smooth PVC plates (20 × 20 × 0.4 cm), rough cement fibre board plates...
with a plastic mesh (1 cm mesh squares) attached to provide more surface structure (Hardiflex, James Hardie Ltd, 20 × 20 × 0.4 cm), polypropylene mussel grow-out rope without mussels (75 mm Mega Loop Standard, Quality Equipment Ltd), mussel grow-out rope with re-seeded 1-year old cultured adult mussels (*Perna canaliculus*) and mussel grow-out rope with small cultured mussels (5 months old) were deployed. The different substrates were deployed at the Papakaraha Bay mussel farm (36° 48” 21.1” S, 175° 27” 07.1” E) and in a site with very high *S. spallanzanii* abundance, the Orakei marina, Auckland (36° 50” 59.8” S, 174° 48” 34.4” E). The aim was to visually assess the timing of larval settlement and establishment of fanworm and their possible preference for different settlement substrates, and comparing settlement within a mussel farm setting and a marina.

Three plates made from PVC sheet (20 × 20 × 0.4 cm) and three cement fibre board plates with a plastic mesh (1 cm mesh squares) were attached back to back and attached with cable ties at random positions along a 3 m rope, that was weighed down with a 2 kg lead weight. The three ropes with three pairs of plates (n = 6) each were then suspended at random intervals along a mussel farm backbone line at Papakaraha Bay and left there from May 2019 until January 2020. Additionally, three empty polypropylene mussel grow-out ropes, three mussel grow-out ropes with re-seeded 1-year old cultured adult mussels (*Perna canaliculus*) and three mussel grow-out rope with small cultured mussels (5 months old), each 2 m in length and weighed down with a 2 kg lead weight, were also suspended from an anchored surface float, vertically attached to a weighted rope at Papakaraha Bay. Simultaneously, three 2 m long polypropylene mussel grow-out ropes, three 2 m ropes with three smooth PVC plates and three cement fibre board plates with plastic mesh each attached back to back to the rope with cable ties and weighed down with a 2 kg lead weight, were deployed at Orakei marina from April 2019 until December 2019. For logistical and biosecurity reasons, ropes with mussels could not be transported from Coromandel and deployed in the marina. To cover approximately one calendar year, a second set of empty polypropylene mussel grow-out rope without mussels, smooth PVC plates and cement fibre board plates with a plastic mesh were deployed in Orakei marina from February 2020 until June 2020. Due to time, logistic constraints and Covid-19 lockdowns, a second set of plates and ropes could not be deployed in the Papakaraha Bay mussel farm.

Additionally, HOBO Water Temp Pro v2 loggers were attached to the ropes both at Orakei marina and Papakaraha mussel farm to record seawater temperature at a frequency of every 30 minutes at 2 m depth.

Upon retrieval of the different substrate materials, all the fanworms on the different substrates were counted and their body length measured. However, due to the large number of fanworms present on the substrates in the marina, a randomly selected subsample of 280 fanworms, were carefully removed with tweezers from the respective substrate in the laboratory and their body lengths whilst remaining within their tubes were measured. There was a lower number of fanworm on the other types of substrates at the mussel farm site and therefore all the retrieved fanworms were measured from this site.
4.2.2 DNA and RNA extraction

In the laboratory each filter mesh (seawater sample) was cut up into small pieces using pre-autoclaved scissors and forceps and put into a tube containing Zymo Research Lysis Buffer (ZR-Duet DNA/RNA MiniPrep Kit, Zymo Research, CA, USA). From the biofilm samples, a subsample of 0.2 g was taken for DNA/RNA extraction. Both the seawater filter and biofilm samples were then lysated in a bead beater with previously autoclaved metal beads for 2 min at 1500 RPM (TissueLyser II, Qiagen). The samples were then centrifuged for 5 min at 13,000 RPM. The supernatant was removed and transferred to a new column. Total DNA and RNA were then co-extracted from each sample using the ZR-Duet DNA/RNA MiniPrep Kit (Zymo Research, CA, USA), following the manufacturers protocol. A negative DNA extraction control was included for every set of 12 samples. The quality and purity of isolated DNA and RNA was checked using a NanoPhotometer N60 (Implen).

4.2.3 Droplet digital PCR (ddPCR) analysis

Droplet digital PCR (ddPCR) assay for detecting *S. spallanzanii* eDNA signal was undertaken on a BioRad QX200 system, using a specific primer-probe set that targets the cytochrome c oxidase I (COI) region of *S. spallanzanii* Sab3-F (5´- GCTCTTATTAGGCTCTGTGTTTG - 3´) and Sab3- R (5´ CCTCTATGTCCAACCTCCTTTG - 3´) (10 µM) with a FAM reporter dye at the 5´end and a Black Hole Quencher-2 at the 3´end (Sab3- Probe (5´- AAATAGTTCCGTCGTCGCCC - 3´)) (Wood et al., 2017). Each ddPCR reaction included 450 nM of each primer and probe, 1 × BioRad ddPCR Supermix for probes (No dUTP), 1 µl DNA and sterile water for a total reaction volume of 22 µl. The BioRad QX200 droplet generator partitioned each reaction mixture into nanodroplets by combining 20 µl of the reaction mixture with 70 µl of BioRad droplet oil for probes. After processing, the reaction mixture of each sample (40 µl), was transferred to a PCR plate for amplification using the following cycling protocol: hold at 95 °C for 10 min, 40 cycles of 94 °C for 30 s, 60 °C for 1 min, and a final enzyme deactivation step at 98 °C for 10 min (Wood et al., 2017; von Ammon et al., 2019). The plate was then analysed on the QX200 instrument using QuantaSoft Analysis Pro software tool (version 1.0.596). For each ddPCR plate run, at least one negative control (i.e., containing all reagents and nucleic acid free water) and one positive control (i.e., genomic DNA extracted from fanworm tissue) were included to ensure there was no contamination throughout the extraction process. Each sample taken from a settlement plate was analysed on the QX200 instrument to establish the threshold value separating negative and positive droplets, and perform copy number quantification of the target DNA. The ddPCR output results (copies/ µl) were converted to copies per sample using the following formula: number of copies per µl × 22 µl (the initial volume of the PCR reaction) × 60 µl (the volume used to elute the DNA during extraction) (Wood et al., 2019).
4.2.4 RNA analyses

Due to time and financial limitations, not all collected eRNA samples were processed for droplet digital PCR (ddPCR) analysis. Based on the eDNA analysis results, and a previous study that indicated that higher eDNA copy numbers show higher probability of positive eRNA signal (von Ammon et al., 2019), a subset of 48 samples with the highest ddPCR eDNA copy numbers were selected for further RNA analysis via ddPCR. The selected eRNA extracts were first transcribed into cDNA before following the protocol described in von Ammon et al. (2019). Briefly, trace DNA in the selected samples was eliminated by two sequential DNase (TURBO DNA-free™ Kit, Thermo Fisher Scientific, Massachusetts, USA) treatments following the protocols of Langlet et al. (2013). Treated RNA was diluted to 10 ng μl⁻¹ equimolar concentrations and reverse transcribed into cDNA using the SuperScript® III reverse transcriptase (Thermo Fisher Scientific, Massachusetts, USA) to then be processed with ddPCR as described above. The ddPCR output results (copies μl⁻¹) were converted to copies per sample using the following formula: number of copies per μl × 22 μl (the initial volume of the PCR reaction) × 60 μl (the volume used to elute the DNA during extraction) (Wood et al., 2019).

4.2.5 Statistical analyses

4.2.5.1 eDNA and eRNA

Since eDNA was extracted and amplified via ddPCR for all the collected samples, it was tested whether eDNA copy number per sample was affected by sampling site (Wilson Bay, Esk Point, Papakarahi Bay), month and the biological matrix it was sampled from (biofilm or seawater sample). A multiple linear regression model with corresponding F-statistic was applied on log-transformed eDNA data (log + 1) using the lm function implemented in R (v 3.6.0. R Core Team, 2019). January 2018 was chosen as the baseline intercept value. All statistical analyses and graphical visualisations were performed in R (v 3.6.0. R Core Team, 2019).

HOBOware software was used to read out seawater temperature data from the HOBO Water Temp loggers and the monthly mean seawater temperature was calculated from all 30 minute measurements logged throughout each month (Excel, 2016).

4.2.5.2 Occupancy model

To assess spatial and temporal patterns in distribution of the molecular detection of fanworm, a likelihood based occupancy estimation modelling approach was applied (MacKenzie et al., 2002). This method estimates the proportion of area occupied by a species (occupancy), accounting for imperfect detection and has recently been shown to be an effective approach for interpreting eDNA-based data and quantifying detection probabilities via different sampling approaches (Lugg et al., 2018; Wood et al., 2019). This method estimates the proportion of sites occupied by a
species (occupancy) when detection probabilities are less than one, i.e., when the data fail to
detect the presence of a species (Pochon et al., 2017; Wood et al., 2019). In this study, the “single
season, multi-method” occupancy model variant was applied to the eDNA based detection histories
of S. spallanzanii, derived from seawater and biofilm samples. The model was implemented in
PRESENCE v12.37 software (Hines, 2006), with sampling events (i.e., unique combination of
sampling site, month, sampling matrix (seawater vs biofilm) and depth for biofilm) treated as
sampling units (N = 66) and sample replicates among the same month as the repeated surveys. A
few a priori models were defined with sampling month, depth and site as covariates, to test for their
effect on target occupancy and detection probabilities estimated from the model ranked the best
based on Akaike Information Criterion (AIC) (Burnham & Anderson, 2004). The naïve occupancy
estimate was calculated as the proportion of sample units where the positive detection was
reported over all sampled units. This model estimates fanworm occupancy based on detection/
non-detection of the collected samples and makes a prediction about probability of detection.

4.2.5.3 Number and body length of fanworms on different substrate materials

To test if substrate type had an effect on the number of settled fanworms, several glm models were
evaluated to establish best fit with sampling site (Papakaraha mussel farm or Orakei marina) as a
covariate factor. Model fit was evaluated by looking at null and residual deviance and log-likelihood
between different model variations. A negative binomial generalized linear model was applied with
all the zero counts removed (glm.nb, MASS package, v3.6.0. R Core Team, 2019). This model was
chosen to account for over-dispersion (Zuur et al., 2009; Hilbe, 2011). The number of fanworms on
the empty grow-out rope without mussels was chosen as the intercept value. Estimated coefficients
were used to infer how likely it would be to find fanworms settling on the different substrate
materials. Tukey pairwise comparisons were applied to compare abundance of fanworms on the
different substrate materials.

To test sampling site (Papakaraha mussel farm or Orakei marina) and substrate material (PVC
plates, cement board plates with plastic mesh, empty grow-out rope without mussels, grow-out
rope with adult mussels, grow-out rope with small mussels) effect on the body length of fanworms,
a two-way analysis of variance (ANOVA) was applied following square root transformations to
normalize the data. The body length of fanworms on grow-out rope without mussels was chosen as
the intercept value. Post-hoc Bonferroni pairwise comparisons were applied for multiple
comparisons to identify differences in fanworm body length among the different substrate materials.
All statistical analyses and graphical visualisations were performed in R (v 3.6.0. R Core Team,
2019).

HOBOware software was used to read out seawater temperature data from the HOBO Water Temp
loggers and the monthly mean seawater temperature was calculated from seawater temperature
readings that occurred every 30 minutes for a month. The data were analysed and plotted,
comparing monthly mean seawater temperatures for Orakei marina and the Papakaraha mussel
farm (Excel, 2016).
4.3 Results

4.3.1 Patterns of S. spallanzanii eDNA and eRNA signal around Hauraki Gulf mussel farms

From a total of 276 analysed eDNA samples, 96 seawater and 180 biofilm samples showed either a positive fanworm eDNA detection, (45% positive signal, of which 47% were seawater samples and 53% were biofilm samples) and 55% of all samples resulted in no fanworm eDNA detection (zero counts). Contamination was not detected in the control samples. Descriptive statistics showed that Esk Point (n = 96) and Papakarahi Bay (n = 92), had more positive eDNA detections, 36% and 35% respectively, compared to the 29% positive detections in Wilson Bay (n = 88). Among sampling months, the highest eDNA signal occurrence was observed in seawater samples in January, March and April. Signal strength (eDNA copy numbers) varied highly among the different months (Figure 4.2). Overall, the strongest eDNA signal was detected in seawater in January, September and October and for biofilm samples the strongest eDNA signal was detected in February 2019 (Figure 4.2). Multiple linear regression modelling showed that the considered predictor variables (i.e., sampling site, month, sampling matrix (biofilm vs seawater)) had a significant effect and explained around 24.3% of the variance in the eDNA copy numbers data ($R^2 = 0.243$, $F_{(14, 261)} = 6.48$, $p < 0.001$). Specifically month and sampling matrix (seawater or biofilm) contributed significantly to predicting the eDNA signal strength ($p < 0.001$). However, eDNA copy numbers were not affected by sampling sites ($p = 0.77$; Fig. 4.1 map A: Wilson Bay, Esk Point and Papakarahi Bay).

Figure 4.2. Log10 transformed DNA copy number of S. spallanzanii from all collected biofilm and seawater samples throughout the year. Each box plot represents the upper and lower quartiles (edges), median (horizontal line), the maximum value of the data that is within 1.5 times the
interquartile range over the 75th percentile (the upper whisker), the minimum value of the data that is within 1.5 times the interquartile range under the 25th percentile (the lower whisker). Due to weather and boat restrictions, no samples were collected in May, June and November 2018.

Compared to the eDNA detection rate for fanworm, eRNA detection was rather low and did not allow proper quantitative comparisons with the eDNA signal. From the 48 selected samples that were tested for eRNA which were characterized by the highest eDNA copy numbers, only four out of 19 biofilm samples and six out of 29 seawater samples yielded positive detections (Table 4.1). The eRNA signal strength in these ten samples ranged from 113 (biofilm) to 144,047 (seawater) cDNA copies per sample, being generally higher in the seawater samples with an overall maximum of 144,047 copies per sample reported in a seawater sample taken in December 2018 (Table 4.1). The calculated monthly mean seawater temperatures among the mussel farms varied slightly from each other ranging between 0.3 – 1 °C. However, in most months the seawater temperature at the three sites was the same or very similar. Esk Point had the warmest mean seawater temperature from January until July, ranging from 22.1 °C ± 0.9 s.d. in January, 22.5 °C ± 0.4 s.d. in March to 14.3 °C ± 0.2 s.d. in July. Whereas Wilson Bay and Papakarahi had overlapping slightly cooler mean seawater temperatures that ranged from 22 °C ± 1.2 s.d. in January, 21.8 °C ± 0.5 s.d. in March to 14.0 °C ± 0.2 s.d. in July. The coldest monthly mean seawater temperature was measured in July with 14 °C ± 0.3 s.d. at both Wilson Bay and Papakarahi, and the warmest mean seawater temperature was measured in February with 23.1 °C ± 0.8 s.d. at Esk Point.

