INVESTIGATING VECTORS FOR AQUATIC INVASIVE SPECIES OF TUNICATES IN THE CANADIAN SHELLFISH AQUACULTURE INDUSTRY

BY

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ABSTRACT

The potential spread of *Didemnum vexillum* through aquaculture transfers of Pacific oyster (*Crassostrea gigas*) was investigated on the Sunshine Coast and the West Coast of Vancouver Island, BC. Pacific oysters from infested areas are regularly transferred for processing to non-infested areas thereby posing a risk of spreading *D. vexillum* to new areas. This observational study used concepts of the Hazard Analysis Critical Control Point (HACCP) methodology to identify points (control processes, CPs) where the clusters of oysters received manipulations that could alter the amount of epibiont fouling, notably *D. vexillum*. Three control processes (CPs) were identified: 1) the harvesting procedure, 2) transportation from the harvesting area to the processing plant, and 3) shucking of the oysters and depositing fouled shells in non-infested waters. The percentage coverage of *D. vexillum* on oyster clusters was evaluated at each CP for product originating from two aquaculture sites, Lemmens Inlet and Okeover Inlet. The results from the assessment demonstrated a significant loss of fouling on *C. gigas* clusters at both sites from Post-Harvesting (CP1) to Post-Shucking (CP3). Initial tunicate coverage was significantly higher in Okeover Inlet (60.8 %) compared to Lemmens Inlet (34.5 %). This was attributed to cleaning efforts by the farmer at the time of harvest. At the final CP, oyster shells were still covered with a substantial amount of *D. vexillum* (13 % and 20 %) and disposed into areas exposed to tidal waters of un-infested bays. Thus, the risk of secondary introduction related to shellfish aquaculture practices remains high. This observation led to the creation of a protocol using the

vital stain Neutral Red (NR) to determine whether tunicates were being disposed alive or dead.

A NR viability assay was developed using *Botrylloides violaceus*, which is an invasive colonial tunicate in PEI, as a proxy to the colonial tunicate *D. vexillum* as it is not locally found on PEI. *B. violaceus* was collected from various locations around PEI and mortality could be determined under light microscopy by assessing filtering and reaction to tactile stimuli as clinical markers. A total of 32 *B. violaceus* segments (3 cm^2) were assessed for viability by comparing a treatment (dead – acetic acid immersion) and a control (alive) group by evaluating NR uptake. Determination of viability in *B. violaceus* was successful in 100 % of experiments. All of the control group were alive, demonstrating a response to tactile stimulus and a noticeable amount of stain inside the zooids. All treatment groups were dead, demonstrated by the lack of staining and non response to tactile stimulus. A total of 27 fouled clusters containing *C. gigas* and *D. vexillum* were collected from an aquaculture lease, Lemmens Inlet, BC, for an aerial exposure comprised of 9 equally divided treatments (0 (control), 0.5, 1.5, 3, 6, 12, 24, 48 and 72 hours). Three segments of *D. vexillum* were removed from *C. gigas* and evaluated with the NR stain protocol. Preserved tissues were analyzed and showed no stain uptake for all segments, including the control. This finding demonstrates that *D. vexillum* has a high tolerance for uncommon situations and may reflect their ability to enter a dormant state during adverse environmental conditions.

In the Montague River estuary, PEI, *Ciona intestinalis* is the dominant tunicate AIS and is now present in adjacent bays. Removal of *C. intestinalis* from mussels in processing plants can release gametes into effluent water. Two separate trials in this experimental study focused on the effect of turbidity and flow found in effluent water on *C. intestinalis* egg fertilization. The first trial evaluated the effect of turbidity (0, 300, 600 and 1 200 NTU), and the second evaluated the effect of turbidity (0, 300, 600 and 1 200 NTU) combined with water flow (0.9583 L/s). Unfertilized eggs were then exposed to viable sperm and incubated at 21^{0}C for 48 h. The two trials had three repetitions and the total propagules (eggs, larvae and recruits) were counted under a dissecting microscope. Turbidity levels did not change the fertilization rate (65.88±2.95 %). Water flow decreased fertilization rates but there was no difference between turbidity levels. The control had a mean fertilization rate of 63.14 % (±1.75 %), while the mean of the treatments was 50.25 % (±2.85 %). These trials illustrate that regardless of turbidity and flow rate a potential propagule pressure still exists when removing tunicates from mussels within processing plants.

Tunicates have a detrimental effect on the mussel industry by fouling aquaculture gear, product and by increasing labour time and cost of culture. Management to maintain an economically sustainable aquaculture industry via optimized husbandry practices, from a grower and processor perspective, is required to lessen the impact of current tunicates and to reduce the potential impact from new AIS threats. The continued monitoring, education and communication to

minimize, if not stop, the ongoing spread is a necessity, especially for shellfish growers in PEI and BC who are already combating tunicate invaders.

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LIST OF ABBREVIATIONS

AAFC	Agriculture and Agri-Food Canada
ANOVA	analysis of variance
AIS	aquatic invasive species
ASW	artificial seawater
BC	British Columbia
^0C	degree Celsius
CAISN	Canadian Aquatic Invasive Species Network
cm	centimeter
CP	control process
DNA	deoxyribonucleic acid
DFO	Department of Fisheries and Oceans
FAO	Food and Agriculture Organization
HACCP	Hazard Analysis Critical Control Points
L/s	liters per second
µm	micrometer
M	million
mL	milliliter
mm	millimeter
NR	neutral red
NTU	nephelometric turbidity units
pH	potential of hydrogen
PEI	Prince Edward Island
RNA	ribonucleic acid
RPM	revolutions per minute
SE	standard error
WHO	World Health Organization

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Chapter 1

General Introduction

1.1 Introduction

The increasing world population and the decreased possibility of producing land based protein has lead to an increased interest in aquatic resources through the development of aquaculture worldwide to supplement this desire/need for protein (Avault, 1996). For example, Canadian shellfish aquaculture increased production from 10 590 tons in 1991 to 31 693 tons in 2009 with landed values of $12.2 to $60.5 M, respectively (Statistics Canada, 2002, 2010). Presently in Canada, shellfish aquaculture production occurs mainly in the provinces of British Columbia (BC) and Prince Edward Island (PEI) with production levels aimed at both national and international markets (Statistics Canada, 2002, 2010).

In 2009, 5,400 tons of Pacific oysters, *Crassostrea gigas* (Thunberg, 1793), were produced in BC (Statistics Canada, 2010). Over 90 % of this production, with a total value of $10 M, was exported to the United States and Hong Kong (AAFC, 2010). Similarly, over 90 % of the blue mussel, *Mytilus edulis* Linnaeus, 1758, production on PEI was exported to the US and European markets. The total production of mussels on PEI has a sale value of $36.3 M contributing $106 M to the province's Gross Domestic Product (DFO, 2006). Both provinces' shellfish aquaculture industries, along with developing industries for clams and scallops could potentially continue to increase if they are able to overcome setbacks such as diseases and Aquatic Invasive Species (AIS).

1.2 Aquatic Invasive Species

The worldwide increase in aquaculture and international commerce is contributing to an increased introduction and spread of AIS which are significantly impacting aquaculture growing areas. The majority of AIS have been transported unintentionally by humans, but intentional introductions also have occurred for commercial purposes (Ruiz and Carlton, 2003; Keller and Lodge, 2007). As an example, the introduction and the high frequency of transport of Pacific oysters (*Crassostrea gigas*) associated with the aquaculture industry along the Pacific coast has provided an effective dispersal vector for marine plants (Verlaque, 2001). International commerce has served as a vector for many recognized invasive species and the importance of this vector has been revealed by the number of introductions in regions with increased international trading frequency (Levine and D'Antonio, 2003).

Currently several species of invasive tunicates are of concern in Canada, particularly in relation to the impact on shellfish aquaculture production. In BC, four invasive tunicate species have been reported on shellfish farms and could become an economic nuisance for the industry: the clubbed tunicate, *Styela clava* Herdman, 1881; the golden star tunicate, *Botryllus schlosseri* Pallas, 1766; the violet tunicate, *Botrylloides violaceus* Oka, 1927 and *Didemnum vexillum* Kott, 2002 (DFO, 2007). PEI currently has four invasive tunicates that have severely impacted the mussel industry. The clubbed tunicate, *Styela clava*, was first identified in 1997 followed by the golden star tunicate, *Botryllus schlosseri*, in 2001; the violet tunicate, *Botrylloides*

violaceus in 2002; and the vase tunicate, *Ciona intestinalis* (Linnaeus, 1767), in 2004 (Gill *et al.*, 2007; Locke *et al.*, 2007). These tunicates impact the mussel industry negatively by fouling aquaculture gear, boats and product (Darbyson *et al.*, 2009) while also increasing labour time and cost of culture which cuts into growers and processors profit margins (DFO, 2006).

1.3 Invasion

Invasions follow a process involving a series of stages (Williamson and Fitter, 1996; Kolar and Lodge, 2001). Generally, a minimum of three stages are delimited: arrival, establishment, and spread (Freckleton *et al.*, 2006) or establishment, spread, and integration (Marchetti *et al.*, 2004). Because mature tunicates are sessile, their natural dispersal takes place either through spawning, fragmentation or rafting (Birkeland *et al.*, 1981). Therefore, long distance spread of these organisms occurs naturally over long time periods. However, the long distance spread of invasive tunicates (and other taxa), is occurring more frequently due to anthropogenic effects (Lambert, 2005).

1.3.1 Recent Invasion Trends

The higher frequency in international trade aids the number of introductions in two ways. First, the increase in number of transport events brings a variety of species introduced and secondly, the repeated introduction of the same species augments the chance of establishment, i.e. propagule pressure (Lockwood *et al.*, 2005). This has contributed to a growing number of AIS throughout the world,

leading to AIS becoming the second biggest threat to biodiversity after the loss of habitat (Lowe *et al.*, 2000; Sala *et al.*, 2000).

The West Coast of North America has incurred over 100 introduced invertebrate species. The majority are native to the northern hemisphere and were unintentionally introduced (Wonham and Carlton, 2005). The primary vectors include shipments of Pacific oyster (*C. gigas*) and Atlantic oyster (*Crassostrea virginica*) from other regions, and also hull fouling and ballast water transport of cargo ships. Although all these pathways are important, half of the introduced species are thought to be associated with ballast water (Wonham and Carlton, 2005). Approximately 3 to 5 billion tons of ballast water is transferred in international waters annually (Raaymakers, 2002). It has been estimated that more than 10,000 different species are being transported worldwide in the ballast water of ocean vessels in any 24 hour period (Carlton, 1999a). This includes species that can be concealed within the large amounts of sediment located in ballast tanks (Gramling, 2000).