Table 4.1. Ten out of 48 samples with a positive eRNA signal with their corresponding sampling site (mussel farms), sampling method, month of sampling and their respective eDNA and eRNA copy numbers.

<table>
<thead>
<tr>
<th>Month</th>
<th>Sampling Site (mussel farms)</th>
<th>Sampling method</th>
<th>DNA copy number</th>
<th>RNA copy number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jan</td>
<td>Papakarahi</td>
<td>Biofilm</td>
<td>353,701</td>
<td>113</td>
</tr>
<tr>
<td>Jan</td>
<td>Esk Point</td>
<td>Seawater</td>
<td>27,921</td>
<td>28,259</td>
</tr>
<tr>
<td>Jan</td>
<td>Wilson Bay</td>
<td>Seawater</td>
<td>60,789</td>
<td>437</td>
</tr>
<tr>
<td>Mar</td>
<td>Esk Point</td>
<td>Seawater</td>
<td>1302</td>
<td>793</td>
</tr>
<tr>
<td>Jul</td>
<td>Wilson Bay</td>
<td>Biofilm</td>
<td>890</td>
<td>41,552</td>
</tr>
<tr>
<td>Sep</td>
<td>Papakarahi</td>
<td>Seawater</td>
<td>2346</td>
<td>230</td>
</tr>
<tr>
<td>Oct</td>
<td>Wilson Bay</td>
<td>Biofilm</td>
<td>53,402</td>
<td>7907</td>
</tr>
<tr>
<td>Dec</td>
<td>Papakarahi</td>
<td>Seawater</td>
<td>1222</td>
<td>144,047</td>
</tr>
<tr>
<td>Dec</td>
<td>Wilson Bay</td>
<td>Seawater</td>
<td>7273</td>
<td>1589</td>
</tr>
<tr>
<td>Feb-19</td>
<td>Wilson Bay</td>
<td>Biofilm</td>
<td>18,716</td>
<td>114</td>
</tr>
</tbody>
</table>
4.3.2 DNA occupancy and detectability

Among the fitted occupancy models, the one accounting for constant large-scale occupancy (not dependent on sampling site, depth or month), was ranked the highest compared to those assuming constant small-scale occupancy (i.e., the probability that the target DNA is present in the direct vicinity of sampling, given the sampling unit is occupied) and method-dependent detection probability. The relative likelihood of this model (i.e., AIC weight) model was 0.997. The parameters in the best-ranked model were estimated with reasonably good precision, as the standard error values did not exceed 12% of the estimate values. According to the model, occupancy of the eDNA signal for fanworm, was 0.896 (±SE 0.065), indicating the eDNA is present and detectable in around 90% of the sampled locations, regardless of month or depth. The naïve occupancy estimate was only 78.1%, showing potential underestimation of species presence if not accounting for imperfect detection. The modelled probabilities of detection for water samples were twice as high compared to biofilm samples 0.576 (±SE 0.065) and 0.271 (±SE 0.038) respectively. No effect of month, depth or site factors on detection probabilities were identified.

4.3.3 Presence, settlement and growth of fanworm

There was a significant relationship between the abundance of fanworms on the settlement substrate and the explanatory variables (i.e., the substrate materials and the sampling sites), negative binomial glm model, p < 0.05 (Table 4.2). There was higher overall abundance of fanworms reported on suspended material in the marina compared to the mussel farm site (Figure 4.3). More fanworms were on empty grow-out ropes without mussels compared to cement board plates with plastic mesh in the marina (p < 0.001), and more fanworms were on rope with re-seeded adult mussels compared to cement board plates with mesh in the mussel farm (p < 0.001) and more fanworms were on the rope with re-seeded adult mussels compared to rope with pre-seeded small mussels in the mussel farm (p < 0.01).

Table 4.2. Results of negative binomial GLM comparing abundance of fanworm on four types of substrate at two sites, and using empty grow-out rope as intercept for the analysis, with ** indicating p < 0.01 and *** p < 0.001.

<table>
<thead>
<tr>
<th>Effect of substrate material and location on fanworm abundance</th>
<th>Estimate</th>
<th>SE</th>
<th>Pr (&gt;Chi)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept (empty grow-out rope)</td>
<td>2.706</td>
<td>0.44</td>
<td>&lt; 0.001 ***</td>
</tr>
<tr>
<td>Substrate rope with adult mussels</td>
<td>1.014</td>
<td>0.60</td>
<td>0.112</td>
</tr>
<tr>
<td>Substrate cement board plates with mesh</td>
<td>-1.448</td>
<td>0.41</td>
<td>0.002 **</td>
</tr>
<tr>
<td>Substrate rope with small mussels</td>
<td>-0.915</td>
<td>0.65</td>
<td>0.178</td>
</tr>
<tr>
<td>Sampling site marina</td>
<td>2.214</td>
<td>0.47</td>
<td>&lt; 0.001 ***</td>
</tr>
</tbody>
</table>
At the Papakarahi mussel farm none of the smooth PVC plates had fanworm on them and only two of the six cement board plates with plastic mesh had attached fanworm (Figure 4.3). Most fanworms were found amongst the mussel rope with re-seeded adult mussels (41 ± 24 s.d.), on empty grow-out rope (17 ± 6 s.d.) and on the rope with pre-seeded small mussels (6 ± 4 s.d.) (Figure 4.3). However, many adult mussels had detached from the rope, leaving the rope empty for other biofouling species to settle on. A closer examination of the specific point of attachment of the established fanworm identified that the foot of the fanworm tubes was strongly attached either directly to the cement board plate through the plastic mesh or on the grow-out ropes the fanworm foot was directly attached to the broader core rope below the coarse fibre threads.

In contrast, most Mediterranean fanworms in the marina were settling on the empty grow-out rope (129 ± 4 s.d.) or on the cement board plates with the plastic mesh (33 ± 31 s.d.) (Figure 4.3).

The mean body length of fanworms varied among the different substrate materials ($F_{(3,662)} = 29.57$, $p < 0.001$) and for the two study locations (Papakarahi mussel farm and Orakei marina) $F_{(1,662)} = 105.07$, $p < 0.001$ (Figure 4.4). Fanworms on cement board plates with plastic mesh in the marina were significantly smaller than fanworms on empty grow-out rope for the mussel farm site ($p = 0.006$). However, fanworms in the marina on empty grow-out rope (5.7 cm ± 1.6 s.d.) had similar body length compared to fanworms on cement plates with a plastic mesh (5.6 cm ± 2 s.d.) at both locations.
Most fanworms on the different substrates at the mussel farm were also of similar length, except for fanworms on the empty grow-out rope, which reached almost double the length (10.5 cm ± 3.6 s.d.) compared to the fanworms on ropes re-seeded with adult mussels (5.7 cm ± 1.7 s.d.), ropes pre-seeded with small mussels (6.2 cm ± 2.7 s.d.) and the few fanworms on the cement board plates with plastic mesh (5.2 cm ± 0.9 s.d.) (Figure 4.4).

Interestingly, no fanworms were observed on the second sampling set of empty grow-out rope and cement board plates with plastic mesh that were deployed in the marina from 20 February 2020 until 23 June 2020 but their surfaces were overgrown by calcareous sponges.

The calculated monthly mean seawater temperature at Orakei marina and Papakarahi mussel farm varied slightly from each other ranging between 0.3 - 1.3 °C, with Orakei marina being slightly colder than Papakarahi mussel farm in May, June and July, having very similar mean seawater temperatures in August, September and October and Orakei marina being slightly warmer in November and December than Papakarahi mussel farm. The lowest mean seawater temperature was measured in August with 13.2 °C ± 0.5 s.d. at Orakei marina and the highest mean seawater temperature was measured in December with 21.9 °C ± 1.0 s.d at Orakei marina.

Figure 4.4. Mean (± s.d.) fanworm length (cm) on different substrates at Papakarahi mussel farm (n = 159) and Orakei marina (n = 502).
4.4 Discussion

4.4.1 Fanworm detection via droplet digital PCR

Environmental DNA can be present in the environment as extracellular, cellular, vesicular or free form, separate from the organism (Barnes & Turner, 2016). Hence, the source of fanworm eDNA detected in this study could have come from fanworm larvae, shed tissue, mucous, faeces or pseudo faeces (Wood et al., 2017; Zaiko et al., 2018; von Ammon et al., 2019). This could explain why eDNA copy numbers were detected to various degrees in every monthly sample, since fanworms are known to be present at varying densities throughout the Hauraki Gulf, including most mussel farms in the vicinity of the sampling sites (Skelton & Jeffs, 2019). This was also supported by occupancy modelling, which supported the presence of a constant and large scale occupancy (90%) of fanworm from sampling at the three farm sites for over a year. Furthermore, it could be expected that mussel harvesting that occurs throughout much of the year, results in fanworm material being fragmented and released into the environment. In general, the seawater samples detected higher eDNA copy numbers compared to biofilm samples (i.e., means of 24,299 and 3506 copy numbers respectively), although more biofilm samples than seawater samples detected an eDNA signal, (i.e., 65 versus 58 respectively). It is possible that the material containing the extracellular fanworm eDNA is accumulated on the biofilm on the PVC plates or the elevated eDNA signal on these plates could originate from settled fanworm larvae, despite the visible presence on PVC plates not being detected at any time during this study, even when they were left in place for periods of four to eight months, including the Orakei marina, which had high fanworm abundance (Floerl et al., 2012; von Ammon et al., 2019). The abundance and quality of eDNA found in the environment, even in densely populated areas, can highly vary depending on the local biotic and abiotic factors (i.e., temperature, microbial activity, metabolic rate, biomass, excretion, secretion, reproduction, decomposition, salinity, pH and UV radiation) as well as diffusion and resuspension, which can all affect the production and persistence of eDNA in the environment (Barnes et al., 2014; Barnes & Turner, 2016; Bowers et al., 2021). In contrast to eDNA, eRNA is potentially a more reliable indicator of live target organisms, since it degrades within minutes to hours in the environment (Darling et al., 2017; Pochon et al., 2017; Cristescu, 2019; Wood et al., 2019; Wood et al., 2020). Therefore, looking at both eDNA and eRNA together, has the potential to provide a more reliable indication of the presence of live organisms in a particular area and reduces the risk of making false assumptions about the presence or absence of the targeted species (Pochon et al., 2017; Cristescu, 2019; Wood et al., 2020; Bowers et al., 2021). However, in this study, even though samples with the highest eDNA signal were selected for eRNA analysis, only very few of these samples had a positive eRNA signal and there was no clear relationship between the copy numbers of eRNA and eDNA for fanworm (von Ammon et al., 2019). This suggests that most of the detected molecular signal originates from released material, rather than living fanworms, including larvae. However, eRNA signal was observed in December to January across sampling locations, potentially coinciding with a spawning or recruitment event. While it is assumed that the release of extraneous material from fanworm would increase the amount of free-floating molecular material that is detectable via ddPCR (Jute, 2015; von Ammon et al., 2019), it is currently unknown if this is
also the case for eRNA (Barnes & Turner, 2016; Sassoubre et al., 2016; Wood et al., 2018; Murakami et al., 2019; Wood et al., 2020). It is conceivable that “live” branchial crown or body fragments from the fanworm may be present in the environment, possibly as a result of release from mussel harvesting activities (Jute, 2015; Sassoubre et al., 2016; von Ammon et al., 2019). The wide variation in sample detectability and concentration for both eDNA and eRNA could be due to a number of factors. The detectability and concentration of eDNA and eRNA signal can be affected by the target species morphology and life cycle, as well as the state of the environment (i.e., species interactions, microbes, temperature, salinity, UV radiation) (Pilliod et al., 2013; Barnes & Turner, 2016; Sassoubre et al., 2016; Murakami et al., 2019; von Ammon et al., 2019). However, the monthly mean seawater temperature among the mussel farms only varied slightly from each other (0.1 – 0.9 °C), with Esk Point having the warmest mean seawater temperature in January until July, whereas Wilson Bay and Papakarahi mussel farm had slightly colder mean seawater temperatures but all three mussel farm sites overlapped closely in their mean seawater temperature from August 2018 to March 2019 with only slight differences. Although, temperature might be a contributing factor in triggering spawning and eDNA and eRNA degradation, it is likely not the main or only factor as it is well within the temperature tolerance range of S. spallanzanii (Giangrande & Petraroli, 1994; Clapin, 1996; Currie et al., 2000; Giangrande et al., 2000; Fletcher, 2014). Additionally, it is unlikely that all organisms release equal amounts of eDNA at the same rate and time, hence the detected variation in eDNA signal among samples might just be natural variation in the release of the material (Beng & Corlett, 2020). Furthermore, inhibition of detection resulting from interactions with organic material could be a possibility for low ddPCR signal (Schrader et al., 2012; Hunter et al., 2019).

Using eDNA, the presence of fanworm was detected amongst mussel farms in the Hauraki Gulf in every month over a whole year, indicating its presence in the vicinity, which was further confirmed by occupancy modelling. This indicates that species-specific molecular tools can pick up an eDNA and eRNA signal even when there is high turbidity and exposure to various environmental factors, which further dilute and degrade the molecular material. However, there are still limitations with this method, as it does not give detailed indications from what source the material originates from or its abundance. Hence, molecular-based analyses should not be used as the only detection method for marine surveillance purposes (Wood et al., 2018; Rose et al., 2019; Beng & Corlett, 2020). Instead, eDNA methods should be used in addition to traditional visual surveys since they are complementary of each other’s limitations (Rose et al., 2019; Beng & Corlett, 2020). Further investigation is needed into eDNA and eRNA shedding and decay rates for specific invasive species, such as fanworm, and how external factors such as turbidity, temperature, salinity, pH and sun exposure affect decay rates and distribution of detectable target molecules (Takahara et al., 2012; Pilliod et al., 2013; Sassoubre et al., 2016; Wood et al., 2018; Murakami et al., 2019). Prior knowledge of the life history of the target organisms may also assist in determining the timing and location of deployment of molecular detection techniques to improve their reliability in detection (Barnes & Turner, 2016; Franklin et al., 2019; Qu & Stewart, 2019; Takeuchi et al., 2019).
4.4.2 Fanworm recruitment and growth

Fanworm larvae showed a strong preference for settling and establishing on both fibrous grow-out rope without attached mussels and among adult mussels re-seeded onto fibrous grow-out rope. However, fanworms were never observed to be attached to the shell of the mussels. The base of the fanworm tube was always attached directly to the core rope below the fibres of the grow-out rope or occasionally the fanworms were attached to the tube of another fanworm.

On the cement board plates with plastic mesh the fanworms were attached to the plate beneath the plastic mesh. This pattern of fanworm attachment suggests that the mesh on top of the plate and the fibres sticking out from the grow-out rope served as a shelter and protection from competitors and predators, allowing fanworm larvae to become established (Walters & Wethey, 1996). In contrast, fanworm were never observed to establish on smooth PVC plates in this study. High numbers of fanworms established on the grow-out rope with re-seeded adult mussels, many of them on areas of the rope left vacant after patches of adult mussels had been lost from the ropes. It is likely that if fanworms settle on rope with smaller mussels, they directly have to compete with the mussels for food and space. The mussels grow very tightly together on the mussel rope and if the fanworms cannot outgrow the mussels, they would lose access to food and potentially be smothered (Dunham & Marshall, 2012). This suggests that maintaining higher densities and more even distribution of mussels on grow-out ropes is likely to reduce the settlement and establishment of fanworm on mussel grow-out ropes (Ramsay et al., 2008b; Woods et al., 2012). Another option could be to explore alternatives to using fibrous grow-out ropes, which appear to favour the settlement and establishment of fanworm. Another alternative would be to move the mussel grow-out ropes to a more exposed location, where currents could make it more difficult for the fanworm to settle on, since it previously has been found that offshore mussel farm sites had a lower number of biofouling taxa compared to inshore mussel farms (Woods et al., 2012; Atalah et al., 2016b). However, it is currently unknown how well this would work against fanworm, since they are capable of strong attachment that can withstand up to 800g of weight before tearing, hence this strategy might not be very effective against fanworm and could also lead to increased mussel loss (Giangrande et al., 2014b; Atalah et al., 2016b; Atalah et al., 2017).

The results from this current study suggest that more complex substrates provide shelter and structure for the larvae to settle and successfully establish, possibly avoiding potential predators as more vulnerable juveniles (Walters & Wethey, 1996; Nozawa et al., 2011; Nozawa, 2012; Whalan et al., 2015). Rougher surfaces might also reduce the risk of the larvae or settled juveniles being removed by water currents as the post-settled fanworm may be able to form stronger attachment to the more complex surfaces versus smooth surfaces, such as the PVC plates (Walters & Wethey, 1996; Howell & Behrends, 2006; Nozawa et al., 2011; Floerl et al., 2012; Giangrande et al., 2014b; Whalan et al., 2015; Chase et al., 2016; Skelton & Jeffs, 2020). However, the chemical composition of substrate could also contribute to settlement patterns or affect the corresponding composition of biofilm communities that then in turn could attract different secondary macrofouling organisms, such as fanworm (Bavestrello et al., 2000; Chase et al., 2016). The PVC plates are commonly used as a standard method for the passive biofouling surveillance of non-indigenous species in high risk
areas like ports or marinas (Floerl et al., 2012; Tait & Inglis, 2016; Oberbeckmann et al., 2018; Tait et al., 2018; Cai et al., 2019; von Ammon, 2019; von Ammon et al., 2019). Even though, fanworm eDNA and eRNA signal was detected from the biofilm sampled from PVC plates, visible fanworm were never observed on the plates. This indicates that either fanworm material had attached to the biofilm on the plates and or that the fanworm larvae actually settled on the plates but never successfully established (Gosselin & Sewell, 2013; Chase et al., 2016; Tait et al., 2018; Wood et al., 2018; von Ammon et al., 2019).

In addition to surface structure, invertebrate larvae often use chemical cues from bacterial biofilms or adult conspecifics to select sites for settling and metamorphosis (Hadfield & Paul, 2001; Hadfield, 2011; Hadfield et al., 2014). The combination of biofilm on the tube of adult fanworms and the adult fanworms themselves could attract new fanworm recruits (De Gregoris et al., 2012). Further studies are needed to investigate if fanworm larvae directly react to specific bacteria biofilms, adult conspecific cues, substrate morphology or a combination of all three factors.

Analysis of fanworm growth showed differential increase in body size between sampling locations, which can be related to food availability and environmental factors such as weather, temperature, disturbance and competition (Olive, 1995; Alfaro & Jeffs, 2003; Alfaro et al., 2006; Gribben et al., 2011; Gosselin & Sewell, 2013). The fanworms on grow-out ropes without mussels at the mussel farm grew to a larger size compared to the fanworms on cement board plates with plastic mesh at the marina. However, mean monthly seawater temperatures at Orakei marina and the Coromandel mussel farm were very similar, differing only slightly from 0.2 – 1.4 °C from May to December, with Orakei marina being slightly cooler in May to August and slightly warmer in November and December compared to Papakarahi mussel farm. Therefore, temperature might only be one of a number of factors influencing spawning, settlement and growth in this species (Battaglene et al., 2002; Watson et al., 2003; Murray et al., 2011; Nelson et al., 2017). It has been observed that fanworms are in higher density in the Orakei marina compared to the mussel farm area (Jute, 2015; Skelton & Jeffs, 2019). Space is a major limiting resource for sessile marine invertebrates and faster growth in order to outcompete other sessile species could be advantageous (Lohse, 2002; Burgess & Bueno, 2021). The overall larger size of fanworm at the mussel farm compared to the marina could be the results of lower crowding stress and relate to reduced competition for space and food with their neighbouring conspecifics (Svensson & Marshall, 2015). The mussel farm sites are also more exposed to tidal currents, which may also help to supply more phytoplankton; however, this would need further investigation. Additionally, mussel waste released at the mussel farm site may provide an additional food source for fanworms and thus contribute to increased growth of the fanworms (Giangrande et al., 2005; Giangrande et al., 2014c).

From February to the end of June 2020, no fanworms settled or grew on either the cement plates with the plastic mesh or the grow-out ropes without mussels in the marina. It is possible that a period of 4 months was insufficient for the fanworm larvae to settle and establish to reach a size that is visible. This is consistent with previous deployments of materials during this current study, when plates and ropes were retrieved after 4 months, they were typically overgrown with various
biofouling but no fanworms were visible, even though the presence of fanworm was detected with the molecular methods. In contrast, when settlement substrates were deployed for 8 months, considerable numbers of fanworms had established and grown up to ~ 5 cm long on the more complex substrates. This suggests that the initial establishment and growth of fanworm to a visible size (i.e., ~ 2 cm in length) takes at least more than four months within a mussel farm setting. Given that fanworm do not mature until > 6 cm in length, there would be potential to reduce fanworm proliferation on mussel farms through chemical treatment (e.g., freshwater exposure) or stripping, washing and re-seeding mussel lines at intervals of around 4 - 6 months.

4.5 Conclusion

Monthly eDNA signal, occupancy modelling and visual observation of fanworms settling on some of the provided substrate materials confirmed the widespread presence of fanworms in the environment around mussel farms in the Hauraki Gulf. However, it is unknown whether the detected molecular signal originates from extracellular fanworm eDNA molecules (i.e., mucus, branchial crown fragments) that stick to larger biofilm particles on the PVC plates or whether the signal originates from settled fanworm larvae. Since fanworm never visibly grew on the smooth PVC plates, it is likely that even if larvae initially settled on these surfaces, they were unable to establish and grow to a visible size. This raises important consideration for the selection of settlement substrates for monitoring invasive species for biosecurity. On morphologically complex substrates (i.e., fibrous mussel rope, cement board plates with plastic mesh) fanworm could grow to 5 - 6 cm in body length within 8 months, with fanworms reaching slightly larger sizes among mussel farms and wider open areas compared to fanworms in the more sheltered marina. Hence, it is likely that the settlement process of fanworm larvae and subsequent establishment needs at least 6 – 8 months to grow to a size that is visible on monitoring plates, when used for biosecurity detections. By which time fanworms might already be more widely established in the area. Future studies should further investigate what attracts fanworm larvae to specific substrate materials and how eDNA and eRNA shedding and decay rates are affected by external factors such as turbidity, temperature, salinity and bacterial exposure to help develop more effective methods for eDNA/eRNA based surveillance.
Chapter 5 - Regeneration of *Sabella spallanzanii* following fragmentation during green-lipped mussel harvesting

5.1 Introduction

Biofouling significantly reduces the efficiency of marine aquaculture production through competition with farmed species and causes damage to the farm infrastructure (Adams et al., 2011; Fitridge et al., 2012; Bannister et al., 2019). The increasing spread and proliferation of non-indigenous species (NIS) are further exacerbating biofouling in shellfish farms and increasing production costs (Pannell & Coutts, 2007; Adams et al., 2011; Fletcher et al., 2013; Atalah et al., 2016b). Furthermore, it is suspected that cleaning or washing off fragmented pieces of some NIS at sea can facilitate their proliferation in coastal waters by releasing seed fragments of species capable of regeneration or triggering the release of gametes (Floerl et al., 2010; Hopkins et al., 2011; Aldred & Clare, 2014; Johnston et al., 2017). For example, the Mediterranean fanworm, *Sabella spallanzanii*, is a NIS biofouler of shellfish farms in parts of New Zealand, for which laboratory studies have shown it has an ability to regenerate after fragmentation (Licciano et al., 2012; Murray & Keable, 2013; King et al., 2017). Biofouling can reach significant densities on suspended longline farming systems for Greenshell™ mussel in New Zealand, costing the industry up to US $16.4 million per year (Forrest & Atalah, 2017). For example, fanworm is estimated to cost Greenshell™ mussel aquaculture operators over NZD 0.6 million a year in additional production costs (Soliman & Inglis, 2018). Cultured Greenshell™ mussels are typically harvested from suspended culture on coastal farms when they reach about 90 - 120 mm in shell length (Aquaculture New Zealand, 2020; AQNZ, 2020). The suspended longlines are retrieved from the water with a harvesting barge and the mussels are stripped from the lines and put through a de-clumping machine to separate and clean the mussels of the attached biofouling (Stenton-Dozey & Broekhuizen, 2019; Aquaculture New Zealand, 2020). The biofouling can make up over half of the total harvested biomass, although this can vary greatly among sites, depth and the time of initial seeding of the mussel growing line (Atalah et al., 2016b). The wastewater and detached biofouling organisms from harvesting the mussel lines, are almost always discarded overboard (Woods et al., 2012). The fate of this material, especially the fanworms is uncertain, but laboratory studies show that fanworm have a high tolerance to wounding and can regenerate from fragments (Licciano et al., 2012; King et al., 2017; King, 2017), creating the potential for proliferating fanworm in the vicinity. The four studies that have reported that the Mediterranean fanworm can re-grow body segments after fragmentation have all used clean cutting equipment and ideal holding conditions for the resulting fanworm fragments (Read et al., 2011; Licciano et al., 2012; Murray & Keable, 2013; King et al., 2017; King, 2017). However, it is unknown if these fragments could re-grow all the necessary body parts and survive in nature, when exposed to bacteria, water movement, starvation and potential predation, as could be expected to occur in the wild after release from mussel harvesting vessels.
Previous laboratory studies of fragmentation of fanworm have found differences in the ability of different body regions of fanworm to regenerate when fragmented, with abdominal and posterior regions showing greater propensity for regeneration following fragmentation compared to anterior thorax regions (Bely, 2006; Licciano et al., 2012; Murray et al., 2013). Therefore, the aim of this study was to investigate whether 1) there would be a difference in the survival and regeneration between anterior (head fragment without tail) and posterior (tail fragment without branchial crown) fragments of the fanworm, 2) fanworm fragments could survive in the natural environment when exposed to varying natural conditions, bacteria and potential predation, while using laboratory conditions as a control, 3) the size of fanworm fragments determines their subsequent survival, 4) fanworm fragments are predated by fish shortly after being discharged into the sea from a mussel barge during harvesting, when large schools of predatory fish typically gather to feed on the released biofouling. The results of this study will be useful to biosecurity management agencies and the mussel aquaculture industry by helping to determine the potential for the proliferation of fanworms from current harvesting practices.

5.2 Methods

5.2.1 Fragmentation and regeneration in the field

Initially an attempt was made to collect fragmented fanworms directly from a mussel harvesting barge for experimental assessment of subsequent survival and regeneration. A hand-held mesh net was placed under the de-clumping machinery on a mussel harvesting barge, whilst operating and also around the point where biofouling waste was discharged overboard (Figure 5.1b). However, only a small number of fanworms were found underneath the de-clumping machinery, while most fanworms stayed attached within their tubes on the mussel grow-out rope after passing through the device used for stripping the biofouling and cultured mussels from the line. The mussel grow-out rope, with the fanworms remaining attached, was placed into a bulk handling bag on board the harvesting barge for return to shore facilities for cleaning and drying before reuse (Figure 5.1a). A small proportion of fanworms were caught in the machinery or directly discharged back into the sea via the waste chutes (Figure 5.1b). The few fanworms that were found underneath the de-clumping machine had sustained considerable trauma (blunt trauma, several wounds along the body) and were barely moving or classified as dead (no movement, no reaction). Hence, since insufficient live fanworm fragments could be found or recovered from beneath or around the de-clumping machinery for experimentation, intact fanworms were collected directly from the grow-out mussel rope during harvesting, prior to being run through the de-clumping machine.
On 16 December 2020 a total of 140 intact fanworms were collected at random from a mussel grow-out rope, while the rope was hauled onto a mussel harvesting barge. The mussel farm was located at Papakarahi Bay, Coromandel Harbour, New Zealand (36° 48" 21.1" S, 175° 27" 07.1" E). The fanworms were then gently extracted from their protective outer tube and their body length measured (without the branchial crown) from the head to the tip of the posterior end and cut in two fragments (anterior, posterior) with 10 fanworms cut at each of 10 different distances from the head of the fanworm to obtain ten different size categories (i.e., 2, 3, 4, 5, 6, 7, 8, 9, 10, 12 cm). The fragments were based on the size of the anterior fragment, hence the anterior pieces would be standardized into size categories but the remaining posterior fragments have different sizes. The fanworms were cut with blunt and rusty scissors to mimic cuts/fragmentation from the de-clumping machine used during harvesting, which comprises a series of blunt rotating metal blades through which the harvested mussels and biofouling are passed. The different sized anterior fragments from the 10 different individual fanworms were then placed together in a mesh bag (n = 10; 33 x 52 cm with 4 mm diameter mesh) and all the posterior fragments from the same 10 fanworms were placed in a second mesh bag (n = 10). This procedure was replicated eight times to obtain a total...
of 80 anterior and 80 posterior fragments (Total N = 160) that were held in a total of 16 mesh bags. Additionally, 60 control fanworms, were extracted from their tubes but not cut and were separately placed in mesh bags with 10 fanworms per bag (N = 60). One bag of each treatment (anterior, posterior and control) were then attached with a cable tie at random positions along a 3 m rope that was weighed down with a 1.5 kg lead weight. The eight ropes with three bags each were suspended at random intervals along a mussel farm backbone line and left in position for 28 days. After 28 days, the mesh bags were retrieved and placed in seawater in an insulated bin and transported to the laboratory for detailed measurement and microscope analysis. Each fanworm fragment was examined and categorized for wound closure, regeneration and survival (i.e., yes or no for each attribute), and the body length of the living fragments were measured. A fanworm fragment was determined to have wound closure, if the cut end of the fragment was covered by a new thin layer of epithelium and was not releasing blood cells when gently prodded (Licciano et al., 2012). Regeneration of fanworm fragment was considered to have occurred with either the formation of a new branchial crown or posterior chaetiger segments (Licciano et al., 2012). The survival of the fanworm fragments was assessed by gently poking the fanworm fragment with tweezer tips, if there was a reaction, such as movement, retraction or coiling, the fragment was classified as alive, when there was no reaction, movement or retraction and the fragment started to disintegrate, the fanworm fragment was classified as dead (King, 2017).