1.3.2 Secondary Invasion

Invasion processes also occur on smaller (local or regional) scales. Similarly, the frequency of AIS transported from infested to uninfested areas, and the network of an invaded centre determines the risk and potential rate of spread of AIS (Padilla *et al.*, 1996; Buchan and Padilla, 1999). This secondary dispersal determines the extent of the invasion range within a region. It also determines the local impact of an invasive species on an economic and ecological scale (Lodge *et al.*, 1998). Regionally,

the spread of an invasion can be associated with a natural and/or anthropogenic effect.

1.3.2.1 Natural Spread of AIS

Emphasis on spread of invasive tunicates are mainly related to anthropogenic effects while negligible attention has been directed to natural vectors (Bernier *et al.*, 2009). Tunicates can spread naturally through the organism's normal life cycle (i.e. planktonic dispersal stages), migration or via hitchhiking on animals or plants. Botryllids, d6idemnids and *C. intestinalis* have all been reported attached to free-floating eelgrass blades (Highsmith, 1985; Jackson, 1986; Worcester, 1994; Petersen and Svane, 1995; Edlund and Koehl, 1998; Thiel and Gutow, 2005; Carman and Grunden, 2010). Other plant material in coastal environments have also been reported as vectors. For example, *Ecteinascidia* have been observed on fragments of mangrove roots (Bingham and Young, 1991) and *Symplegma* on drifting plant material (Dias *et al.*, 2006). Bernier *et al.* (2009) indicated that the lobster and rock crab are vectors for regional transport and spread of tunicates in the southern Gulf of St. Lawrence.

1.3.2.2 Anthropogenic Effect on the Spread of AIS

The transportation via anthropogenic vectors, however, remains the main route of successful invasions (Lambert, 2005). Additionally, any artificial substrate within the water column potentially represents a significant anthropogenic site for successful AIS establishment. In the marine and the estuarine environments, human

activities can facilitate the spread of AIS (Wasson *et al.*, 2001; Minchin *et al.*, 2005). Regionally, the most important activities include recreational vessel movements (Floerl and Inglis, 2003), and the aquaculture industry (Naylor *et al.*, 2001): both ranking as the highest potential for AIS spread in an expert survey (Therriault and Herborg, 2008).

1.3.2.2.1 Recreational Boating

Although few studies investigated small boats as vectors for secondary spread, recreational boating is considered an important vector for AIS (Floerl *et al.* 2005). In California, 70 % of the exotic species, in estuaries sheltered from international shipping, may have been introduced by hull fouling of recreational boats (Wasson *et al.*, 2001). Australian coastal water recreational boating activity has accounted for the introduction of 38 fouling species (Floerl and Inglis, 2005). The secondary spread of invasive macrophytes and invertebrates, in North American fresh waters, has been mainly attributed to recreational boating (Les and Mehrhoff, 1999; Johnson *et al.*, 2001). In New Zealand, the spread of these invasive macrophytes has been linked to plant fragments carried on boats and on boat trailers (Johnstone *et al.*, 1985).

Mature adults of tunicates can attach to the vessel hull and are considered the main cause of infestation on docks and marinas (Carlton and Geller, 1993). Focusing on external hull fouling alone, however, can lead to an underestimation of the risk of recreational vessels (Acosta and Forrest, 2009). As part of the recreational boating network, marinas can provide the first entry point for AIS via

international boating and a network of suitable habitats for the secondary invasion of a species via local commercial or recreational boating activities. Floating marinas are described as "sheltered islands" for AIS (Bax *et al.*, 2002). They represent substantial habitats for marine organisms (Connell, 2000).

1.3.2.2.2 Aquaculture

The importance of aquaculture as a vector for the spread of AIS has been mentioned previously (Carlton, 1992; Naylor *et al*,. 2001; Streftaris *et al.*, 2005). The spread of AIS can be associated with either the introduced bivalves or facilitated by aquaculture activities (Carlton, 1989; Carlton, 1999b). These AIS may include both fouling organisms and/or organisms that may cause diseases and impact both the host and possibly other species (Kuris and Culver, 1999). In PEI, the main concern is that aquaculture vessels frequently move between estuaries since many lease owners have sites in more than one location (Locke *et al.*, 2007). Aquaculture shares similarities with recreational boating as the use of boats brings the same fouling opportunities as the boat hull acts as shelter and/or substrate, but additionally many other vector possibilities in the form of gear (rope, cages, floats, etc.) and supporting processing industries.

Other related vectors in the aquaculture industry (e.g., processing plant) are also of importance at a regional scale. The aquaculture industry acts as a regional vector but also an interregional one, as the transfer of stock among sites is a possible and important factor in the spread of AIS (Bourque *et al*, 2003). A processing plant, near an estuary in the New London area (PEI), has discharged

wastewater from processed infested mussel socks, but no invasions have occurred locally (Locke *et al.*, 2007). This pathway still remains a possible vector for further invasion.

The new habitat, offered by the aquaculture species and the environment where it grows, may aid in the establishment of AIS by providing suitable unexploited substrates (Carver *et al.*, 2003). AIS have been shown to negatively impact the mussel industry by fouling aquaculture gear and product (Darbyson *et al.*, 2009). As an example, the estuaries of PEI have mud or sandy bottoms, but the gear used for aquaculture provides a hard substrate that favours settlement of invasive tunicates (Tyrrell and Byers, 2007).

1.4 Conclusions

The importance of the aquaculture industry keeps growing worldwide. This is not only because aquaculture is a relatively new sector of the agri-food industry, but also because of its spatial opportunity and the increased demands in providing the growing human population with an inexpensive protein source. For example, the mussel aquaculture industry in PEI contributes to the local economy through production sales, employment, and the contribution to the gross domestic product and government tax revenues.

Production continues to increase in commercial farming of mussels, oysters, and other bivalves on Canada's coast to meet the increasing market demands. The shellfish product that is brought by the industry gives a novel substrate that is

constantly renewed at a higher density. After establishment, it is unlikely that the tunicates can be eliminated from this newly invaded habitat. It then becomes an ever present nuisance that once integrated has to be continuously managed. Taking into account the successful spread of AIS, bivalve aquaculture has to be considered as an important vector.

Unfortunately, this industry is the most affected by the spread of AIS that they may also be contributing to. Tunicates raise maintenance costs for growers as equipment and product needs to be treated or sprayed. The cost is also reflected in the additional labour to harvest mussels and by the additional trucking costs due to larger volumes. The higher costs are also reflected in the necessity for processors to install effluent treatment and control systems, high labour intensity, more waste of which to dispose. Thus, tunicates are cutting into the profit margins of growers and processors.

The implementation of an internationally recognized preventive approach could help in the reduction of these constraints in a processing plant setting. Gunderson and Kinnunen (2004) took the Hazard Analysis Critical Control Point (HACCP) and adapted it to AIS introductions, AIS-HACCP. This approach reduces the risk of establishment to a new location and aids in maintaining economical stability of aquaculture. With this approach, knowledge of the viability of the invasive tunicate is important. In order to manage the risk of introduction, it is essential to ensure that individuals are dead when leaving the processing site. This does not limit itself to mature tunicates, but also other life stages. Phases in the procedure of

processing can contain an average of 1000-5000 eggs of *C. intestinalis* in the effluent waters (Bourque *et al.*, 2007).

The spread of AIS needs to be continually monitored to reduce the risk that can come with new species introductions. The rapid assessments, risk assessment and development of mitigation strategies and management strategies will help lessen the impact felt by the industry and the communities economically affected. The following objectives gathered knowledge to fill in gaps on the impact and effect of tunicates, and also bring data to help with future management strategies.

1.5 Objectives

The first objective of this study was to apply the Hazard Analysis Critical Control Points (HACCP) concept to evaluate the risk of incursion of *D. vexillum* from infested *C. gigas* culture sites to non-infested processing areas. This concept, which has been applied to the blue mussel industry in Prince Edward Island (PEI) in relation to *C. intestinalis*, is now being applied to evaluate the decrease in percentage coverage of *D. vexillum* infested *C. gigas* clusters from culture sites to non-infested processing areas. Rather than critical control points, this study will use control processes (CPs) when identifying a possible source for the decrease of *D. vexillum* coverage in the handling of *C. gigas* from the harvest to the final processes. These CPs will aid in the identification of areas where the fouling is reduced or eliminated from the aquaculture product.

Secondly, vital stains were considered as an approach to accurately assess the viability (or mortality) of *D. vexillum.* More specifically, the second objective of this study was to assess different methods for determining the viability of *D. vexillum* under controlled experimental conditions by simulating the air exposure encountered during the normal transportation and processing of oysters. Validation experiments were conducted on the violet tunicate (*Botrylloides violaceus*) to compare the effectiveness of Neutral Red (NR) stain. *B. violaceus* was selected as a proxy because *D. vexillum* was not available locally on PEI. The violet tunicate has similar biological features to *D. vexillum* that include colony formation and colour morphs (Carver *et al.*, 2006; Daniel and Therriault, 2007). *B. violaceus'* mortality however can be determined using microscopy by observing its filtering action or its response to tactile stimulation (Dijkstra *et al.*, 2008; Rinkevich *et al.*, 1992; Chapter 3). This should provide critical information to assess the risk of *D. vexillum* spread associated with the current oyster harvesting and processing activities in BC. Furthermore, this study will assist with risk assessment, rapid response and development of mitigating measures in the event of *D. vexillum* introduction on the Atlantic coast of Canada.

The last objective of this project was to assess whether the eggs of *C. intestinalis* would still be fertile after exposure to environmental factors similar to those encountered in mussel processing plant conditions. This study specifically focused on the effect of water turbidity and flow. These two parameters were mimicked under controlled experimental settings. Two separate trials were conducted: one to evaluate the effect of water turbidity alone, and the other to

evaluate the interaction between water turbidity and water flow. The results of this study could provide information to the mussel processing industry that would be helpful in exploring future tunicate mitigation management strategies.

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Chapter 2

Didemnum vexillum: Invasion Potential via Harvesting and Processing of the Pacific Oyster (*Crassostrea gigas*) on Vancouver Island

2.1 Abstract

Pacific oysters from infested areas are regularly transferred for processing to non-infested areas thereby posing a risk of spreading *D. vexillum* to new areas. Three control processes (CPs) were identified in existing aquaculture practices where clusters of *C. gigas* received manipulation or stress that could alter the amount of covering epibionts, notably *D. vexillum*. The CPs were: 1) the harvesting procedure, 2) transportation from the harvesting area to the processing plant, and 3) shucking of the oysters. The percentage coverage of *D. vexillum* on oyster clusters was evaluated at each CP for product originating from two aquaculture sites, Lemmens Inlet and Okeover Inlet. A total of 60 clusters were sampled from Lemmens Inlet and 46 from Okeover Inlet. Results demonstrated a significant loss ($P<0.05$) of *D. vexillum* coverage on *C. gigas* clusters from CP1 to CP3 for both sites. Although variations existed between the sites (discussed in 2.5.2), the percentage coverage varied from 48 % (mean of Post-Harvest) to 30 % (mean of Post-Transportation) to finish around 17 % (mean of Post-Shucking) At the final CP, oyster shells were still covered with a substantial amount of *D. vexillum* (13 % and 20 %) and disposed in areas exposed to tidal waters of un-infested bays. Thus, the risk of secondary introduction related to shellfish aquaculture practices remains high.

2.2 Introduction

Aquatic invasive species (AIS) are introduced into coastal regions of the world at a high frequency (Carlton, 1989). The main vector for introduction is the transportation and release of ships ballast water (Carlton, 1987; Carlton and Geller, 1993). Carlton and Geller (1993) estimated that more than 3000 species are potentially in movement around the world in large vessels, likely resulting in more invasions in coastal environments (Carlton, 1996). Including the movement by large vessels, AIS are transported by humans unintentionally and also, intentionally (Ruiz and Carlton, 2003; Keller and Lodge, 2007). Other vectors are found at regional scales, which results in secondary spread of AIS within a region (Lodge *et al.*, 1998). Although the natural dispersion of AIS occurs on a smaller scale, (water currents or attached to floating objects) (Carlton, 1987), anthropogenic transportation has been the main cause of the successful spread and invasions of AIS (Lambert, 2005). Among regional vectors, the movement of recreational boats (Floerl and Inglis, 2003) and the gear associated with the aquaculture and fishing industries (Naylor *et al.*, 2001) have been well documented. Herborg *et al.* (2009) identified aquaculture transfers as a vector of high importance for invasive tunicate spread based on a survey of experts.

As an example, intentional introductions have been made possible by the high frequency of transport of Pacific oysters (*Crassostrea gigas* (Thunberg, 1793)) associated with the aquaculture industry along the Pacific coast (Verlaque, 2001). This intentional introduction of oysters has unintentionally brought over 100

introduced and identified invertebrate species to these coastal waters. The majority, of these unintentionally introduced species, are native to the northern hemisphere (Wonham and Carlton, 2005). In 2008, 5,300 tons of Pacific oysters were produced in British Columbia (BC), Canada. In 2009, 90 % of the $10 M exported Pacific oysters were shipped to the United States and Hong Kong (AAFC, 2010). The export encompasses several markets, mainly half-shell and shucked.

For the shucked market, the oyster growers string old oyster shells suspended on lines in the bays and inlets to collect naturally settling larvae. Oyster seed (settled larvae) are usually bought from a commercial hatchery for remote settling and reared in nurseries until ready for planting in designated inlets. Shells holding the spat, from 10 to 20, are strung in double twine rope at 12 inch intervals (Quayle, 1969). The length of the line depends on the depth of the water column. The oysters are grown in suspension until they attain the desired length, taking approximately 2 years (Quayle, 1969). The mass of oysters that has grown on the original mother shell is then referred to as a cluster. At harvest, the clusters are separated and the undersized oysters and old shells are returned to the beds. In processing plants the market size oysters are then shucked and the meat is cleaned and packed for shipping.

Currently several AIS are of concern in BC, including four tunicate species: the clubbed tunicate, *Styela clava* Herdman, 1881; the golden star tunicate, *Botryllus schlosseri* (Pallas, 1766); the violet tunicate, *Botrylloides violaceus* Oka, 1927 and *Didemnum vexillum* Kott, 2002 (DFO, 2007). *D. vexillum* has been identified on

Pacific oyster (*C. gigas*) farms on the Sunshine Coast and West Coast of Vancouver Island (Valentine, 2003; Cohen, 2005). All oysters grown on Vancouver Island are processed on the East Coast of Vancouver Island where *D. vexillum* has not yet been reported, thus exposing the area to a potential incursion.

D. vexillum is a colonial tunicate that has the capability of overgrowing aquaculture gear and potentially causing mollusc mortality (Valentine, 2003). The colony is composed of many small zooids that grow in ropey forms or mats, draping off the attached substrate (Kott, 2002; Lambert and Lambert 2005). Didemnids are capable of both sexual and asexual reproduction that allows them to rapidly disperse naturally within newly invaded habitats. The larvae are brooded within the tunic below the zooids (Berrill, 1975; Svane and Young, 1989; Monniot *et al,.* 1991). Once the tadpole larvae are fully developed they are typically released in the water column after sunrise and they then swim for varying times ranging from minutes to hours before settling (Svane and Young, 1989; Cohen, 2005; Valentine *et al.*, 2007b). Once settled, the tadpole larvae undergo metamorphosis to a zooid and begin feeding (Burke, 1983). After settling, the first zooid of the colony begins to rapidly reproduce by asexual budding to increase the colony size (Berrill, 1975; Kott, 2001; Monniot *et al.,* 1991; Nakauchi and Kawamura, 1990; Tyree, 2001). Disturbance of the colony when scraped or torn from the substrate helps the spread of *D. vexillum* as the displaced zooids and colony fragments have a high success rate of reattachment (Osman and Whitlatch, 2007). Valentine *et al.* (2007a) found that colony fragments could increase up to 11 times the original size in 15 days, by budding.

There has been a steady increase in the global range of *D. vexillum* and to date it has been recorded in Australia, New Zealand, North America, Japan, and Europe (Valentine, 2003; Cohen, 2005). Other factors may contribute to its successful spread and establishment. Anthropogenic disturbance, for example, creates a favourable habitat for AIS colonization (Monniot *et al.*, 1991; Cohen, 2005; Valentine *et al.*, 2007b). In areas where shellfish aquaculture has developed, *D. vexillum* can cover and overgrow bivalves to the point that their siphons become occluded (Monniot *et al.*, 1991; Valentine, 2003; Cohen, 2005; Gittenberger, 2007; Valentine *et al.*, 2007a and 2007b). Blue mussel, *Mytilus edulis* Linnaeus, 1758, farms provide additional substrate for the establishment of invading tunicates such as *D. vexillum.* Further, the *D. vexillum* tunic prevents settlement of larvae of some species, including the blue mussel and bay scallop, due to its acidity that ranges in pH from 1-2 (Morris *et al.*, 2009). Also, when the tunicate is injured, the contents of the acidic bladder are released making it impossible for the settlement of other marine species on its tunic (Stoecker, 1980; Carman, 2007). *D. vexillum* has also been shown to outcompete other colonial tunicates, such as *Botrylloides* and *Botryllus,* even if its peak in recruitment occurs a month after the peak recruitment of these other species (Auker and Oviatt, 2008).

This observational study uses the Hazard Analysis Critical Control Points (HACCP) principle. HACCP is recognized as a preventive approach in food and pharmaceutical safety to identify potential hazards in processing or manufacturing (FAO/WHO, 2001). For the aquaculture industry, the key management practices are: (1) identify critical points where AIS can be controlled, (2) monitor critical control

points, and (3) establish corrective actions to prevent AIS introductions. These key practices are components of the seven HACCP principles (Gunderson and Kinnunen, 2004). The additional four principles were not taken into account in this project as they ensure continued monitoring and management following a preliminary assessment.

The objective of this study was to apply the HACCP concept to evaluate the risk of incursion of *D. vexillum* from infested *C. gigas* culture sites to non-infested processing areas. This concept, which has been successfully incorporated into the blue mussel industry on Prince Edward Island (PEI) in relation to *Ciona intestinalis* (Linnaeus 1767), was applied to evaluate the decrease in percentage coverage of *D. vexillum* infested *C. gigas* clusters from culture sites to non-infested processing areas on Vancouver Island BC. Rather than critical control points, this study used control processes (CPs) when identifying a possible source for the decrease of *D. vexillum* coverage in the handling of *C. gigas* from harvest to final processing and discard of fouled oyster shells. These CPs will aid in the identification of areas where the fouling is reduced or eliminated from the aquaculture product and identify potential areas for management intervention.

2.3 Materials and Methods

CPs for the reduction in coverage of *D. vexillum* were identified for the shucked oyster market. CPs were harvesting/processing practice where the aquaculture product received a manipulation or a stress that could alter the amount of fouling on the clusters. The CPs were 1) the harvesting procedure, 2)

transportation from the harvesting area to the processing plant, and 3) processing of the oysters.

C. gigas culture sites fouled with *D. vexillum* and their corresponding processing plants were identified after site visits. These visits lead to the identification of two inlets with a significant amount of fouling, Lemmens Inlet (near Tofino, BC) and Okeover Inlet (near Powell River, BC). These sites were found to employ different aquaculture and harvesting systems thereby increasing the robustness of this project.

2.3.1 Assessing *Didemnum vexillum* Coverage

Assessment resulted in an estimation of the percent coverage of *D. vexillum* on oyster clusters at each CP. Since the clusters are not the same size, the percentage of coverage was used instead of weight. A single observer (recorder) estimated the percentage coverage of *D. vexillum* on each cluster used in the trial with the assistance of a handler. The clusters were passed by the growers to the handler. While maximum coverage was sought, some clusters that were assessed had a low amount of coverage. The recorder collected the data and associated a tag number with the clusters. The handler placed the assessed cluster in an identified mesh bag which was then handed back to the crew members who returned them to the harvest process.

A total of 60 clusters were sampled from Lemmens Inlet and 46 from Okeover Inlet. The lower number of observations from Okeover Inlet was due to

conflicts with the fast paced harvesting process. Each cluster was followed and assessed at the three CPs. Damaged clusters were reconstructed by assembling the different sections based on colour and shell shape. Although not common, the reconstructions were seamless. A mesh filter under the effluent grate showed no visible signs of colonial tissue leaving the processing plants, thereby eliminating the possibility of dispersal via effluent water.

2.3.2 Site Variation within the CPs

2.3.2.1 Control Process 1 – Post-Harvest

The initial assessment of the clusters (CP1) was conducted at the time of harvest. The site in Lemmens Inlet uses a long line system with clusters grown on a double twine rope. At harvest, the lines were pulled onto the boat and cut between each cluster. The grower on this site removed excess epibionts, including *D. vexillum*, and dead shells to reduce the weight per shipment. These clusters were then dumped in mesh bags for shipment.

The site in Okeover Inlet also uses a long line system, but the clusters are grown on PVC pipes (French tubes). Harvesting at this site differed from the first. Here, ropes were cut and the pipes were loaded onto a tray. The clusters on the pipes were removed via a sloughing device. The device pushes a slightly larger diameter pipe over the end of the smaller diameter pipe used as a substrate to grow the oysters, which allows the clusters to be removed. A conveyor belt catches the

oysters and dumps them into a mesh bag for shipment. No manual cleaning was done in this system.