5.2.2 Fragmentation and regeneration in the laboratory

Due to biosecurity and logistical restrictions, it was not possible to transfer live fanworms from the Papakarahi Bay mussel farm to the laboratory for a parallel assessment of fragmented fanworm held under ideal laboratory conditions. Consequently, on 17 December 2020 a total of 90 adult fanworm were collected at random from piers at 0.5 m depth at Orakei marina, Auckland, New Zealand (36° 50" 59.8" S, 174° 48" 34.4" E) and transported in seawater to the University of Auckland. The different sized anterior pieces from 10 different individual fanworms were then placed together in a 5.5 L plastic tank (n = 10; 17 x 17 x 19 cm) and all the posterior pieces from the same fanworm individuals were placed in a second plastic tank (n = 10). This procedure was replicated six times to obtain a total of 60 anterior and 60 posterior fragments (Total N = 120). In addition, to mimicking the fragmentation process by using blunt, rusty scissors, a set of fanworms were cut with surgical blades (Swann-Morton, Sheffield, UK) to determine whether the method of cutting affected the regeneration capabilities or survival of the resulting fragments. Except for the surgical cut posterior fragments (n = 28), there were a total of 30 fragmented fanworms in each position, i.e., blunt cut anterior (n = 30), blunt cut posterior (n = 30), surgical cut anterior (n = 30), and intact fanworms as control (n = 30). Five intact control fanworm were kept together in a 5.5 L tank with seawater and aeration at room temperature at 23 °C in a 12: 12 light: dark regime. The seawater was 5 µm filtered and UV treated. Every three days the seawater in the tanks was manually exchanged and all the fanworm fragments were fed with a mixed diet (1:1:1:1) of axenic microalgae (Tisochrysis lutea, Diacronema lutheri, Tetraselmis suecica and Chaetoceros muelleri).
At 3, 6, 8, 11, 14, 17, 19, 22, 25 and 28 days each fanworm fragment was examined under a microscope and categorized as previously described for the field experiment.

5.2.3 Assessment of natural predation

To determine whether fanworms are predated by fish when discharged among biofouling from a mussel harvesting vessel, various sized fanworms (N = 10) were directly collected from a mussel farm grow-out rope during harvesting operations on a mussel barge (13 January 2021; Moturua Island (Rabbit Island), Coromandel, New Zealand (36° 41" 55.0" S, 175° 23" 55.0" E). Fanworms were then gently extracted from their protective tubes and prepared in different arrangements for threading on a metal fishhook (Burnsco, beak size: 3/ 0), which was attached to a monofilament fishing line (25 kg breaking strain). Each baited hook was deployed into the plume of the biofouling material discharged from a mussel harvesting barge whilst in operation. Ten different sized fanworm pieces, five with their branchial crown removed and five with their branchial crown intact, were threaded on the hook and deployed one at a time for up to 5 min with a stopwatch being used to determine the time to fish strike (N = 10). As a control, a strip of squid mantle (n = 3) or a whole mussel bait (n = 2) was threaded on the hook and deployed for up to 5 min with a stopwatch being used to determine the time to fish strike (n = 5). Upon fish being caught on the hook, the fish were immediately recovered to the mussel barge by retrieving the monofilament line and the fish immediately identified and euthanized. The research was conducted according to animal ethics approved (Ref. No. 002254) under New Zealand’s Animal Welfare Act 1999.

5.2.4 Statistical analyses

5.2.4.1 Mussel farm experiment

Statistical analyses were performed to determine whether cutting positions (i.e., anterior, posterior and control) or fragment size had an effect on the fragments regeneration or survival capabilities. To determine whether fanworm regeneration and survival were affected by the fragmentation position (i.e., anterior, posterior and control) Chi square tests with Yates continuity correction and post-hoc Hochberg pairwise comparison were performed (Benjamini & Hochberg, 1995). To make further comparisons among fragmentation position (i.e., anterior and posterior) and their capability for regeneration in relation to fragment length (i.e., 2, 3, 4, 5, 6, 7, 8, 9, 10, 12 cm body length from the anterior end of the fanworm), a binomial generalized linear model and Fisher’s exact test were applied (glm, R Core Team, 2019, version 3.6.0).
5.2.4.2 Laboratory experiment

A poisson generalized linear model followed by Tukey’s comparisons (glm, R Core Team, 2019, version 3.6.0) was used to identify differences among the number of alive fanworm fragments for the different fragmentation treatments (i.e., blunt anterior, blunt posterior, surgical anterior, surgical posterior and control) over the course of this experiment (i.e., days 3, 6, 8, 11, 14, 17, 19, 22, 25 and 28). Cutting method (blunt versus surgical) was added as a covariate factor but since there was no significant effect or interaction, the factor was removed from the model to improve model fit. A quasibinomial generalized linear model followed by Tukey’s pairwise comparisons was used to identify differences in regeneration capabilities among the different fragmentation treatments (i.e., blunt anterior, blunt posterior, surgical anterior, surgical posterior and control) over the course of this experiment (i.e., days 3, 6, 8, 11, 14) using the proportion of fragments that have regenerated on a given day in relation to the number of fragments that were still alive.

To make further comparisons among fragmentation position (i.e., blunt posterior, surgical anterior, surgical posterior) and their capability for survival in relation to the different fragment length categories (i.e., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 cm body length from the anterior end of the fanworm), a Fisher’s exact test was applied (R Core Team, 2019, version 3.6.0). The one remaining bluntly cut anterior fragment that survived was excluded from the analysis to reduce analytical data imbalance.

A quasibinomial generalized linear model (glm, R Core Team, 2019, version 3.6.0) with Tukey’s post-hoc comparisons was used to compare the proportion of alive fanworm fragments among fragmentation treatments (i.e., blunt anterior, blunt posterior and control) between the laboratory and the mussel farm at the conclusion of both experiments at 28 days and included the interactive effects. A quasibinomial model was used due to under dispersion and because of the proportional nature of the survival data.

5.3 Results

5.3.1 Fragmentation and regeneration on a mussel farm

The fanworm fragments did not grow during the course of this experiment, hence the fragment length measured at the end of the experiment is the same as at the beginning of the experiment when fanworms were cut into two fragments.

Upon retrieving the fanworm fragments in the mesh bag after 28 days in the field, it was noticed that a number of them had regrown a new, thin tube but they had not regenerated the bluntly cut anterior or posterior structure and these structures had less advanced regeneration compared to the laboratory fanworm fragments after 28 days (Figure 5.3).

After 28 days in the field, 20 out of a total of 80 anterior fragments, regardless of length, were alive (25.0%) and 41 out of 80 posterior fragments were alive (51.3%), and of the 60 whole fanworms (control), 49 were alive (81.6%). The type of fanworm fragment position (anterior or posterior) significantly affected its survival after 28 days in the field ($\chi^2(2) = 44.12, p < 0.001$), where survival
was higher for the whole uncut fanworm (control) versus both the anterior and posterior fragments, and survival was higher for posterior versus anterior fragments (p < 0.001). Of the surviving fragments after 28 days, there was no difference in wound closure between anterior (22.5%) and posterior fragments (35.0%) ($X^2(1) = 2.47$, $p = 0.11$), or in the regeneration of fanworm between anterior (21.3%) and posterior (27.5%) fragments ($X^2(1) = 3.09$, $p = 0.078$). The length of the fanworm fragment had no effect on the subsequent regeneration of either anterior or posterior fragments among the fanworm fragments that survived to 28 days (Figure 5.2). Furthermore, there was no difference in the survival or regeneration of anterior or posterior fragments among the different size categories after 28 days (Figure 5.2).

![Figure 5.2](image_url)

Figure 5.2. A) The number of anterior fanworm fragments in the different size categories (2, 3, 4, 5, 6, 7, 8, 9, 10, 12 cm) and their subsequent survival and regeneration after 28 days in the field. B) the number of posterior fanworm fragments in the different size categories (2, 3, 4, 5, 6, 7, 8, 9, 10, 12 cm) and their subsequent survival and regeneration after 28 days in the field.

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5.3.2 Fragmentation and regeneration in the laboratory

The fanworm fragments did not grow in size during the course of this laboratory experiment, hence the fragment size measured at the end of the experiment is the same as at the beginning of the experiment when fanworms were bisected. However, the regeneration of new tail segments and formation of new branchial crown structures were observed during the course of this experiment (Figure 5.8).

There was a marked difference in the survival of fanworm fragments among the four fragmentation treatments (i.e., blunt anterior, blunt posterior, surgical anterior, surgical posterior and control) and the number of live fragments over the course of the experiment as well as an interactive effect between treatment group and duration of the experiment (day) (glm, p < 0.001; Figure 5.4; 5.5). The survival of bluntly cut anterior fragments varied over the course of the experiment but regardless of the day of inspection, their survival was lower compared to bluntly cut posterior, surgically cut anterior and surgically cut posterior fragments at day 28 (glm, Tukey’s pairwise comparisons p < 0.05). From sampling during the course of the experiment, bluntly cut posterior fragments had higher survival compared to bluntly cut anterior, surgically cut anterior and whole control fanworms (p < 0.01) but none of these differed significantly from surgically cut posterior fragments. The anterior fanworm fragments in the laboratory sometimes could not stem the bleeding from the posterior wound or close the wound within the first few days, resulting in their subsequent death.

The lowest survival rate was for the bluntly cut anterior and the surgically cut anterior treatments, which were 43% and 57% respectively on day 14 (Figure 5.4; 5.5a; c). Surgically cut anterior fragments expired up until day 14 when mortalities stopped, with 57% of fragments remaining alive at day 28 (Figure 5.5c). The bluntly cut anterior fragments continued to gradually expire throughout the experiment with only 3% remaining alive at 28 days.

In contrast to the anterior fragments, the posterior fragments, both blunt and surgically cut, had a much lower mortality (Figure 5.5b; d). All of the bluntly cut posterior fragments stayed alive until day 19, with survival declining to 97% at day 28. Of the 93% surgically cut posterior fragments that
were alive on day 3, all these fragments stayed alive until day 28 (Figure 5.4; 5.5d). Fisher’s exact test showed that amongst the fragments that survived, their survival was not affected by the fragment size (Fisher’s exact test, $p = 0.22$).

Figure 5.4. The total number of alive fanworm fragments when assessed every 3 days over a 28 day period in the laboratory for four fragmentation treatments i.e., blunt bisection of fanworm anterior ($n = 30$), blunt bisection of fanworm posterior ($n = 30$), surgical bisection of fanworm anterior ($n = 30$), surgical bisection of fanworm posterior ($n = 28$), whole fanworms as control ($n = 30$).

During the first 3 days after fragmentation of fanworm in the laboratory, wound closure was observed both in the anterior and posterior fragments, both in surgically and bluntly cut fragments, whereby the fanworms formed a thin new layer of epithelial cells over the wound. An exception was one fanworm fragment in the surgical posterior group that took 6 days to complete wound closure. Those fragments that died, did so within the first 14 days, whilst fragments that survived beyond this time, began regenerating posterior or anterior structures within that timeframe.

There was a difference in the proportion of fanworm fragments that were able to regenerate, either a new branchial crown or tail segment, among the four fragmentation treatments (i.e., blunt anterior, blunt posterior, surgical anterior and surgical posterior) and between the different days of observation (glm, $p < 0.001$; Figure 5.5; 5.6). Tukey’s pairwise comparison showed that up until day 14 the bluntly cut anterior pieces regenerated more slowly compared to the bluntly cut posterior, surgical anterior and surgical posterior fragments ($p < 0.05$). The fastest to regenerate were the bluntly cut posterior fragments, reaching 100% regeneration of all the surviving fragments by day 11, closely followed by surgically cut anterior and surgically cut posterior fragments that reached 95% and 96% regeneration respectively on day 11 and 100% on
day 14 (Figure 5.5). The slowest to regenerate were the bluntly cut anterior fragments, reaching
100% regeneration of the surviving 43% fanworm fragments on day 14 (Figure 5.5a; 5.6).
All 43% of the surviving bluntly cut anterior fragments on day 14 showed wound closure and had
started regenerating tail segments. However, most of these remaining anterior fragments died
within the next 14 days, even though they had started the wound closure and regeneration
process, until only 3% of the anterior fragment remained alive on day 28 (Figure 5.6).
Figure 5.5. A) Blunt bisected anterior fragments (n = 30) regeneration and simultaneous survival every 3 days over the first 14 day period in the laboratory. B) Blunt bisected posterior fragments (n = 30) regeneration and simultaneous survival every 3 days over the first 14 day period in the laboratory. C) Surgical bisected anterior fragments (n = 30) regeneration and simultaneous survival every 3 days over the first 14 day period in the laboratory. D) Surgical bisected posterior fragments (n = 28) regeneration and simultaneous survival every 3 days over the first 14 day period in the laboratory.

Figure 5.6. The percentage of regeneration in fanworm fragments in the laboratory as assessed at days 3, 6, 8, 11 and 14 for the four experimental fragmentation treatments at day 1, i.e., blunt bisection of fanworm anterior (n = 30), blunt bisection of fanworm posterior (n = 30), surgical bisection of fanworm anterior (n = 30), surgical bisection of fanworm posterior (n = 28).

5.3.3 Field versus laboratory experiment

Upon comparing the proportions of fanworm fragments that survived in the laboratory against the proportions of fanworm fragments that survived in the field experiments at 28 days, it was found
there was an interactive effect between fragmentation position (i.e., anterior, posterior and control) and the laboratory versus field conditions (glm, p < 0.01).

In the field experiment, the control fanworms had the highest survival rates (80%), followed by the bluntly cut posterior (51%) and lastly the bluntly cut anterior fragments (25%), whereas in the laboratory the bluntly cut posterior fragments had the highest survival rates (97%) followed by the control fanworms (77%) and barely any anterior fragments survived (3%; Figure 5.7). In the field experiment, significantly more of the intact control fanworms survived compared to the anterior cut fragments (p < 0.05), whereas in the laboratory experiment significantly more posterior fragments and intact control fanworms survived compared to the anterior fragments (p < 0.05).

Bluntly cut posterior fragments in the laboratory experiment had significantly higher survival (97%) compared to the bluntly cut posterior fragments in the field experiment (51%; p < 0.05). The intact control fanworms had similar survival rates between the field (80%) and the laboratory (77%; p = 0.19) and even though only one bluntly cut anterior fragment survived in the laboratory (3%), the bluntly cut anterior fragments also had the smallest number of surviving fragments in the field (25%) (p = 0.17; Figure 5.7).