2.3.2.2 Control Process 2 – Post-Transportation

The second assessment (CP2) was conducted after transportation to the processing plant on the East Coast of Vancouver Island. The shipment from Lemmens Inlet was sent to the processing plants via land with a 5 hour transit time. The Lemmens Inlet clusters were shucked 2 days after their arrival at the processing plant. The shipment from Okeover Inlet was sent to the processing plants via boat with a 6 hour transit time. These transit time represent the typical time taken for the shipment. The Okeover Inlet clusters were shucked 2 days after their arrival at the processing plant. For the clusters from both points of origin, the clusters were kept in the mesh nets covered with a tarpaulin sheet while in transit and awaiting the shucking process.

2.3.2.3 Control Process 3 – Post-Shucking

The third assessment (CP3) was conducted after the shucking of the oysters. The mesh bags were emptied in an exterior chute bringing the clusters into the shucking room. After being shucked, the shells and fouling were pushed aside in a wheelbarrow. Once filled, the wheelbarrows were wheeled out to the shell pile located in the intertidal zone outside the processing plants. No variation in the shucking and disposal method was noticed between the two sites.

2.3.3 Statistical Analysis

Percentage coverage was analysed using the General Linear Model procedures of Minitab 15.1.0.0 (© 2007 Minitab Inc., State College, Pennsylvania, USA) and Stata SE 10.0 (Stata Corporation, College Station, Texas). A split plot design was used to assess the differences in the two sites for the different CPs. Additionally; an Analysis of Variance (ANOVA) was used to analyze each CP for differences between sites. The outcome was transformed using the arcsine of the square root of the percentage. This data transformation was performed to obtain normality and to follow the model assumptions for the residual analysis. Individual clusters were used as random effects between the subjects in the sites. In all statistical models, the significance level was set at 0.05.

2.4 Results

2.4.1 Variations in Control Processes

The percentage coverage of *D. vexillum* on clusters of *C. gigas* decreased during the harvesting and processing procedure used at both sites (Figure 2.1). The split plot (Table 2.2) showed that the sites (P=0.147) followed the same overall trend. The similarities are depicted (Figure 2.1) by the overall tendencies in the reduction of the percentage coverage through the CPs. When taking CPs without sites as a factor, this tendency demonstrated a significant decrease from the Post-Harvest to Post-Shucking (P<0.05). The abrupt decrease in coverage between each CP averaged ~15 % (Figure 2.1). The split-plot analysis also demonstrated that the

variation in coverage for both sites followed the same trend ($P<0.05$) (Tag (site) in Table 2.2).

2.4.2 Site Variation within the CPs

The following utilizes the one-way ANOVA (Table 2.1) which displays the differences in each CPs between the sites. Each corresponding percentage value, with its standard error, can be found in Figure 2.1.

After the first CP, the sites were significantly different in the Post-Harvest assessment ($P<0.05$). The percentage coverage of *D. vexillum* (mean ±SE) on the cluster of *C. gigas* was significantly higher in Okeover Inlet (60.80 ±4.15 %) than in Lemmens Inlet (34.57 ±3.67 %).

In the second assessment (CP2), the difference of coverage between the two sites (4.75 %) Post-Transportation was non-significant ($P=0.3539$). The percentage (mean ±SE) on the cluster of *C. gigas* was 32.07 ±4.43 % in Okeover Inlet which was comparable with the coverage in Lemmens Inlet (27.32 ±3.02 %).

The final assessment in the timeline (CP3 - Post-Shucking) was marginally significant between the two sites ($P<0.05$). This time, the percentage coverage (mean ±SE) in Okeover Inlet (13.04 ±2.68 %) was lower than what was found in Lemmens Inlet (20.50 ±2.72 %) by 7.46 %.

2.5 Discussion

2.5.1 Control Processes Variations

This observational study, demonstrated a significant loss of coverage on *C. gigas* clusters from Post-Harvest to Post-Shucking for both sites. Although variations existed between the sites (discussed in 2.5.2), the percentage coverage varied from 48 % (mean of Post-Harvest) to 30 % (mean of Post-Transportation) to finish around 17 % (mean of Post-Shucking). Possible causes for this decrease included physical removal during each stage of the process or fall-off due to mortality. The mortality could be caused by exposure to air or sunlight during transportation to processing plants (further investigated in Chapter 3). Katayama and Ikeda (1987) noticed mortality in *Didemnum moseleyi* (later confirmed as *D. vexillum* by Lambert, 2009) after 5 hours or more of air drying in direct sunlight dependent on ambient temperature.

It is a possibility that the wind that resulted from traveling decreased the temperature and removed humidity from transported product. However, clusters that were transported in nets were covered by a tarpaulin. This canvas blocks direct sunlight, but keeps humidity and temperature high in the core area of the shipment. The effect from the wind would reduce the perimeter temperature and humidity for the length of the travel, thus possibly having a mitigating effect on *D. vexillum* by desiccation. The tunicates located in the centre of the cluster mass, while in transit, would receive an effect leading to believe that *D. vexillum*'s chances of survival would be compromised.

2.5.2 Site Variation within the CPs

Post-Harvesting was shown to have a significantly higher amount of *D. vexillum* in Okeover Inlet (60.8 %) compared to Lemmens Inlet (34.5 %). The difference between these two sites was likely due to the added manipulation by the grower in Lemmens Inlet. The added manipulation reduced the weight associated with shipping non-oyster product (hence financial gains) to the processing plant. This reduces the number of tunicates in transit by half, therefore lowering the potential for additional spread in comparison with the other site. Although reduced, the threat of invasive species introduction via transportation is still possible.

The significant difference in Post-Harvest was followed by smaller differences between the two sites (4.74 % for Post-Transportation and 7.46 % for Post-Shucking). This demonstrated an abrupt decrease of *D. vexillum* coverage on the clusters of Okeover Inlet in comparison to Lemmens Inlet. And, it also possible that pre-cleaning the clusters maintained a stable decrease in the level of coverage from Post-Harvest to Post-Shucking reducing the chance of spread along the way.

Although not truly tested, the transit time and different transportation methods are two possible factors to take into account for the difference in coverage from Post-Harvest to Post-Transportation between the two sites. Although receiving the largest reduction, the additional hour of transport and the water mode of transport was not likely the determining factor in this reduction of coverage in Okeover Inlet. Being able to track the loss of *D. vexillum* highlighted that the major loss of tunicates in transit represents a possible spread between the two

geographical locations. The important factor to consider is the higher amount of substrate left on the clusters. This could help weigh down the coverage as a higher amount of *D. vexillum* is weakened during travel.

2.6 General Conclusions

Although the harvesting and processing procedures have occurred for a number of years, *D. vexillum* has still not been reported in the effluent waters of processing plants. Reasons for *D. vexillum's* absence in these waters are unknown; however, it is possible that *D. vexillum* does not survive the procedures from the initial harvest. It is still critical to determine if *D. vexillum* is dead before it leaves the processing site as this would prevent its establishment in surrounding intertidal areas. In order to manage the risk of introducing *D. vexillum* into new areas, it is essential to ensure the death of the entire colony since establishment can occur from small fragments (Bullard *et al.*, 2007; Valentine *et al.*, 2007a). Even with a decrease in the percent coverage of *D. vexillum* on the clusters, a substantial amount (13 % and 20 %) was still present and disposed attached to shucked shells in the intertidal zone. This represents a potential for invasion. Further investigations in *D. vexillum's* viability were conducted in Chapter 3.

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Table 2.1 One-way analysis of variance of the percentage coverage of *D. vexillum* on *C. gigas* clusters for individual control processes.

		CP 1		CP 2		CP 3	
Source	df	*F*	*P*	*F*	*P*	*F*	*P*
Site	1	21.30	<0.05*	0.87	0.3539	3.99	<0.05*
Error	104						

*= significant p-values at the 5% cut off point.

Table 2.2 Split-plot design analysis of variance results for the site and Control Process effect on the *D. vexillum* coverage on *C. gigas* clusters.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Site	1	0.40393	0.38804	0.38804	2.13	0.147
CPs	2	5.35478	5.97097	2.98549	118.14	<0.05
Interaction	2	1.75683	1.80361	0.90181	35.69	<0.05
Tag(Site)	104	18.92778	18.92778	0.18200	7.20	<0.05
Error	207	5.23104	5.23104	0.02527		
Total	316	31.67436				