Figure 5.7. The proportion of alive fanworm fragments from three fragmentation treatments (i.e., blunt anterior, blunt posterior and control) for the laboratory and field experiments after 28 days, with ** indicating p < 0.01 and * p < 0.05.
Figure 5.8. A-F. Regeneration process of the bluntly cut anterior and posterior fragments in *Sabella spallanzanii* held in the laboratory. A) Bluntly cut anterior fragment on day 1 forming blastema over wound, scale bar = 10 mm. B) Bluntly cut anterior fragment on day 19, having formed a new tail segment visible in darker coloration compared to the rest of the fragment, scale bar: 10 mm. C) Posterior fragment on day 6 forming bilobed new branchial crown, scale bar = 1 mm. D) Posterior fragment on day 8, the length of the branchial crown has increased and the radioles are visible, scale bar = 1 mm. E) Posterior fragment on day 11 with new branchial crown, now pigmented, scale bar: 10 mm. F) Fully formed new pigmented branchial crown on day 28, scale bar = 10 mm.
5.3.4 Natural predation

From the different sized fanworm fragments deployed on hooks with the branchial crown remaining intact (n = 5) and without a branchial crown (n = 5) none were eaten by fish after each being presented for 5 minutes in the water (Total = 50 min). However, it was confirmed that predatory fish were present and consuming material from the discharged biofouling plume, as five fish (three snapper, Pagrus auratus, two trevally, Pseudocaranx dentex) were readily captured with squid (n = 3) or mussel flesh bait (n = 2) deployed on the hooks at Moturua Island (Rabbit Island), Coromandel, New Zealand.

5.4 Discussion

There are biosecurity concerns that the handling or removal of biofouling in aquaculture may lead to proliferation of biofouling species through regeneration from fragmentation and the release of propagules (Forrest & Blakemore, 2006; James & Shears, 2016; King, 2017). In this study, it was observed that when Greenshell™ mussels were harvested, many of the fouling fanworms remained attached to the mussel grow-out rope, even after being run through the de-clumping machine (Figure 5.1a). The processed grow-out rope with attached fanworms was placed in bulk handling bags for return to shore facilities for cleaning prior to reuse, precluding their release back into the sea. Close examination of the recovered fanworms found that their posterior end of their tubes were firmly attached to the core rope beneath the mussel fibres of the grow-out rope. Therefore, only a small proportion of fanworms were removed from the grow-out rope or evacuated their tube during the de-clumping processing. Those whole fanworms and fanworm fragments released in the de-clumping machinery that were recovered had all sustained severe trauma (i.e., several injuries along the body, ripping, tearing, cuts), probably as a result of high pressure water jets and rotating blades. Once discharged into the sea, they were mostly dead or unlikely to survive, (i.e., not reacting to touch and did not show any movement) or were inviable fragments, especially detached branchial crowns. Rupturing of the coelomic cavity of the fanworm would release any contained gametes, however, it is known from previous studies that unfertilized fanworm eggs that come into contact with seawater become inviable, while in contrast sperm starts moving rapidly (Giangrande et al., 2000; Stabili et al., 2009; Chapter 3; Appendix 1).

Upon retrieval of the fanworm fragments in the field experiment, it was observed that some of them had started to rebuild a new paper thin outer tube, whereas this was not observed in the laboratory experiment and may have been due to the greater availability of suspended detritus for tube construction in the field (Dales, 1961; Stabili et al., 2010; Licciano et al., 2012). However, some of the fanworms within these newly built tubes did not have newly regenerated structures or their regeneration was far less progressed compared to those in the laboratory experiment (Figure 5.3). The tube is likely to be important for the survival of fanworms in the wild, since it provides protection from potential predators and environmental exposure and keeps the fanworm in an upright position that facilitates filter-feeding activity (Giangrande et al., 2014b; Giangrande et al., 2014c). It could be that rebuilding a protective and feeding facilitating structure might be more
important to the survival of fanworms in the field situation, compared to regenerating a new branchial crown or closing and re-building the posterior structure. Furthermore, the slower regeneration rate of exposed fanworm fragments in the field situation compared to those in the laboratory indicates that exposure to environmental factors (i.e., currents, temperature, food sources, storms and bacteria) affects both the survival of fanworm fragments and the pace at which they can regenerate and re-grow body segments (Henry & Hart, 2005; Lindsay, 2010). Regardless, the results of this study indicate that fanworm fragments that are washed back into the sea, do have the potential to survive. However, proving whether they can survive when released at sea from a mussel harvesting vessel would require further and more challenging research to trace their exact fate in the natural environment.

The ability to regenerate lost body fragments is widespread among annelids, where most species can regenerate the posterior end of the body but not many can regenerate the anterior end (Bely, 2006).

In this current study, anterior fanworm fragments had lower survival compared to posterior fragments or control intact fanworms, which is in agreement with previous studies on S. spallanzanii but has also been found in the fanworm Sabella pavonina and Sabellastarte sp. (Henry & Hart, 2005; Licciano et al., 2012; Murray et al., 2013; King, 2017; Planques et al., 2019). Increased mortality in anterior fragments of S. pavonina and Sabellastarte sp. have previously been linked with loss or degradation of the branchial crown and thorax segments, which is an indicator of acute exposure to environmental stressors (Fitzsimons, 1965; Murray et al., 2013). A previous study investigating regeneration after fragmentation in S. spallanzanii and Branchiomma luctuosum found there was no difference in survival among anterior and posterior fragments in S. spallanzanii, however, most anterior and posterior fragments of B. luctuosum died soon after wound healing and regeneration (Licciano et al., 2012). Contrary to that, King (2017) found lower survival in anterior fragments in uncut control fanworms compared to posterior fragments. In the current study, several of the uncut control fanworms also died during the field and laboratory experiment. This could be due to increased stress levels from handling (i.e., some of the control fanworms also lost their branchial crowns) or due to the loss of their tubes, impairing their filter feeding capacity, which resulted in higher mortality. Another explanation might be that in this current study, too many fanworms were held together and it was too crowded (i.e., ten control fanworms were held together in the mesh bags in the field and five control fanworms in the tanks in the laboratory), whereas, in the study by Licciano et al. (2012), where 100% of intact control fanworms survived, the fanworms were held individually in 1 L tanks. Since for this current experiment, the fanworms were extracted out of their tubes, they were floating on the bottom of the tanks in the laboratory or pushed together in one corner of the mesh bag in the field. The uncut control fanworms were larger in body length compared to the cut fragments and were often observed laying on top of one another or their branchial crowns being intertwined with each other, without any opportunity to retreat or avoid contact. This might have functioned as an additional stress factor and could have resulted in increased mortality in the uncut control fanworms.
In the current study, it was observed that the anterior fragments in the laboratory deteriorated in two ways: 1) the branchial crown was lost and sometimes but not always re-grown, or 2) the thoracic collar segments were gradually degenerating (i.e., body tissue turning green and black, without movement of the chaetae), which in both scenarios ultimately resulted in the death of the fanworm after a few days (Murray et al., 2013; pers. observation). It could be that the individual fanworm that shed the branchial crown or showed thoracic tissue degradation had fewer initial energetic reserves with which to utilise for wound closure or regeneration (Murray et al., 2013). Although, the radiolar or branchial crown is the primary source for respiration and feeding in Sabellidae, they can also use auxiliary respiration by generating water currents through the tube; hence losing the branchial crown does not mean immediate mortality, since many posterior and also anterior fragments were able to re-grow a new branchial crown (Rouse & Pleijel, 2001). In S. pavonina higher mortality in anterior fragments compared to posterior fragments was observed as well as higher concentrations of ammonia in the water with holding anterior fragments and intact control worms (Murray et al., 2013). Higher mortality in the anterior fragments and control worms compared to the posterior fragments was associated with the quicker deterioration of the tank water conditions due to presence and filtering of the branchial crowns (i.e., highest levels of complete ammonia and the lowest concentrations of dissolved oxygen). However, further research would be needed to determine if water quality actually deteriorates among the different fragment groups and if anterior and posterior fragments vary physiologically and therefore have different internal energy storages and resources that would affect regeneration and survival.

Fragment size did not have an effect on fanworm survival or regeneration in either the field or the laboratory experiments, with posterior fragments that were less than < 1 cm in length commencing the formation of a new branchial crown. Hence, larger fanworm fragment size does not necessarily mean that it retains more resources or is more capable of regeneration or survival. The ability of S. spallanzani to regenerate new body segments, even though, they are small, is in contrast to the regeneration abilities of many corals and sponges, where size directly affects regeneration capacity, and where smaller individuals have less healthy tissue or resources available for regeneration (Henry & Hart, 2005). Furthermore, cutting method (surgical versus bluntly cut using rusty scissors) did not affect survival in posterior fragments. However, bluntly cut anterior fragments deteriorated and died throughout the course of the laboratory experiment, whereas surgically cut anterior fragments died only within the first 14 days of the experiment. It is likely that the blunt trauma generated through bisection with the rusty scissors generated more tissue damage and a greater area for subsequent microbial infection, whereas the surgical blade used was sterile and gave a clean cut. Hence, the wound location and severity appears to affect the subsequent chances of survival of fanworm (Kramarsky-Winter & Loya, 2000). Some of the fanworms that were observed to be discharged from the de-clumping machinery on the mussel barge had sustained several wounds in contrast to just one clear or blunt cut, and were heavily bleeding and showed no movement, indicating death. Hence, it appears likely that fanworms sustaining several injuries during mussel harvesting will have greatly reduced potential for survival.
and regeneration once released back into the sea, in contrast to the single experimental transverse bisection used in this study.

Although, predatory fish were present and readily consuming material from the discharged biofouling plume of the mussel harvesting vessel as indicated by their rapid capture with squid or mussel flesh bait, no fish consumed the fanworm fragments when they were released from the mussel harvesting vessel. When the fanworm fragments are discharged from the de-clumping machine, they are most likely to sink to the seafloor, where they could be consumed by benthic organisms (Gambi et al., 2000; Fattorini et al., 2004; Fattorini & Regoli, 2004; Giangrande et al., 2014b). The branchial crown of fanworms contains dimethylarsinic acid (DMA), which is slightly toxic and could deter some predators such as fish (Fattorini et al., 2004; Fattorini & Regoli, 2004; Notti et al., 2007; Ricevuto et al., 2016). Likewise, the fanworms mucous is known to have cytotoxicity and lysozyme-like activity, which agglutinates bacteria, pathogens and other biofouling, potentially making the fanworm less palatable to predators (Canicatti et al., 1992; Stabili et al., 2009; Stabili et al., 2011; Giangrande et al., 2014b; Cammarata et al., 2019).

Suspended aquaculture structures may provide optimal conditions and surfaces with good light conditions, sufficient currents, plankton and reduced sediment accumulation for many invasive species (James & Shears, 2016; Atalah et al., 2020). Often the coverage of invasive species is higher on mussel farms compared to adjacent coastal habitats. Although, this current study showed that S. spallanzanii proliferation is not necessarily facilitated by mussel harvesting, it is unknown what happens to other biofouling species (i.e., tunicates, seaweed) on the grow-out mussel lines when the mussels are harvested and whether or not these species are also able to regenerate and further proliferate or mostly get eaten by fish or other benthic predators (Henry & Hart, 2005; Lindsay, 2010; Morris & Carman, 2012; Fletcher et al., 2013). Although, long-line mussel harvesting might not significantly add to the regular proliferation of invasive species, regular maintenance and cleaning of farm infrastructure should be performed to limit the secondary dispersal of invasive species that like to grow on aquaculture infrastructure. For example, models indicate that reducing the density of the invasive species S. clava and S. spallanzanii on mussel longlines in New Zealand’s coastal waters (i.e., 500 to 100 individuals m⁻¹ of longline) could reduce economic impacts by 60% and 44% respectively (Soliman & Inglis, 2018).

5.5 Conclusion

This study confirmed, that even though some fanworms are capable of regenerating after fragmentation both in the laboratory and in the field, the regeneration capability is affected by wound position (i.e., anterior or posterior), the nature of the wound (i.e., clean cut or blunt cut) and subsequent exposure to environmental conditions. A large proportion of the fanworms that were attached to the mussel grow-out rope remained attached during harvesting operations, even after being run through the de-clumping machinery. The small proportion of fanworms that were
released into the sea, were observed to be mostly damaged or dead. Based on these observations and the results from the field experiment, there are relatively low chances of survival for those fanworm fragments released alive from the harvesting vessel. However, the subsequent fate of these fanworm fragments requires further investigation. Currently, the best course of action for the mussel farmers to further reduce spread and proliferation of marine invasive species is to regularly clean their equipment, including potentially mussel grow out ropes, especially when moving between locations.
6.1 Research aims

Some marine non-indigenous species (NIS) become established in new territory and can go largely unnoticed, while the proliferation of other NIS can become a serious threat to local biodiversity by outcompeting or completely displacing native species and becoming a pest by overgrowing harbour and aquaculture structures (Bax et al., 2003; Floerl & Inglis, 2005; Dodgshun et al., 2007; Molnar et al., 2008; Clarke Murray et al., 2011; Bates et al., 2013; Campbell et al., 2017). Once, marine NIS have established, it is extremely difficult and costly, if not impossible, to eliminate them, hence, early and accurate detection is probably the best policy to keep NIS from establishing (Hayes et al., 2005; Ardura, 2019). The Mediterranean fanworm, *Sabella spallanzanii*, was accidentally introduced to New Zealand in 2008 and has subsequently become a nuisance biofouler on wharves, marinas and aquaculture infrastructure in some parts of the country (Read et al., 2011; Murray & Keable, 2013; Fletcher, 2014; Jute, 2015; Ahyong et al., 2017; Campbell et al., 2017; Skelton & Jeffs, 2019). Over the last decade, the fanworm has become increasingly abundant on longline mussel farms in the Hauraki Gulf, increasing the costs of mussel production and creating biosecurity concerns. Therefore, the overall aim of this thesis was to investigate the reproduction, gametogenesis, presence, settlement, growth and regeneration after fragmentation of the Mediterranean fanworm specifically in relation to mussel farming activity. The comparison of these results to existing information on fanworm from previous research done in the Mediterranean and Australia provides insights into how the species has adjusted its biology to the conditions in New Zealand. It was anticipated that by looking specifically at reproduction and spawning, specific periods or seasons could be identified where fanworm are more prevalent and a shift in aquaculture and mussel seeding practices might reduce biofouling and associated costs for aquaculture operators. The efficacy of detecting fanworm via eDNA and eRNA as a potential tool in fanworm biosecurity detection or monitoring, was assessed in infested mussel farming area in the Hauraki Gulf, where the prevalence of fanworm was increasing at the time of the study. Lastly, aquaculture harvesting operations were observed to see if they could potentially contribute to the proliferation of fanworm by fragmenting fanworms harvested with mussels and then releasing the fragments back to the marine environment where they could regenerate if not consumed immediately by predators.

6.2 *Sabella spallanzanii* – tolerant invasive species and biofouler

Biofouling on aquaculture infrastructure leads to increased maintenance, production and processing costs, as well as product loss (Dodgshun et al., 2007; Adams et al., 2011; Fitridge et al., 2012; Forrest & Fletcher, 2015; Atalah et al., 2016b; Atalah et al., 2017; Campbell et al., 2017). A common mitigation strategy that is employed to reduce the impact of biofouling in aquaculture is to adjust the timing of deployment of aquaculture infrastructure to avoid the peak reproductive season of nuisance biofouling species (Fitridge et al., 2012; Sievers et al., 2014; Forrest &
Research for Chapter Three of this thesis investigated whether *S. spallanzanii* have a distinct seasonal spawning or reproductive period that could be used as the basis for a mitigation strategy for aquaculture. Within the native range of the Mediterranean fanworm in coastal Italy in the Mediterranean Sea, the species is known to have specific spawning months (January - February at 11-14 °C) (Giangrande et al., 2000). Whereas in Port Phillip Bay, Australia spawning in fanworm was firstly reported during August under similar environmental conditions (Currie et al., 2000). However, a subsequent study found that spawning appeared to occur continually throughout the year (Lee et al., 2018). The results of this current study indicate that, in contrast to the Mediterranean Sea, fanworm are likely to spawn all year round in the Hauraki Gulf, since eggs of various sizes were continuously present within individual fanworms throughout the year. Hence, a biofouling mitigation strategy for the aquaculture industry based on avoiding seasonal periods of peak settlement for this species is unlikely to be an effective option.

Both monthly dissection of fanworms and environmental DNA and RNA samples could not identify particular months, where there were more or less fanworm larvae present in the water, indicating that moving mussel grow-out lines to other locations or changing the time of seeding the mussels onto grow-out ropes would not reduce biofouling by fanworm. Additionally, an examination of the breeding biology of the fanworm showed a skewed sex ratio with more females to males, which would serve to further enhance the species’ ability to disperse and establish.

Successful invasive species are considered to be physiologically or genetically pre-adapted to adjust quickly to new conditions and habitats (i.e., differing water temperature, salinity, oxygen levels and anthropogenic disturbance), which is an important factor in determining their invasion success (Piola & Johnston, 2008; Prentis et al., 2008; Hänfling et al., 2011; Lenz et al., 2011; Clarke Murray et al., 2012; Jofré Madariaga et al., 2014; Tepolt & Somero, 2014). The invasion and expansion risk of non-indigenous species is highest in those areas where environmental conditions are well within the existing tolerance range of these species, prior to any acclimation or adaptation. NIS can further successfully spread and establish if they have high reproductive flexibility, high fecundity and are no longer controlled by predators in their new environment (Piola & Johnston, 2008; Lenz et al., 2011; Bates et al., 2013; Jofré Madariaga et al., 2014; Kelley, 2014; Tepolt & Somero, 2014; Wang & Feng, 2016; Geburzi & McCarthy, 2018). The Mediterranean fanworm has a biological profile which makes it well suited to invade new habitats by having wide environmental tolerances, including temperature (surviving in temperature ranges between 4 – 29 °C; Giangrande & Petraroli 1994; Clapin, 1996), and salinity (between 26 – 39 PSU) (Giangrande & Petraroli, 1994; Currie et al., 2000). *Sabella spallanzanii* can filter feed on a broad range of phytoplankton and bacteria ranging from 5 - 11 µm (Clapin, 1996), and can occupy a wide range of habitats occurring in depths between 0.5 – 30 m (Fletcher, 2014) and more recently they have also been observed in intertidal zones in a number of locations in New Zealand (Fletcher, 2014; pers. comm. K. James, University of Auckland). Furthermore, the Mediterranean fanworm has multiple biological defences including, a leathery protective outer tube, a branchial crown containing toxic dimethylarsinic acid (Fattorini et al., 2004; Fattorini & Regoli, 2004; Notti et al., 2007), as well as the ability to produce conspicuous amounts of mucus with lysozyme-like activity as an antibacterial substance (Stabili et
Additionally, the Mediterranean fanworm is able to regrow both anterior and posterior segments after multiple fragmentation and regrow its outer tube in natural environment conditions (Licciano et al., 2012). All these traits give the fanworm advanced protection from predators and competitors and the capacity to recover rapidly from disturbance events. Since they do not appear to have a natural predator in their newly invaded territories, their populations can expand without predatory limitations that would be experienced by other species. The reproductive flexibility of the *S. spallanzanii* appears to allow them to mature and spawn on a continuous basis in both its invaded territories within Australia and New Zealand. All of these biological traits, such as a generalist filter feeding diet, habitat generalist, greater dispersal potential due to high fecundity and reproductive output, give this NIS species an advantage when introduced to a new habitat (Kinlan & Gaines, 2003; Lester et al., 2007; Lenz et al., 2011; Bates et al., 2013; Wang & Feng, 2016). This would also help to explain why attempts to eradicate the Mediterranean fanworm have mostly been unsuccessful.

6.3 Detecting the presence of fanworm

Since the fanworm is highly fecund and appears to be able to produce eggs on a continuous basis, other aspects of the biology of fanworms were investigated to contribute to the efforts to identify possible mitigation strategies for this species. In this study (see Chapter Four), although eDNA and eRNA assays did not allow inferring specific seasonal spawning patterns for the fanworm, they could aid in informing on the presence of invasive species in the area (Ficetola et al., 2008; Foote et al., 2012; Takahara et al., 2013; Pochon et al., 2017; Ojaveer et al., 2018; Taberlet et al., 2018; Zaiko et al., 2018; Ardura, 2019; Murakami et al., 2019; von Ammon et al., 2019; LeBlanc et al., 2020; Wood et al., 2020). If an invasive species is detected early enough, there is a higher chance of successful containment or eradication, since proper management actions can be taken before the invasive species has spread or even produced a new generation (Hewitt et al., 2004; Floerl & Inglis, 2005; Floerl et al., 2005; Hewitt & Campbell, 2007; Floerl et al., 2010; Johnston et al., 2017; Giakoumi et al., 2019). Early detection of marine larvae and small juveniles is a significant challenge in marine biosecurity and consequently molecular tools can be useful for improving environmental monitoring of marine species that are inconspicuous or at low abundance (Floerl & Inglis, 2005; Dodgshun et al., 2007; Takahara et al., 2013; Pochon et al., 2017; Ojaveer et al., 2018; Taberlet et al., 2018; Zaiko et al., 2018; von Ammon et al., 2019; Bowers et al., 2021).

Despite current mitigation efforts, the Mediterranean fanworm has further spread around many parts of the New Zealand coast since its initial arrival in Lyttelton, it is highly likely that this pattern of invasion will continue (Atalah et al., 2017; Pande et al., 2017; Soliman & Inglis, 2018; Skelton & Jeffs, 2019; Tait et al., 2020). However, the timing and course of future incursions are hard to anticipate. The spread of this species is dependent on various factors such as the proximity and frequency of suitable transport vectors, the number of larvae released at infested locations, the dispersal range of larvae and the suitability of environmental conditions at new locations infected with founder individuals (Floerl et al., 2009; Floerl et al., 2012; McCann et al., 2013; Forrest &
Fletcher, 2015; Soliman & Inglis, 2018; Tait et al., 2018). Secondary spread via transport vectors for NIS, such as Mediterranean fanworm, include boat hulls, sea chests, bilge and ballast water of commercial and recreation vessels, the translocation of aquaculture gear and product, as well as anthropogenic marine debris (Carlton, 1996; Hewitt et al., 2004; Floerl & Inglis, 2005; Forrest et al., 2007; Hewitt & Campbell, 2007; Floerl et al., 2009; Tsolaki & Diamadopoulos, 2010; Werschkun et al., 2014; Roche et al., 2015; Campbell et al., 2017; Pochon et al., 2017). Therefore, further vigilance is needed to ensure all of these potential transport vectors are monitored and cleaned on a regular basis, especially if they are being moved out of locations infested with fanworm. However, currently not much is known about the dispersal range of fanworm larvae or how long fanworm can survive when attached to boat hulls or held in sea chests or ballast water. These knowledge gaps need further investigation and would be valuable in trying to identify potential locations that are at risk from fanworm invasion.

Molecular (eDNA and eRNA-based) tools could be useful for surveying and monitoring regions, ports and marinas where the fanworm is not yet established but are determined as high-risk areas due to boat traffic and other maritime activities potentially involved in transmission of NIS (Morrisey et al., 2007; Growcott et al., 2017; Campbell et al., 2018; Ojaveer et al., 2018; Taberlet et al., 2018; Zaiko et al., 2018; Ardura, 2019; von Ammon, 2019; Beng & Corlett, 2020; LeBlanc et al., 2020; Skinner et al., 2020). One key advantage of molecular tools for surveillance purposes is their sensitivity (Doi et al., 2015; Wood et al., 2018; Ardura, 2019; Doi et al., 2019; Wood et al., 2019; Beng & Corlett, 2020). Hence, when a NIS signal is detected with these methods it should trigger further investigations using other methods, such as diving and remotely operated vehicle surveys (Sassoubre et al., 2016; Darling et al., 2017; Collins et al., 2018; Murakami et al., 2019; von Ammon et al., 2019; Beng & Corlett, 2020; Skinner et al., 2020; Bowers et al., 2021). In order to reduce false positives and false negatives from eDNA and eRNA monitoring for marine invasive species, proper sampling methodologies with cross-contamination controls and sufficient field sample replication and sample volumes are needed (Darling & Mahon, 2011; Darling et al., 2017; Beng & Corlett, 2020; Mathieu et al., 2020; Bowers et al., 2021). Consequently, molecular tools have a lot of potential for early detection of NIS provided their sampling methodology and processing are sufficiently robust (von Ammon et al., 2018; Wood et al., 2018; Rose et al., 2019; von Ammon, 2019; von Ammon et al., 2019; Beng & Corlett, 2020). Regardless, molecular tools are not yet considered as the sole method for biosecurity monitoring purposes and should instead be used in combination with other methods such as visual inspection of high risk infrastructure and settlement collectors (Barnes & Turner, 2016; von Ammon et al., 2018; Wood et al., 2018; Carvalho et al., 2019; Franklin et al., 2019; Murakami et al., 2019; Shelton et al., 2019; von Ammon, 2019; von Ammon et al., 2019; Beng & Corlett, 2020).

6.4 Mitigation of fanworm in aquaculture

The results of this current study confirm that the Mediterranean fanworm is capable of regeneration after being fragmented, even after being released as fragments from a mussel harvesting vessel.
(see Chapter Five). However, the release of viable fragments of fanworm from mussel harvesting appeared to be less extensive than might have been expected. This was due to the tenacity of the fanworm attachment to the mussel grow-out line, with most fanworms remaining attached to the grow-out line during harvesting, resulting in them being retained in bulk harvesting bags with the grow-out rope. The fate of the fanworms and fanworm fragments that were still alive and were washed overboard during harvesting is unknown as it is logistically impossible to follow the fate of these fragments once they are released into the marine environment. However, many of the fanworms that are released from their tubes during harvesting were severely injured or killed. The experiment with fanworm fragments being held at sea indicated that a small proportion of these fragments have the potential to survive and regenerate. While fanworm were used experimentally to assess whether they would be consumed by fish predators immediately after being discharged from a barge during mussel harvesting, further studies would be needed to determine the fate of fanworms that are released from mussel harvesting, i.e., whether they survive and regenerate, succumb to their injuries and die, or get eaten by marine scavengers when dropped to the seafloor. It could be that the native fish fail to recognize the fanworm as a viable food source or it might be that because of the outer protective tube, toxins in the branchial crown or exuded mucus of the fanworm, they are not palatable to New Zealand predators (Fattorini et al., 2004; Fattorini & Regoli, 2004; Notti et al., 2007; Stabili et al., 2009; Stabili et al., 2011; Giangrande et al., 2014b).

Mussel spat (0.3 – 1 mm) on grow-out lines in mussel aquaculture appear to be most vulnerable to being overgrown by biofouling, being displaced or being washed away compared to intermediate or larger mussels (Fletcher, 2014; Forrest & Atalah, 2017; Skelton & Jeffs, 2020). Current mussel industry practice involves stripping and re-seeding mussels onto new ropes at lower densities once they reach approximately 40 to 50 mm shell length (Woods et al., 2012). This re-seeding serves a dual purpose as it not only thins the mussels out to a more productive stocking density, but also removes or reduces the effects of biofouling (Woods et al., 2012). Fanworms took approximately 8 months to reach 5 - 10 cm in length and were found on settlement structures with more morphological complexity and less densely clustered mussels (see Chapter Four). Hence, to reduce biofouling pressure and competition for mussels, mussel grow-out ropes could be cleaned or stripped and re-seeded in between stages, when fanworms are still below 5 cm in length and have not yet reached reproductive maturity (see Chapter Three). Additionally, different mussel grow-out rope types and materials could be investigated to determine if they could further reduce biofouling attachment and mussel loss (Dunham & Marshall, 2012; Atalah et al., 2016a; Sievers et al., 2019). Surfaces that are more complex are thought to provide more protection from predators and competitors for the settling larvae of biofouling species, such as fanworm (Toonen & Pawlik, 1996; Walters & Wethey, 1996; Hadfield & Paul, 2001; Gribben et al., 2011; Hadfield et al., 2014; Whalan et al., 2015; Skelton & Jeffs, 2020). For example, observations of fanworm during this study indicated that their ability to remain attached to the grow-out rope during mussel harvesting, when most other biofouling was stripped off, was due to their ability to strongly attach their protective tube foot below the fibres of the grow-out rope to the rope core (Giangrande et al., 2014b).
The Mediterranean fanworm is now well established in parts of New Zealand and eradication in locations such as Auckland or Whangarei will be impossible. However, mitigation strategies can still be applied to help reduce quantities of fanworms on mussel grow-out lines and in doing so, reduce further spread and establishment of this species. Reducing local fanworm abundance can also help prevent the negative environmental effects associated with fanworm, including increased levels of dissolved inorganic nitrogen (ammonium and nitrate), decreased denitrification and promoting the establishment of other biofouling organisms, which can then affect biogeochemical cycling (Tait et al., 2020).

6.5 Future directions and research

A key question raised by the research for this thesis is what attracts fanworm larvae to settle on a specific surface. The results from this current study suggest that fanworm larvae preferred morphologically complex surfaces over smooth surfaces for settlement. Currently it is unknown if S. spallanzanii larvae are attracted to a specific surface because of its structure, biofilm composition, presence of other biofoulers (i.e., Styela clava) or chemical signals from adult conspecifics in the vicinity or most likely a combination of these factors (Zaiko et al., 2016; Soliman & Inglis, 2018; Wood et al., 2018; Skelton & Jeffs, 2019). The larvae of marine biofoulers usually settle, attach and metamorphose on an already established biofilm of bacteria (Hadfield & Paul, 2001; Huang & Hadfield, 2003; Lau et al., 2005; Hadfield et al., 2014). If the signalling cues can be identified, specific compounds could be developed to prevent or at least reduce settlement on target surfaces. Further research is needed into how the fanworm would react to different anti-fouling agents, since it has a protective outer tube and its mucus has lysozyme-like activity that might protect the fanworm from harmful substances (Stabili et al., 2009; Stabili et al., 2011; Giangrande et al., 2014b). Furthermore, fibrous polypropylene grow-out rope with different structures could be tested to see if settlement of fanworm and other biofoulers varies depending on the rope structure. However, mussel retention would also need to be considered in combination with biofouling to see if different structures decrease biofouling but do not affect mussel retention (Skelton & Jeffs, 2020).