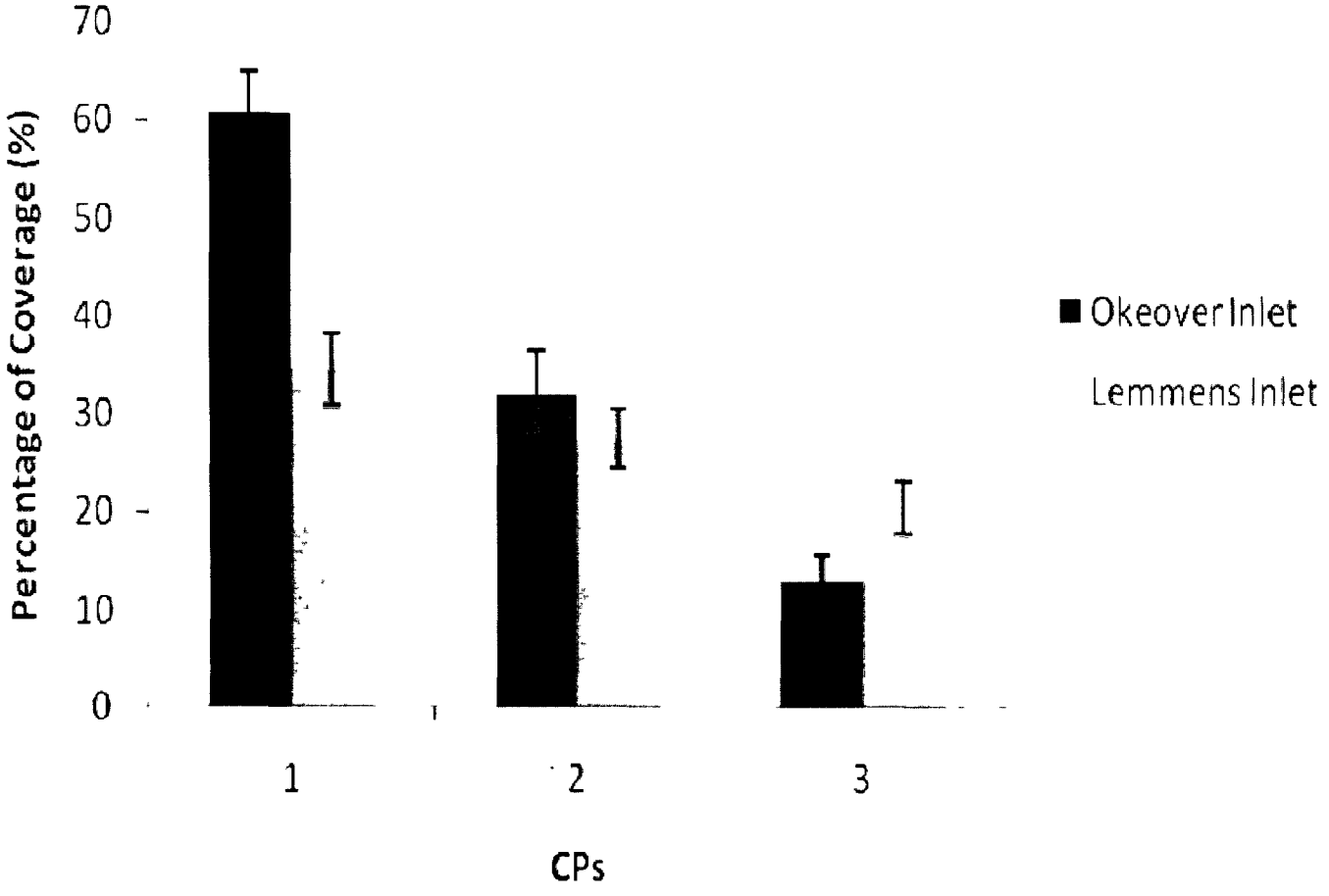

Figure 2.1 Percentage of *D. vexillum* coverage throughout the various processes, (1) Post-Harvest, (2) Post-Transportation and (3) Post-Shucking. The standard errors are displayed.

Chapter 3

Evaluating the Viability of *Didemnum vexillum* to Air Exposure with a Protocol Developed for *Botrylloides violaceus*

3.1 Abstract

A project to determine the risks of introducing *Didemnum vexillum* after the transportation from the harvest site to the processing plant was initiated. The viability of *D. vexillum* was assessed under controlled experimental conditions by simulating the air exposure encountered during the normal transportation and processing of oysters. A NR viability assay was developed using *Botrylloides violaceus,* which is an invasive colonial tunicate in PEI, as a proxy to the colonial tunicate *D. vexillum* as it is not locally found on PEI. Unlike *D. vexillum, B. violaceus'* mortality can be determined under light microscopy by observing changes in the physiological parameters of filtration and reaction to tactile stimulus. A total of 32 *B. violaceus* segments (3 cm^2) were allocated to treatment and control groups. The treatment group was subjected to a mortality model using acetic acid which resulted in 100 % mortality. Viability was then assessed by comparing treatment and control groups response to a Neutral Red stain uptake. All of the control group were alive, demonstrating a response to tactile stimulus and a noticeable amount of stain inside the zooids. All the treatment groups were dead, demonstrated by the lack of staining and non response to tactile stimulus.

A total of 27 fouled clusters containing *C. gigas* and *D. vexillum* were collected from an aquaculture lease, in Lemmens Inlet, BC, for an air exposure trial comprised of 9 equally divided treatments (0, 0.5, 1.5, 3, 6, 12, 24, 48 and 72 hours). Three segments of *D. vexillum* on each cluster were removed and evaluated with a Neutral Red stain protocol used for *B. violaceus.* Preserved tissues were analyzed and no

sign of stain was noticeable, even with the control segments. The findings are consistent with *D. vexillum* demonstrating a high tolerance in situations they are not accustomed to and may reflect their ability to enter a dormant state during adverse environmental conditions. Further studies are required to determine the viability of *D. vexillum* after air exposure.

3.2 Introduction

The colonial invasive tunicate, *Didemnum vexillum* Kott, 2002, is found in the Pacific oyster (*Crassostrea gigas* (Thunberg, 1793)) aquaculture sites on the West Coast of Vancouver Island and the Sunshine Coast of British Columbia (Valentine, 2003; Cohen, 2005). Although its detailed distribution is not known and keeps evolving, some areas on Vancouver Island and along the BC coast are considered to be free of *D. vexillum* and therefore vulnerable to future introduction or spread (Cohen, 2005; Daniel and Therriault, 2007).

On Vancouver Island, transfers of oysters colonised with *D. vexillum* to processing plants in non-infested areas were observed. After processing, the empty shells, along with the attached tunicates, are discarded on the intertidal zone near the processing plants. The potential risk of introducing *D. vexillum* to non-infested areas is real and mainly relies on its viability after the oyster transfer and process. One of the critical factors in determining this threat is to assess the viability of *D. vexillum* related to husbandry activities, particularly air exposure from transportation and processing activities.

The viability of colonial tunicates can be evaluated by several clinical markers including siphoning (filtration) activity, circulation (heartbeat) activity and morphological (discolouration) changes (Dijkstra *et al.*, 2008; Rinkevich *et al.*, 1992). Previous use of vital stains has proven valuable in assessing the health of marine species, including tunicates. For example, Hirose (2001) used Neutral Red (NR) stain to detect the presence of acidic bladder cells in different species of the suborders *Stolidobranchia, Phlebobranchia and Aplousobranchia.* Bradway (1936) suggested that NR stains tunicates in areas containing proteolytic enzymes. Although used in the past, NR has not been evaluated as a viability marker on tunicates. In order to conduct the treatment trials, a validation was deemed necessary.

The objective of this study was to assess different methods for determining the viability of *D. vexillum* under controlled experimental conditions by simulating the air exposure encountered during the normal transportation and processing of oysters. Validation experiments were conducted on the violet tunicate (*Botrylloides violaceus* Oka, 1927) to determine and compare the effectiveness of NR. *B. violaceus* was selected as a proxy because *D. vexillum* was not available locally on PEI. The violet tunicate has similar biological features to *D. vexillum* (colony formation and colour morphs) (Carver *et al.*, 2006; Daniel and Therriault, 2007). However, its mortality can be determined under microscopy by observing its filtering action or its response to tactile stimulation (Dijkstra *et al.*, 2008; Rinkevich *et al.*, 1992). The development of a way to determine *D. vexillum* would provide critical information to assess its risk of spread associated with the current oyster harvesting and

processing activities in BC. Furthermore, it will assist with risk assessment, rapid response and development of mitigating measures in the event of *D. vexillum* introduction on the Atlantic coast of Canada.

3.3 Material and Methods

3.3.1 Viability Markers for Tunicates

3.3.1.1 Clinical Viability Markers

In this study, a positive result for filtration consisted of the detection of movement of zooids and/or movement of particles in the water by monitoring using a dissection microscope. The particles in the water came from the substrate attachment point of the tunicate segment. For the tactile stimulation, positive results consisted of zooid contraction followed by a relaxation to the previous state. The tactile stimulus was applied by the light contact of a dissecting probe. If no reaction occurred, this was repeated five times. A negative result occurred when both filtration and tactile response were absent.

3.3.1.2 Vital Stains

To be used as an indicator of tunicate viability, the vital stain needs to be integrated to the organism by filtration. The requirements for a positive and negative stain are explained below.

3.3.1.3 Procurement and Preparation of *B. violaceus*

Thirty-two colonies of *B. violaceus* were obtained from different natural substrata; blue mussels (*Mytilus edulis*), on eel grass (*Zostera marina*) and on rockweed (*Fucus vesiculosus*) at various locations on PEI. Colonies were removed from substrate, cleaned and a 3 cm^2 segment was dissected carefully to avoid unnecessary tissue stimulation. The segments were observed under a dissecting microscope to ensure that the zooids were siphoning and responding normally to tactile stimulation prior to the treatments. To ensure viability, the segments were kept in fresh artificial seawater (ASW), 28 ‰, and the experiments were conducted upon arrival at Atlantic Veterinary College.

3.3.1.4 Development and Validation of a Neutral Red Staining Protocol for *B. violaceus*

Acetic acid has been shown to be an effective way of controlling fouling by killing tunicates (DFO, 2006; Forrest *et al.,* 2007; Piola *et al.,* 2010). A previous study reported that an exposure to a 15 seconds spray of 5 % acetic acid was sufficient to impact *B. violaceus* (Carver, 2006). Therefore, complete submersion of tunicate segments for 15 minutes was chosen to obtain mortality. The tunicate segments were allocated to 16 treatment and control groups. The treatment group was exposed to 5 % acetic acid (household strength) by submerging the tunicate segments for 15 minutes followed by monitoring of the clinical markers to ensure 100 % mortality. After exposure, the tissues were then soaked with light agitation in ASW for 10 minutes to remove residual acetic acid.

The segments of *B. violaceus* were subjected to NR stain trials. A 0.01 % concentration was prepared from 0.01 g of NR (Acros Organic, New Jersey, USA) per 100 mL of filtered ASW (63 µm) (Cook, 1974). Segments were then submerged in 0.01 % staining solution for 15 minutes. All tissues were subsequently bathed in ASW for 5 minutes to remove excess stain. Viability of segments was then assessed under a dissecting microscope (Zeiss Stemi 2000C, Carl Zeiss Jena GmbH, Zeiss Group, Jena, Germany) at a 6.3 x and a 12.6 x magnification. A segment was considered positive when stain was present inside the zooids wall combined with siphoning and response to tactile stimulation. The opposite was considered a negative result. Fresh live zooids (Figure 3.1a) were used as baseline to compare and describe zooids from control and treatment groups (Figure 3.1b&c).

3.3.2 Viability Trial on *D. vexillum* After Air Exposure

3.3.2.1 Trial Protocol

Twenty-seven clusters of Pacific oysters (*Crassostrea gigas*) fouled with *D. vexillum* were collected in March 2009 from an aquaculture site in Lemmens Inlet, BC. The site cultures Pacific oysters (*C. gigas)* on a long line system in clusters, intertwined in a two stranded rope (Figure 3.3). The clusters are composed of a mother shell (clutch) with several live oysters attached and epibionts, including *D. vexillum*. Clusters were transported from the site to a holding area (wharf) near the laboratory in Tofino, BC. The duration of the transit was 20 minutes. The air exposure from transportation and processing activities were simulated by suspending the fouled clusters, with *D. vexillum,* in a field laboratory. The clusters

were transported in buckets containing seawater from the holding area. This laboratory offered a controlled environment where the suspended specimens were sheltered from the variable environmental factors (wind, rain, sun exposure...), allowing a uniform air exposure at temperatures that ranged from 9^0C (mornings and nights) to 13^0C (mid-afternoons). The exposure durations were set at 0 (control), 0.5, 1.5, 3, 6, 12, 24, 48 and 72 hours.