To improve the speed of biosecurity responses to new fanworm incursions, the accuracy of early detection methods, such as molecular tools (i.e., eDNA and eRNA detection), needs further research and improvement. Specifically, the shedding and decay rates for specific invasive species, such as fanworm, need to be determined, including how environmental factors such as turbidity, temperature, salinity, pH and sun exposure affect decay rates and distribution of detectable molecules (Takahara et al., 2012; Pilliod et al., 2013; Sassoubre et al., 2016; Wood et al., 2018; Murakami et al., 2019). Additionally, the standard method of sampling biofilm from the smooth surfaces of settlement plates should potentially be adjusted to the settlement preferences of the target species. Since the results of this current study suggest that the seawater filtration method detected higher eDNA copy numbers compared to the biofilm method, it would seem
logical for eDNA surveillance to filter larger quantities of seawater to improve the likelihood of the detection of the target species (Beng & Corlett, 2020; Bowers et al., 2021).

To successfully prevent future marine invasions and manage already established NIS, recent studies advocate for a more holistic approach to biofouling management, where anthropogenic transport vectors are better managed (i.e., commercial and recreational vessel hulls, ballast water, sea chests, marine debris, artificial structures, aquaculture and recreational gear and equipment) (Hewitt et al., 2004; Wotton & Hewitt, 2004; Hewitt & Campbell, 2007; Hopkins & Forrest, 2008; Roche et al., 2015; Hoey et al., 2016; Campbell et al., 2017; Growcott et al., 2017; Pochon et al., 2017; Giakoumi et al., 2019; Beng & Corlett, 2020). Public education and public awareness are also important in helping to prevent further anthropogenic spread by reducing fanworm numbers through physically removing them, reporting new sightings to environmental agencies and through the better management and attentiveness of biofouling on vessels (Hewitt et al., 2004; Hewitt & Campbell, 2007; Hoey et al., 2016; Giakoumi et al., 2019).

Better understanding of long-term effects of the fanworm on local communities and ecosystems is still needed, since some NIS species can lead to community shifts and can affect nutrient cycles (Sorte et al., 2010a; Sorte et al., 2010b; Padilla et al., 2011; Geburzi & McCarthy, 2018; Tait et al., 2020). This will also help to predict the risks of possible future incursions of taxonomically or functionally close species (i.e., Branchiomma luctuosum, invading the Mediterranean Sea and competing with S. spallanzani) (Mastrototaro et al., 2015; Giangrande et al., 2020). In the future, it is possible that another invasive species displaces S. spallanzani or the current expansion of the fanworm populations is followed by declines, which has been an observed phenomenon in other invasive species. For example, the invasive Mayan cichlid (Cichlasoma urophthalmus) has declined in the Everglades, however, similar declines have not been documented for many marine species (Simberloff & Gibbons, 2004). Even if a decline in the population of fanworm might occur in the future, this invasive species might already have caused considerable ecological and economic damage (Simberloff & Gibbons, 2004). Consequently, there is merit in continuing to investigate and test different mitigation tactics to reduce further dispersal of the fanworm and suppress the already established populations if at all possible.

The research for this thesis has contributed new knowledge about the reproduction, settlement, growth and regeneration capabilities of the Mediterranean fanworm. The fanworm has adapted well to New Zealand conditions and is highly fecund. With the insight gained from the various experiments and effective collaboration between biosecurity agencies, mussel farmers and scientists, useful mitigation strategies and further research can be advanced to reduce spread and impact of this invasive species to better protect native marine biodiversity and marine ecosystems.
Appendices

7.1 Appendix 1 - Enforced spawning induction in *S. spallanzanii* in the laboratory

To fully understand the reproduction cycle and output of *S. spallanzanii*, it would be beneficial to observe fertilization of gametes and larval development. Once viable larvae are obtained their subsequent development and settlement could be documented in detail, and their tolerances and sensitivities towards temperature and settlement surfaces could be further tested. Obtaining greater knowledge about larval development, settlement and metamorphosis would be greatly beneficial in understanding the biological limitations of *S. spallanzanii* and would help with developing ideas to further manage the proliferation of this invasive species in New Zealand. For example, potential interventions based on the larval settlement behaviour of this species could include the use of appropriately coated surfaces on artificial structures to be placed in the sea that are known to discourage the settlement of fanworm larvae.

In vitro fertilization of *S. spallanzanii* has been attempted previously by both mixing sperm with eggs inside the coelomic cavity of Mediterranean fanworms and within a Petri dish, but both methods were unsuccessful (Giangrande et al., 2000). Viable larvae have only been reported to be obtained in captivity, when individual Mediterranean fanworm spontaneously spawned in the aquarium (Giangrande et al., 2000). It was observed that the female *S. spallanzanii* released fertilized eggs in long mucous strings. Previous attempts to manually remove eggs from female fanworm and place them into the seawater to mix with live sperm have failed because the eggs wrinkled and collapsed once placed in seawater and were no longer viable and were unable to be fertilized (Giangrande et al., 2000; Read et al., 2014; pers. observation). Therefore, it is assumed that fertilization in fanworm is most likely to occur internally or within the tube and that upon fertilization, the eggs form a protective membrane or rely on a mucous covering to protect them upon their subsequent release into seawater (Giangrande et al., 2000; Stabili et al., 2009).

The aim of this research was to attempt to induce spawning in *S. spallanzanii* to enable the in vitro production of viable larvae so that larval development and duration could be closely observed. An in vitro supply of larvae would facilitate research to explore factors that affect larval survival and settlement, including different temperatures and different settlement substrates. Only one study has examined the larval development and duration of Mediterranean fanworm in any detail (Giangrande et al., 2000). However, many factors that influence *S. spallanzanii* larval development and settlement remain unknown, despite their potential usefulness in more effectively managing this invasive species, including in mussel aquaculture.

There are no available reports of effective methods for inducing spawning in *S. spallanzanii*. Therefore, a number of other methods that have induced spawning in other marine invertebrates, such as strip spawning and temperature shock treatment, were attempted with adult fanworm for this current study (Battaglene et al., 2002; Watson et al., 2003; Nelson et al., 2017). Strip spawning is a technique, where male and female gametes are extracted from adult animals, either by
dissecting out the reproductive tissues and washing out gametes, or squeezing the animals to promote the release of gametes. Typically, both male and female gametes obtained by strip spawning are then mixed directly in seawater or a Petri dish to facilitate fertilization. For the current study, a longitudinal incision was made to expose the coelomic cavity of both adult male and female *S. spallanzanii* to release gametes into the seawater as well as ablating the tail (i.e., cutting off 1 cm of the posterior end of the fanworm with scissors) to encourage spawning in conspecifics, which is a technique that has successfully induced spawning in another fanworm *Sabellastarte spectabilis* (Giangrande et al., 2000; Bybee et al., 2006, 2007; Nelson et al., 2017).

Exposing adults to thermal stress by alternating between hot and cold temperatures is another common technique used to induce spawning in marine invertebrates (Morgan, 2000; Battaglene et al., 2002; Nelson et al., 2017). The seawater temperatures used for the current study were based on the environments in which Mediterranean fanworm naturally occur. During winter in the Mediterranean Sea, Australia and New Zealand the seawater temperatures can drop as low as 11 °C and in the summer the seawater temperatures in Australia and New Zealand typically reach around 20 °C, whereas in the Mediterranean Sea it can reach around 27 °C (Currie et al., 2000; Giangrande et al., 2000; "World sea temperature," 2020). Therefore, for the current study, temperatures at the extreme end of the fanworms temperature range were used.

### 7.1.1 Methods

#### 7.1.1.1 Collection and maintenance of *S. spallanzanii*

A first set of adult *S. spallanzanii* was collected from 0.5 m depth on the outer surfaces of floating pontoons from the Orakei marina, Auckland (36° 50’ 59.8” S, 174° 48’ 34.4” E) in December 2019 (N = 150) and were brought back to the seawater laboratory facilities at the University of Auckland and used for experiments 1 – 8 during which fanworm were not fed. A second set of adult fanworms was collected in January 2020 (Total N = 60) and used for experiments 5 - 9 during which time they were fed with a mixture of cultured microalgae, in the event that availability of food was associated with spawning behaviour.

#### 7.1.1.2 Strip spawning

From the first set of *S. spallanzanii*, 20 adult fanworms were taken out of their tubes to clearly differentiate them into male and female fanworms by making a small incision to reveal the colour of the coelomic fluid to indicate gender, i.e., green is female and tan brown is male. Two groups of 10 fanworms, of which 2 -3 were male fanworms and the rest female, were placed together in a bucket containing 10 litres of filtered seawater (10 µm and UV) and held at room temperature of 23 °C for 24 h before the start of any experiment so the fanworms could adjust to the conditions. If gender could not be identified clearly, a few drops of coelomic fluid were spread on a microscope slide and
further examined under a Leica DMR fluorescent microscope to identify gender. The coelomic fluid of some of the fanworms was also examined under the microscope to confirm that mature gametes (~200 µm diameter) were present within the fanworm to confirm that if spawning occurred that it would result in viable larvae. All fanworms were starved for the duration of the strip spawning trial and the temperature stress experiments (both hot and cold temperatures).

Experiment 1 – Strip spawning with incision

From the 20 adult fanworms that were extracted from their tubes to determine their gender, one male fanworm was taken and a 2 cm long incision was made along the abdomen to release coelomic fluid containing sperm into a beaker of 1 L of filtered and autoclaved seawater at room temperature of 23 °C and which contained four female fanworms from the same holding bucket. The male fanworm with the incision was then also placed in the beaker to further release sperm in an attempt to trigger spawning of eggs by the female fanworm to facilitate fertilization of viable eggs.

The water was checked for spawned eggs every hour after the sperm was added to the beaker for a total of 6 h and then again after 24 h. The seawater was then filtered through a 75 µm mesh sieve and the filter residue placed on a microscope slide and examined for fertilized eggs or larvae under a Leica DMR fluorescent microscope. However, after 24 h there were no fertilized eggs or larvae found in the seawater. After 24 h the sperm was no longer mobile and no eggs had been released or been fertilized. The male fanworm was retained and held in filtered seawater and the incision healed within 5 days.

Experiment 2 – Strip spawning by bisection

From the 20 adult fanworms that were extracted from their tubes to determine their gender, one male fanworm was taken and a 1 cm section from the end of the tail of the fanworm was ablated to encourage spawning, a method previously successfully used by Bybee et al., (2006; 2007). The fanworm subsequently released sperm into a beaker of 1 L of filtered and autoclaved seawater at room temperature of 23 °C containing four female fanworms from the same holding bucket. The water was checked for fertilized eggs or larvae every hour after the tail of the fanworm was removed, for a total of 6 h and then again after 24 h. The seawater was filtered through a 75 µm mesh sieve and the filter residue placed on a microscope slide and examined for fertilized eggs or larvae under a Leica DMR fluorescent microscope. However, after 24 h there were no fertilized eggs or larvae found in the seawater.

Experiment 3 – Strip spawning and attempted fertilisation in seawater

From the 20 adult fanworms that were extracted from their tubes to determine their gender was classified, a 2 cm incision was made along the abdomen of one male and one female fanworm and their coelomic fluid was extracted and mixed directly in a Petri dish and covered with filtered
autoclaved seawater at room temperature of 23 °C. The dish was left to stand for 10 minutes and then examined under a Leica DMR fluorescent microscope. However, even though the sperm were mobile and active in the seawater, the eggs had become wrinkled and lost their form in the seawater and no longer appeared to be viable. No fertilised eggs were observed. These observations are in agreement with previous studies that have reported that when unfertilised eggs of *S. spallanzanii* come into contact with seawater they wrinkle, collapse and are inviable (Giangrande et al., 2000; Stabili et al., 2009).

**Experiment 4 – Strip spawning and attempted fertilisation in mucous**

The only observation of spawning *S. spallanzanii* reported the fertilised eggs being released in a mucous string, suggesting the possibility that mucous may provide some protection to eggs from the direct interaction with seawater (Giangrande et al., 2000; Stabili et al., 2009). Mucous was collected from five new adult *S. spallanzanii* taken from the bucket of seawater and left on a tray exposed to air for 5 minutes. Exposure to air, triggers *S. spallanzanii* to produce conspicuous amounts of mucous that was collected and placed in a Petri dish. Eggs and sperm were once again collected from the same incision previously made (1 male and 1 female fanworm) and mixed directly in a Petri dish with the collected mucous but without adding the seawater to prevent the eggs from wrinkling and hence increase chances of fertilization. However, even after allowing the dish to stand for up to 30 minutes with continuous observation, the sperm stopped moving and eggs had wrinkled and collapsed without being fertilized.

### 7.1.1.3 Elevated temperature stress

**Experiment 5 – Elevated heat shock for 3 or 5 days**

A set of ten adult fanworms out of their tubes, containing 2 - 3 males and 6 - 8 female fanworms, and ten adult fanworms with their tubes intact were kept at room temperature at 23 °C in a 10 L aerated bucket of filtered seawater and exposed to the following elevated heat shock treatment for 3 consecutive days. A second set of ten adult fanworms out of their tubes, containing 2 - 3 males and 6 - 8 female fanworms, and ten adult fanworms with their tubes intact were kept at room temperature at 23 °C in a 10 L aerated bucket of filtered seawater and exposed to the same elevated heat shock treatment but for 5 consecutive days.

Approximately 10 L of filtered and aerated seawater was heated to 28 °C in a separate bucket using an Aqua One Heatstick (100 W). Temperature was monitored continually with a thermometer in the seawater. The fanworms with their tubes and without their tubes were then moved between the hot water bucket (i.e., 28 °C) and the ambient water bucket (i.e., 23 °C) each hour for a total duration of 6 h (Figure 7.1). The temperature shock treatment was carried out for three and five consecutive days respectively, exposing the fanworms to the hot and ambient temperatures for 6h each day. Between each day of heat shock treatment the fanworm were held in seawater at ambient room temperature. The seawater was examined for the presence of gametes by filtering
the seawater through a 75 µm mesh sieve and putting sample drops of the filtrate on a microscope slide to be examined under a Leica DMR fluorescent microscope. The seawater was examined for the presence of gametes each day immediately after the temperature shock treatment was completed and again in intervals of 24, 48, 72, 96 and 120 h. The fanworms did not spawn and no gametes or larvae were found in the filtered seawater.

**Experiment 6 – Elevated heat shock with incision**

A set of ten adult fanworms out of their tubes, containing 2 - 3 males and 6 - 8 female fanworms, and ten adult fanworms with their tubes intact were kept at room temperature at 23 °C in a 10 L aerated bucket of filtered seawater and exposed to the following elevated heat shock treatment for 3 consecutive days. A second set of ten adult fanworms out of their tubes, containing 2 - 3 males and 6 - 8 female fanworms, and ten adult fanworms with their tubes intact were kept at room temperature at 23 °C in a 10 L aerated bucket of filtered seawater and exposed to the same elevated heat shock treatment but for 5 consecutive days. Approximately 10 L of filtered and aerated seawater was heated to 28 °C in a separate bucket using an Aqua One Heatstick (100 W). Temperature was monitored continually with a thermometer in the seawater. The fanworms with their tubes and without their tubes were then moved between the hot water bucket (i.e., 28 °C) and the ambient water bucket (i.e., 23 °C) each hour for a total duration of 6 h. The temperature shock treatment was carried out for three and five consecutive days respectively, exposing the fanworms to the hot and ambient temperatures for 6 h each day (Figure 7.1). Between each day of heat shock treatment the fanworm were held in seawater at ambient room temperature. The seawater was examined for the presence of gametes by filtering the seawater through a 75 µm mesh sieve and sample drops of the filtrate were placed on a microscope slide to be examined under a Leica DMR fluorescent microscope. The seawater was examined for the presence of gametes each day immediately after the temperature shock treatment was completed and again in intervals of 24, 48, 72, 96 and 120 h. In addition to the elevated heat shock exposure, a 2 cm incision was made on one female and one male fanworm that had their tubes removed, along their abdomens and the free floating sperm and eggs were released into the ambient temperature seawater bucket (23 °C) everyday for three or five consecutive days respectively, to encourage or induce spawning in the other fanworms. A smear of the coelomic fluid was also taken on a microscope slide to examine the size/ maturity state of the gametes. Eggs were found to be between 150 - 200 µm in diameter. However, the fanworms did not spawn and no gametes or larvae were found in the filtered seawater.
7.1.1.4 Lowered temperature stress

Experiment 7 – Lowered heat shock for 3 or 5 days

A set of ten adult fanworms out of their tubes, containing 2 - 3 males and 6 - 8 female fanworms, and ten adult fanworms with their tubes intact were kept at room temperature at 23 °C in a 10 L aerated bucket of filtered seawater and exposed to the following elevated heat shock treatment for 3 consecutive days. A second set of ten adult fanworms out of their tubes, containing 2 - 3 males and 6 - 8 female fanworms, and ten adult fanworms with their tubes intact were kept at room temperature at 23 °C in a 10 L aerated bucket of filtered seawater and exposed to the same elevated heat shock treatment but for 5 consecutive days.

Another bucket with 10 L filtered, aerated seawater was cooled by putting ice packs in the seawater. Temperature was monitored by keeping a thermometer in the water until it reached 10 °C. The fanworms were switched between the cold water bucket at 10 °C and ambient seawater at room temperature of 23 °C and left in each bucket for 1 h for a treatment over a total of 6 hours, i.e., five changes of temperature. The lowered temperature shock treatment was carried out for three or five consecutive days, exposing the fanworms to the 6 h period of cycling between cold and ambient temperatures on each consecutive day. Between each day of cold shock treatment the fanworms were held in seawater at ambient room temperature. The seawater was examined for the presence of gametes by filtering the seawater through a 75 µm mesh sieve and placing sample drops of the filtrate on a microscope slide to be examined under a Leica DMR fluorescent microscope. The seawater was examined for the presence of gametes each day immediately after the temperature shock treatment was completed and again in intervals of 24, 48, 72, 96 and 120 h after the temperature treatment had ended. The fanworms did not spawn and no gametes or larvae were found in the filtered seawater.
**Experiment 8 - Lowered heat shock with incision**

Ten fanworms that had their tubes removed were kept at room temperature at 23 °C in a 10 L aerated bucket of filtered seawater containing 2 - 3 males and 6 - 8 female fanworms. Another bucket with 10 L filtered, aerated seawater was cooled by putting ice packs in the water. Temperature was monitored by keeping a thermometer in the water until it reached 10 °C. The fanworms were switched between the cold water bucket at 10 °C and ambient seawater at room temperature of 23 °C and left in each bucket for 1 h for a treatment over a total of 6 hours, i.e., five changes of temperature. After the lowered temperature treatment lasting 6 h, a 2 cm incision was made on one female and one male fanworm along their abdomen at the beginning of the experiment and the sperm and eggs were released into the ambient room temperature water bucket in an effort to encourage conspecifics to spawn. The seawater was examined for the presence of gametes immediately after the temperature shock treatment was completed and again in intervals of 24, 48 and 72 h after the temperature treatment had ended. The seawater was examined for the presence of gametes by filtering the seawater through a 75 µm mesh sieve and placing sample drops of the filtrate on a microscope slide to be examined under a Leica DMR fluorescent microscope. The fanworms did not spawn and no gametes or larvae were found in the filtered seawater.

**Experiment 9 – Repeated spawning experiments including feeding**

The same lowered and elevated temperature stress treatments (experiments 5 - 8) were repeated for a second time with new S. spallanzanii collected at the end of January 2020 (sample set 2). However, these 60 new fanworms were fed throughout the experiments with a mixed diet of cultured microalgae *Tisochrysis lutea*, *Diacronema lutheri* and *Tetraselmis suecica* that were grown in the laboratory (Figure 7.2). The microalgae were grown in 1 L aerated Schott bottles of autoclaved filtered seawater with light tubes (Aqua One PlantGlo 45, 12V 18W) on each side of the bottles for 24 h continuous lighting with a mean room temperature of 23 °C. F2 media was used for the microalgae culture following standard protocols (Guillard, 1975). After two weeks, when cell cultures should have approximately reached optimum density, 400 mL of any cell culture or a mixture of two cell cultures (i.e., 200 mL *T. lutea* and 200 mL *D. lutheri*) were fed to the fanworms every second day and mixed into the holding seawater. The exact cell number was not measured, as the aim was to just feed the fanworm to excess.

Seawater in the fanworm buckets was changed every 3 days with new filtered seawater. Before discarding the seawater, it was filtered through a 75 µm filter mesh sieve and the filtrate checked under the microscope for any larvae or released gametes.

The new set of 60 fanworms that were continuously fed, were held for two months after the end of the temperature stress trials to see if they would spontaneously spawn afterwards, when being kept at room temperature and fed to excess. All the fed fanworms were still alive two months after collection from the marina and after undergoing the temperature experiments. However, the fanworms did not spawn and no gametes or larvae were found in the filtered seawater. The
fanworms all had to be euthanized in 100% ethanol and were discarded as biological waste due to Covid-19 lockdown in March 2020.

Figure 7.2. Microalgae culture setup.

It was observed that throughout the experiments, all *S. spallanzanii* that were kept in their tubes, lost their branchial crown and started re-growing new ones possibly as a result of handling or emersion. In contrast, most of the fanworms that were removed from their tubes to classify sex kept their branchial crowns but did not rebuild their tubes.

After feeding the fanworms with the microalgae, the seawater became turbid from the added microalgae, indicating that without the branchial crown, fanworms were unable to feed. In contrast, the fanworms with their tubes intact and being stuck into a plastic mesh on the cement plate to have a horizontal position, were able to filter feed well and cleared the seawater of the microalgae within 24 h. However, the fanworms without their tubes were accumulating at the bottom of their buckets and the seawater did not clear until the seawater was exchanged with fresh seawater. Hence, it can be assumed that their feeding ability was impaired by being out of the tube and not being able to extend their branchial crown to filter feed.

7.1.2 Conclusion

This study attempted to spawn and fertilize the gametes of captive *S. spallanzanii* by testing a variety of methods that have been used successfully for inducing spawning in other marine invertebrates, such as thermal shock and exposure to gametes in seawater (Morgan, 2000; Battaglene et al., 2002; Bybee et al., 2007; Murray et al., 2011). However, none of the various methods that were attempted were effective in either inducing spawning or achieving fertilisation despite the presence of mature gametes in the experimental fanworm.
It has previously been observed that *S. spallanzanii* population from the Mediterranean Sea population in Italy spawned in January/February, i.e., Northern Hemisphere winter, (Giangrande et al., 2000) and populations in Port Phillip Bay, Australia were observed to spawn with falling seawater temperatures in July and August, coinciding with Southern Hemisphere winter (Currie et al., 2000). These temperature ranges were simulated in this experiment to see if either hot or cold extremes could encourage spawning in captive *S. spallanzanii*.

Results from Chapter Three indicate that female fanworms in New Zealand contain various sized eggs within their coelomic cavity all year round and therefore instead of an extreme cold or warm temperature limit, the fanworms retain the eggs until conditions are optimal. Therefore, instead of trying to stress the fanworm into spawning, they might have to be kept at optimal conditions with medium to warm temperatures (~15 °C), ambient food and low disturbance until they spawn naturally. It has been assumed that handling or stressing the fanworm might lead to spontaneous release of the gametes. However, this has never been reported in the literature or observed in the laboratory or in the field when fanworms were collected and handled during the course of this four year study (pers. obs.). Additionally, it appears that the mucous of *S. spallanzanii* may play a crucial role in the fertilization process and is needed to maintain the integrity of the eggs (Giangrande et al., 2000; Stabili et al., 2009; Stabili et al., 2011). The results confirmed that most likely unfertilized eggs are not released into the seawater but fertilization occurs in the tube or at the tube opening just when the eggs have been released from the coelomic cavity (i.e., in situ fertilization) (Giangrande et al., 2000; Stabili et al., 2009).

7.2 Appendix 2 - Fecundity of *S. spallanzanii*

An effort was made to develop an effective method for the automated counting of the total number of eggs held by female *S. spallanzanii*. Doing so would enable comparisons of egg output from fanworms for different times of the year and locations and provide a means of gaining a better estimate of overall fecundity. However, since *S. spallanzanii* do not have distinct ovaries or testes and produce their eggs in the coelomic cavity, where they are mixed together with coelomocytes, it is hard to separate the different cells from one another. Furthermore, eggs are quite delicate when unfertilized and prone to rupturing, wrinkling and collapsing once they come into contact with seawater or air. Coelomocytes range in size from 5 – 50 µm in diameter while eggs can range in size from 5 – 250 µm in diameter and therefore there is a size overlap between coelomocytes and eggs, which creates a problem for differentiating eggs from coelomocytes (Dales, 1961). Typically eggs of different sizes are present within the same fanworm at the same time so that for most female fanworms there will be some overlap of similar sized coelomocytes and eggs. Therefore, the aim of this research was to attempt to develop a method to distinguish eggs from coelomocytes in coelomic fluid so the eggs could be counted using a coulter counter.
7.2.1 Methods

Adult *S. spallanzanii* were collected from the Orakei Marina, Auckland (36° 50' 59.8" S, 174° 48' 34.4" E) in December 2019 and in January 2020 (N = 19), from 0.5 m depth from the surfaces of floating pontoons and were brought back to the seawater laboratory facilities at the University of Auckland.

Female *S. spallanzanii* (recognized by their green coloured coelomic fluid) were taken out of their tubes, cut longitudinally along their entire body length and as much of the coelomic fluid as possible was gathered with a 1 mL syringe with a hypodermic needle (0.8 x 38 mm). The coelomic fluid was treated in several ways to determine the best way to preserve the integrity of the eggs so they could be subsequently counted to estimate fecundity. The unfertilized egg cells were very fragile, being prone to bursting, clumping, wrinkling and collapsing or forming dense clumps with other eggs or coelomocytes.

### 7.2.1.1 Egg treatment

To determine whether eggs would be prevented from clumping to one another and collapsing, the efficacy of a dispersant (Tween 80) was evaluated as a pre- and/or post-washing step before storing the egg cells in different fixing solutions (ethanol or formalin).

**Experiment 1 – Pre-washing eggs**

A pre-wash step was applied immediately after the eggs were manually extracted from the female *S. spallanzanii* with the syringe. The extracted eggs were gently washed 1 - 3 times with either a 1, 0.1, or 0.2% Tween 80 solution mixed with filtered seawater. The eggs were then filtered through a 125 µm mesh sieve and were transferred to either 0.1, 0.5, 1, 5 or 10% formalin in seawater solution for fixing. The fixed eggs were then counted on a coulter counter at the Cawthron Institute (Beckman-Coulter Multisizer 4 Coulter Counter).

Prior to running the egg cells through the coulter counter, different washing and screening steps were tested again by washing the cells for a second time with a 0.2% Tween 80 solution mixed with filtered seawater (1 µm) and screening on a 125 µm mesh. However, it was found that after the eggs were preserved in formalin, the eggs were even more prone to bursting and clumping with the coelomocytes and other cell debris.

**Experiment 2 – Reduced pre-washing of eggs**

The second experiment aimed to reduce the washing steps to maintain the integrity of the eggs and prevent them from clumping and bursting. Only one pre-washing step with Tween 80 solution immediately after the egg cells were extracted from the female fanworm was performed instead of the previous three washing steps. The eggs were then stored in either a 0.1, 0.5, 1, 5 or 10%
formalin in seawater solution and enumerated directly on a coulter counter without applying another Tween 80 solution wash.

Experiment 3 – Direct preservation

In an attempt to preserve the integrity of the egg cells and reduce clumping, for the third experiment coelomic fluid samples were not washed but placed directly in either 70, 80 or 100% ethanol solutions. However, it was found that the ethanol did not preserve the eggs effectively, instead they wrinkled up and collapsed altogether, possibly due to the dehydration effect of the ethanol.

Unfortunately, the coulter counter cannot differentiate between eggs and coelomocytes and their overlapping size ranges would limit the accuracy of the egg count (Figure 7.3). The different cells can be differentiated visually under a microscope, since the eggs have a big nucleus in the middle, whereas the coelomocytes have smaller oil globules inside (Dales, 1961).

7.2.2 Conclusion

The results of this current study indicated that washing the coelomic fluid three times with 0.2% Tween 80 solution and then storing it in 0.5% formalin in seawater proved to be the most effective in preserving the eggs integrity before running them through the coulter counter. However, the eggs were still quite delicate and prone to bursting and clumping when run through the coulter counter or washed over the 125 µm mesh sieve. Furthermore, it remained difficult to separate the eggs from the coelomocytes and other cell debris (Figure 7.3).

To get an accurate count from the coulter counter of all the eggs in the different size ranges, the coelomic fluid would have to be run once through the coulter counter with a gating in place to exclude cells smaller than < 60 µm, since these cells could be either coelomocytes, egg cells or other small cell debris, and then the same fluid would have to be washed and screened again to exclude all the larger cells and be re-run through the coulter counter again with a smaller size gating. Subsequently, manual microscope counts of a smaller sample could be used to assign the proportion of the counts to coelomocytes and eggs.

Since the coulter counter cannot differentiate between the egg cells, the coelomocytes and cell debris, an alternative method could be the use of a flow cytometer with a differential staining method in order for the flow cytometer to be able to differentiate between the smaller egg cells that overlap in form and size with the coelomocytes.

Eggs were very delicate and prone to bursting and clumping, even after trialling different preservation methods, concentrations, work flows and washing steps. Therefore, exposing the eggs to several washes and running them firstly through the coulter counter and secondly through
the flow cytometer without them bursting or clumping is highly unlikely and most probably would not
give accurate results of the actual number of eggs present.

Figure 7.3. Example of coulter counter output showing the number of particles (coelomocytes, oocytes and cell debris) per mL of all the cells and their diameter (µm).
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