Three clusters/treatments were allocated to the nine air exposures. Following treatment, clusters were returned to the holding area for an acclimation period (30-60 minutes) to allow surviving zooids to recover filtering activities. Three segments (3 cm^2) per cluster were then observed microscopically for clinical markers (as defined above) and exposed to the NR staining protocol. All segments were preserved in 10 % formalin and filtered seawater (63 μm solution).

The preserved tissues were transported to the Atlantic Veterinary College, in Charlottetown, PEI, where all tissue samples were observed under a dissecting microscope at a 6.3 x and 12.6 x magnifications, for NR stain and any other morphological observations. Changes in morphology were graded by two observers using a blinded protocol along with the scale developed in Figure 3.2.

3.3.3 Statistical Analysis

The agreement between the two observers was assessed using an exact symmetry test and the Stuart-Maxwell test for marginal homogeneity in contingency tables. The symmetry test compared symmetrical cells, whereas the marginal

homogeneity test compared the distribution of test results (Table 3.2). Because there was agreement (both $P < 0.05$), only the observations from the first observer were kept for further analysis. These data were transformed to a binary scale, where 0 and 1 were changed to a negative (0) and 1 and 2 were changed to a positive (1). In order to plot a graph, the natural log of the treatment time was used. A lowess smooth plot of the binary observations (Figure 3.4) demonstrates the relationship between the observations and the time of air exposure. The data was analyzed using Stata SE 10.0 (Stata Corp., College Station, TX, USA, 2007).

3.4 Results

3.4.1 Validation Trials on *B. violaceus*

Clinical assessment of all 32 segments before the trials showed no evidence of compromised viability. The zooids were sensitive to tactile stimulation and were actively filtering.

The control stained colonies showed no difference from the baseline zooids. Figure 3.1b shows that the inside and the circumference of the siphon were stained. Conversely, no siphoning action or movement was observed in the treatment group (Figure 3.1c). All of the control groups were confirmed to be alive, demonstrating physiological activity and a noticeable amount of stain inside the zooids. All the treatment groups were confirmed to be non-responsive to tactile stimulation and did not uptake stain.

The experimental results are summarized in a 2x2 contingency table (Table 3.1). The observations were consistent with a systematic staining of live *B. violaceus.*

3.4.2 Air Exposure on *D. vexillum*

No signs of staining were observed in any of the full and sectioned segments of *D. vexillum,* including the controls. The control showed no clinical signs of viability (siphoning and reaction to tactile stimuli). The only change throughout the hours of exposure was the general morphology of the colonies. Visual observation made by two observers showed agreement (P=0.2150). The trend in Figure 3.4 displays a fast initial degradation in *D. vexillum*. This was followed by a steady decrease in health.

3.5 Discussion

Although there are no published studies on the use of vital stains to assess the living state of colonial tunicates, various experiments have been conducted on these biochemical stains for other purposes. NR stain was used to detect acidic bladder cells in different species of the suborders *Stolidobranchia, Phlebobranchia* and *Aplousobranchia* (Hirose, 2001). It was also suggested that NR stains tunicates in areas containing proteolytic enzymes (digestive tract) (Bradway, 1936). In this study, a protocol was developed to assess the viability of *D. vexillum* in relation to treatments simulating transportation and processing conditions.

Because the trials arose from the assessment of the oyster aquaculture industries on Vancouver Island, the protocol was viewed as a possible future routine validation of the state of *D. vexillum* before they would be discarded on the intertidal

zone. Although the results are not conclusive, the protocol was meant to be time efficient if any management options were to be implemented at the processing level. The option of air exposure to reduce viability of tunicates would possibly be an extra management step at the end process. The shells fouled with *D. vexillum* would be exposed to a known environmentally friendly method of treatment before being discarded on the intertidal zone. This method would only be used if the tunicate species in question showed a positive result for the protocol.

In order to manage the risk of introducing *D. vexillum* into new areas, it is essential to ensure the death of the entire colony since establishment can occur from small fragments (Bullard *et al.*, 2007; Valentine *et al.*, 2007). Denny (2008) noticed that *D. vexillum* controls had a 65 % mortality, which would indicate a negative impact coming from manipulations (cutting and handling). Therefore, handling that occurs during the pre- and post-processing of oysters can enhance the mortality of tunicates. Katayama and Ikeda (1987) observed mortality of *Didemnum moseleyi* (later confirmed as *D. vexillum* by Lambert, 2009) after 30 minutes or more of air drying in direct sunlight in June and July, while it took over 5 hours to detect mortality in March. Even though the method of shell disposal has not changed in the past decades, the zones surrounding these processing plants have not become infested with *D. vexillum* (Cohen, 2005; Daniel and Therriault, 2007). It is still critical however to know that the *D. vexillum* is dead before it leaves the processing site as this would prevent the establishment of this invader in the surrounding intertidal areas. The degradation of the *D. vexillum* segments was scaled over time (Figure 3.2

and 3.4). Although we cannot set a threshold for mortality, the degradation follows a steady slope.

The trials on *B. violaceus* demonstrated that all of the control groups were alive and all the treatment groups were dead. Following the results, NR appears to be a useful method for determining the viability of the violet tunicate. The stain enters the siphon opening during active filtration and stains the surrounding structures. The acetic acid treatment was able to kill the tunicate, but residual NR stain was still present in some specimens within the siphon muscle. This staining deposition likely occurs due to residual acetic acid that was not removed following artificial seawater washing. This is consistent with the NR stain, which is also a pH indicator, changing to a darker red in pH lower than 6.8 and yellow in conditions above a pH of 8.0.

A challenge arose in determining viability of *D. vexillum* using the techniques such as filtering ability and reaction to tactile stimulus that were successful for evaluating *B. violaceus*. No filtration or response to stimuli was noticeable using microscopy even with fresh samples. The trials employing the NR vital stain also proved inconclusive. This result could be explained by the elapsed time between the staining and the analysis of the segments (approximately one month), although the absence of physiological markers of viability are still more plausible. Assessments of these clinical markers in *D. vexillum* indicated a poor applicability. Absence of siphoning in *D. vexillum* was apparently not associated with discolouration.

The reasons for the observed differences between the two tunicate species may be basic to their respective biology. The zooids in *D. vexillum* (1-2 mm) are smaller in comparison with *B. violaceus* (2-4 mm) (Carver *et al.*, 2006; Daniel and Therriault, 2007). An observational trial executed after the negative results of the prior trials used menthol crystals to relax the siphons to facilitate the uptake of NR. The same results were encountered. No stain was taken up even if the menthol crystals relaxed the siphon muscle via analgesia (Galeotti *et al.*, 2002). This method was also used to relax tunicates prior to histological preparation (G Lambert, pers. comm.).

D. vexillum demonstrates a tolerance to uncommon situations in comparison with other colonial tunicates. For example, Forrest *et al.* (2007) showed that 4% acetic acid killed the colonial ascidians *Botryllus schlosseri* (Pallas, 1766) and *Botrylloides leachii* (Savigny, 1816), which are morphologically and functionally similar to *D. vexillum.* However, Forrest *et al.* (2007) found that acetic acid was ineffective at killing 100% of the *D. vexillum*. Similarly, Denny (2008) did not observe any effects of a 10 minutes of fresh-water immersion on the survival of *D. vexillum*. This may be associated with the ability of tunicates to close their siphons for extended periods in order to overcome environmental changes.

Ascidians close their siphons for various reasons, ranging from physical disturbance (involuntary movement), light variation or to avoid obstructions. Coutts and Forrest (2005) found that the solitary tunicate, *Styela clava,* was able to withstand 1 hour of fresh water immersion by presumably closing their siphons for

an extensive period. At salinities lower than 20 ‰, ascidians can close their siphons for long periods of time, which can eventually lead to zooid death (Stoner, 1992). Heavy turbidity (sedimentation) cause ascidians to close their oral siphons to prevent the siphon and brachial filtering wall from clogging and ceasing respiration to avoid suffocation (Monniot *et al.*, 1991). Therefore, *D. vexillum* may employ a similar strategy and might not release the siphon muscles due to adverse or changes in its surrounding area.

The *D. vexillum* colonies, as well as *B. violaceus*, used in the trials were mature. The microscopic examination of the colonies revealed a new formation over the old colony. This budding after winter dormancy seems to be consistent with published data suggesting that ascidian urochordates can produce dormant asexual buds (Stebbing, 1970; Bell, 1982; Marks, 1996). Bell (1982) described these as being reminiscent of bryozoans' sessoblasts and Marks (1996) found that they could remain dormant for up to five months before re-establishing a colony.

D. vexillum is suspected to induce and maintain dormancy when exposed to adverse environmental conditions. Whereas some species remain dormant only as long as conditions are unfavourable, others remain dormant for periods much longer than the unfavourable environmental conditions, with dormancy extending beyond the duration of the environmental hardship which introduces potential costs to the reproductive capacities of the specie (Cáceres, 1997).

This study found that *B. violaceus* was susceptible to acetic acid treatment and that an established protocol that included physiological (filtration and response

to stimuli) and NR vital stains could be used to assess viability. However, these methods were not translatable to *D. vexillum.* Due to this, further studies in the determination of *D. vexillum*'s viability after handling are needed. Other researchers have encountered difficulties in keeping *D. vexillum* alive in a closed circuit and have had problems in accurately determining if it is dead or alive (Forrest BM, pers. comm., Valentine PC, pers. comm., Therriault TW, pers. comm.). Future studies could include molecular analysis to evaluate the degeneration of *D. vexillum.* Quantification of DNA/RNA after performing treatments similar to this trial, could give a measure of internal degradation of nucleic acids up to the time of death or a plateau for a dormant stage. Others studies could include the quantification of particle depletion or the uptake of O_2 marking metabolism rate. The results would provide a better understanding of the biology of *D. vexillum.* The ability to determine mortality in *D. vexillum* would certainly help in the reduction of its spread.

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Table 3.1 2x2 contingency table for the observed stained *B. violaceus* colonies.

	Treatment		
	NR	Acetic Acid + NR	Total
Alive	16	0	16
Dead	0	16	16
Total	16	16	32

Table 3.2 Contingency table comparing the results of the scale based observations from two observers on the pictures of the treated segments of *D. vexillum.*

	Observer 2				
Observer 1	**0**	**1**	**2**	**3**	Total
0	**18**	6	1	0	*25*
1	10	**10**	4	0	*24*
2	2	11	**6**	1	*20*
3	0	1	3	**8**	*12*
Total	*30*	*28*	*14*	*9*	81

a Symmetry test compared symmetrical cells around the agreement diagonal (in bold).
B Marginal homogeneity test compared the marginal distributions of the two observers (italicized).

Table 3.3 Contrast between *D. vexillum* and *B. violaceus* viability markers after staining of the control segments with Neutral Red.

D. vexillum	**Viability Marker**	***B. violaceus***
No particle movement	**Filtration**	Particle movement near siphons
No zooid movement	**Tactile Stimulus**	Zooid contraction and return to relaxed state
No Staining of tissue	**Stain**	Stain inside zooids

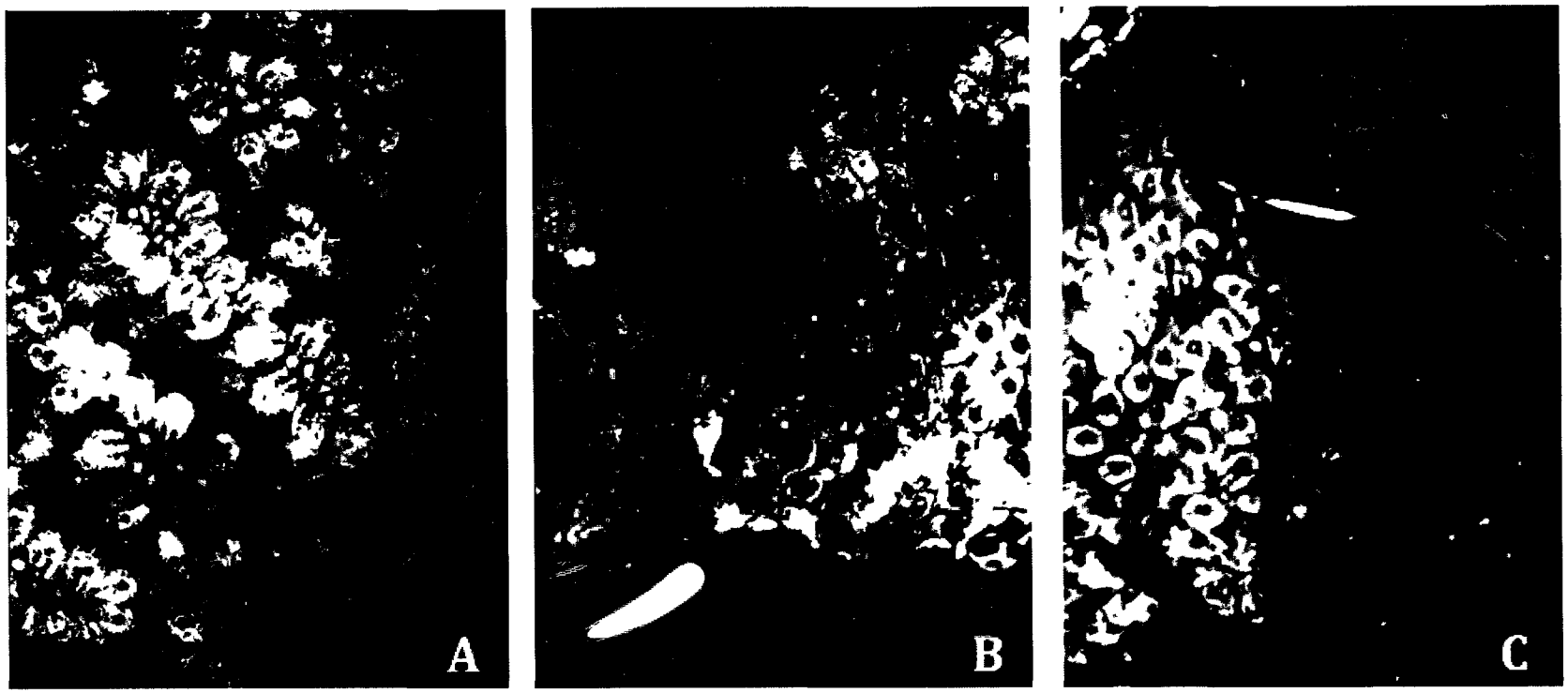

Figure 3.1 The three different zooid states used to compare a dead and a live colony of *B. violaceus*. (A) Intact tunicate showing a healthy zooid. (B) Control group - Healthy zooids after a 15 minute submersion in 0.01 % Neutral Red and ASW solution (C) Treatment Group - Dead zooids after a 15 minute submersion in 5 % acetic acid followed by a 15 minute submersion in 0.01 % NR. All images were at a 6.3 x magnification.

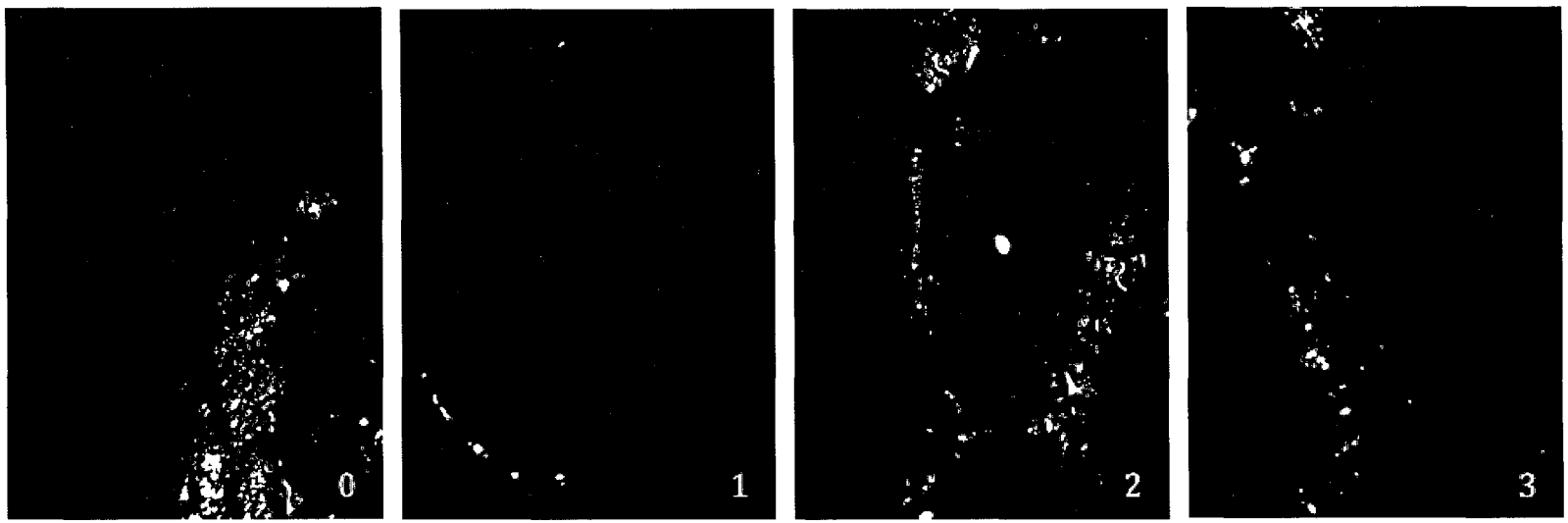

Figure 3.2 Grading scale for the deterioration of the morphology of *D. vexillum* zooids. From left to right, the colonies have a weaker grade on a scale of 0 to 3. (0) Represents a fresh colony (control) were all the zooids show no sign of deterioration. (1) Represents a colony that received a mild treatment effect, a few zooids started to lose their shape and/or degrade. (2) Represents a colony that received a moderate treatment effect, more than half of the zooids are irregular and degraded, (3) Represents a colony that received a severe treatment effect. Almost all the zooids are irregular to the point that they are unrecognizable. All are at a 6.3 x magnification.

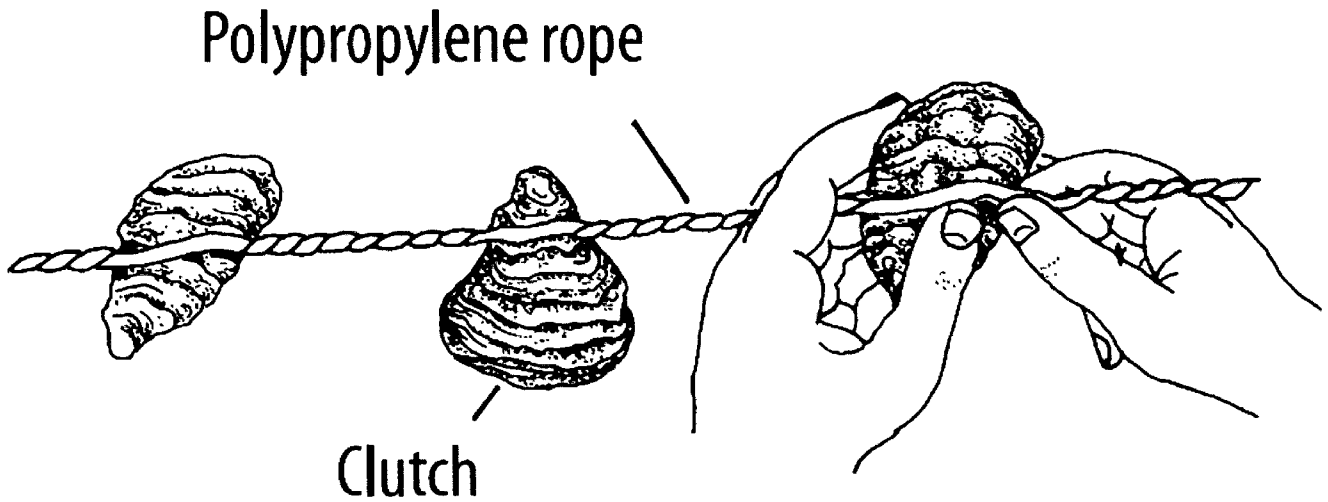

Figure 3.3 Placement of mother shell (clutch) between the two strands of a rope in a suspended long line system. (Illustration from Toba D, 2004)

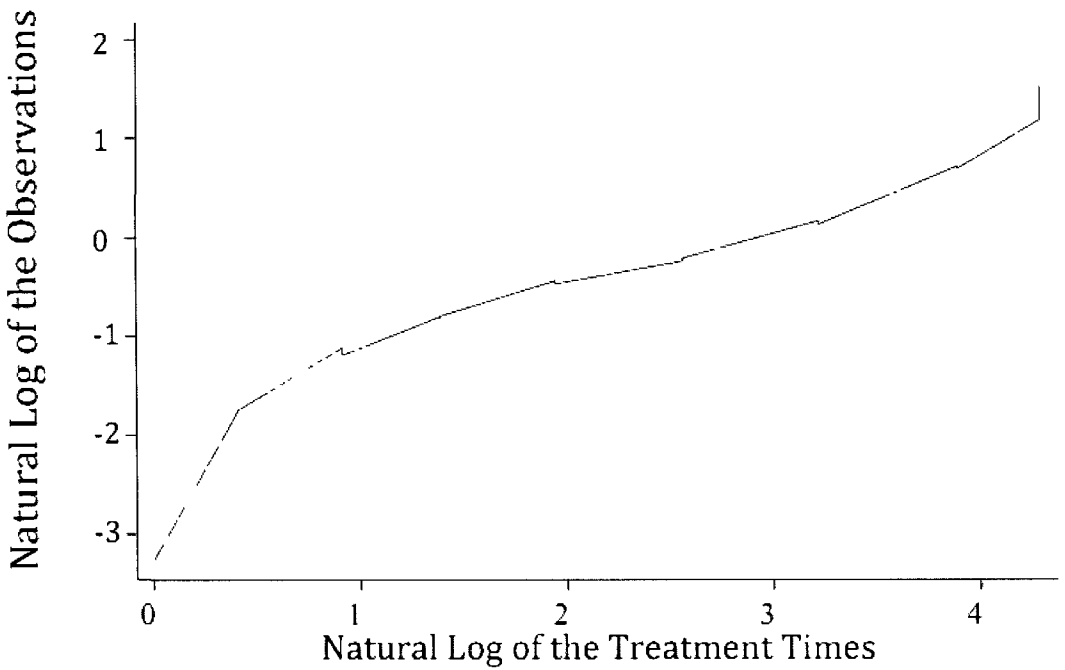

Figure 3.4 The solid line is the lowess smooth curve representing the relationship between the treatment times (using a natural log scale) and the observations on the grading scale for the deterioration of the morphology of *D. vexillum* zooids.

Chapter 4

The Fertility of *Ciona intestinalis* Eggs in Mussel Processing Plant Conditions

4.1 Abstract

Two separate experimental trials focused on the effect of water turbidity and water flow on *C. intestinalis* egg fertilization. The first trial evaluated the effect of water turbidity (0, 300, 600 and 1 200 NTU), and the second evaluated the effect of water turbidity (0, 300, 600 and 1 200 NTU) with water flow (0.9583 L/s). Unfertilized eggs were then exposed to viable sperm and incubated at 21^0C for 48 hours. Total propagules (eggs, larvae and recruits) were counted under a dissecting microscope for three trial replicates. Levels of turbidity did not change the fertilization rate (65.88 ±2.95 %). Water flow decreased fertilization rates but there was no difference between turbidity levels. The control had a mean fertilization rate of 63.14 % (±1.75 %), while the mean of the treatments was 50.25 % (±2.85 %). These trials continue to provide a clear indication of the potential propagule pressure of processing plants. Additional *in situ* experiments are needed to determine potential mitigation measures.

4.2 Introduction

The introduction of aquatic invasive species (AIS) is occurring at a high frequency in coastal regions of the world (Carlton, 1989). This is mainly due to the transportation and release of ships ballast water (Carlton, 1987; Carlton and Geller, 1993). Carlton and Geller (1993) estimate that more than 3000 species are potentially in motion around the world in large vessels and therefore, more invasions are likely to occur in coastal environments (Carlton, 1996). Other vectors are found at regional scales, which result in spread of AIS within a region (Lodge *et al.*, 1998). Although the dispersion of AIS can be natural at smaller scales, (water currents or attached to floating objects) (Carlton, 1987), anthropogenic transportation has been the main cause of successful invasions of AIS (Lambert, 2005). In these, we can find movement of recreational boats (Floerl and Inglis, 2003) and the aquaculture and fishing industries (Naylor *et al.*, 2001).

The blue mussel, *Mytilus edulis* (Linnaeus, 1758), is the most important aquaculture species on Prince Edward Island (PEI) with sales of $36.3 M contributing $106 M to the province's Gross Domestic Product (DFO, 2006). These tunicates negatively impact the mussel industry by fouling aquaculture gear, product (Darbyson *et al.*, 2009) and by increasing labour time and cost of culture (DFO, 2006). The estuaries of PEI mainly have mud and sandy bottoms, but the gear used for aquaculture provides a hard substrate that favours settlement of AIS (Tyrrell and Byers, 2007).

PEI currently has four invasive tunicates that affect the mussel industry. The clubbed tunicate, *Styela clava* Herdman, 1881, was first identified in 1997 followed by the golden star tunicate, *Botryllus schlosseri* (Pallas, 1766), in 2001; the violet tunicate, *Botrylloides violaceus* Oka, 1927 in 2002; and the vase tunicate, *Ciona intestinalis* (Linnaeus, 1767), in 2004 (Gill *et al.*, 2007; Locke *et al.*, 2007).

C. intestinalis was first identified in the Montague River and is now the dominant species of tunicate where it has outcompeted *S. clava* (Ramsay *et al.*, 2008; MacNair, 2005). *C. intestinalis* also is present in adjacent and high traffic bays including Souris Harbour, Cardigan River, Brudenell River, St. Mary's Bay, Murray River, and Charlottetown Harbour (DFO, 2006; Locke *et al.*, 2009b). The spread of this solitary tunicate in PEI is not limited to passive larval dispersal as other vectors can contribute to their spread including boat hulls, flotsam, and the aquaculture industry (Therriault and Herborg, 2008; Dogshun *et al.*, 2007; Locke *et al.*, 2007).

In PEI, tunicate management's primary objective is to remove large masses of tunicates from blue mussel aquaculture lines (Locke *et al.*, 2009a) and secondly, to limit the spread of invasive tunicates by controlling vectors associated with the transfer and harvest of aquaculture species (Locke *et al.*, 2009a; Locke *et al.*, 2009b).

In mussel processing plants, the mechanical removal/manipulation of *C. intestinalis* can lead to crushing and tearing of their tunic thereby releasing gametes into the effluent water (Bourque *et al.*, 2007). The processing plant effluent water is passed through a No. 25 mesh screen (710 μm) which removes adult tunicates very

efficiently but is inefficient in removing eggs and larvae (respectively 150-180 μm and 100-200 μm in diameter) (Carver *et. al,* 2006).

Eggs of *C. intestinalis* were found in all of the processing stages (stripping, holding, processing) with the highest number being found in the stripping phase averaging 1000-5000 eggs per second. The exact amounts are dependent on the tunicate reproductive status which occurs from June until the end of October (Carver *et al.*, 2003; Ramsay *et al.*, 2009).

C. intestinalis reproduces sexually with the larval life stage lasting approximately 12 hours prior to spontaneously settling over a period of minutes (Berril, 1947; Cirino *et al.*, 2002). *C. intestinalis* generally produces 500-1000 eggs daily (Yamaguchi, 1975; Carver *et al.*, 2003). Unfertilized eggs remain functional for 24-30 hours and spermatozoa 16 hours in seawater (Morgan, 1945; Svane and Havendand, 1993).

The mussel processing plant effluent water exposes *C. intestinalis* eggs to various salinity, temperature, water flow, and turbidity conditions. Each variable may individually or through interactions, impact the fertilization process.

Bourque *et al.* (2007) reported that fertilization did not occur when *C. intestinalis* eggs were exposed to low salinity (below 10 ‰) and then subsequently returned to a more favourable 28 ‰. Also, eggs that were fertilized prior to being exposed to water below 10 ‰ for at least 5 minutes, did not developmentally progress to the next stages (Bourque *et al,.* 2007). Natural spawning in *C. intestinalis*

occurs at water temperature greater than 6^0C, and lower water temperatures either inhibit or slow egg maturation and growth (Joly *et al.*, 2007). The tolerance limits of normal embryonic and larval development ranges from approximately 6-24^0C, while the speed at which the development in each of the stages occurs is positively correlated with environmental temperatures (Dybern, 1965). Tung *et al.* (1941) reported that centrifugation of *C. intestinalis* eggs, before fertilization, reduces early development. The majority of eggs centrifuged at a minimum of 2000 rpm for 10 minutes never reached the larval stage. The effect of turbidity on the fertilization of *C. intestinalis* is suspected to interfere with the fertilization process but has never been investigated. Galbraith (2006) reported an inverse linear relationship of suspended sediment concentration on the proportion of successfully fertilized eggs in sockeye (*Oncorhynchus nerka*) and coho (*Oncorhynchus kisutch*) salmon. It was suspected that the suspended solids interfered with the egg-sperm interaction. A recent study by Griffin *et al.* (2009) on Pacific herring (*Clupea pallasi*) showed that the vitelline coat is susceptible to the permanent attachment of sediment particle during the first 2 hours that the eggs were in the water. The sediment did not reduce hatchability, but significantly increased the amount of abnormal larvae.

The aim of this project was to assess if the eggs of *C. intestinalis* would still be fertile after exposure to environmental factors similar to those encountered in mussel processing plant conditions. This study specifically focused on the effect of water turbidity and flow. These two parameters were mimicked under controlled experimental settings. Two separate trials were conducted: one to evaluate the effect of water turbidity alone, and the other to evaluate the interaction between

water turbidity and water flow. The results of this study could provide information to the mussel processing industry that would be helpful in exploring future tunicate mitigation management strategies.

4.3 Materials and Methods

Preliminary work from Bourque *et al.* (2007) included an assessment of the various conditions encountered by *C. intestinalis* eggs in the effluents of processing plants. The average amount of time that eggs could be exposed to these variables in the processing plants was established at 5 minutes. The flow rate of the effluent water in mussel processing plants varied from 0.05 L/s to 67.7 L/s and the turbidity ranged from 50 to 1100 Nephelometric Turbidity Units (NTU).

4.3.1 Procurement and Selection of *C. intestinalis*

C. intestinalis were collected, in September 2009, from mussel socks on a lease in Montague River, PEI and held in a recirculation system prior to the start of the trials. Sexually mature tunicates were selected for the trial based on the criteria of a full sperm duct and total body lengths from 8-10 cm (Dybern, 1965; Cirino *et al.*, 2002).

4.3.2 Effects of Turbidity on Egg Fertility

4.3.2.1 Trial Conditions

Turbidity was simulated by adding silt collected from the bottom of a mussel lease, to artificial sea water (ASW) in an 125 mL Erlenmeyer flask. All ASW used in

the trials was at 28 ‰ and filtered with a 0.45 μm bottle top filter. Turbidity levels used in the trials were verified spectrophotometrically (HACH DR/400V, Loveland, USA). The four turbidity levels used in the trial (Table 4.1) corresponded with the range of levels found in typical mussel processing plant effluent water. The trial was repeated three times.

4.3.2.2 Fertilization

The fertilization protocol was modified from that designed by S. Stewart-Clark (Appendix A). Twenty-four mature *C. intestinalis* were used in each of the repetitions. Eggs from 16 tunicates were collected in a beaker containing 200 mL of ASW. Sperm was gathered from the remaining 8 tunicates in a beaker containing 100 mL of ASW.

The unfertilized eggs were then divided equally by volume (50 mL) into four beakers corresponding to the four turbidity levels; 0, 300, 600 and 1200 NTU (Table 4.1). The sperm was divided in four parts (25 mL) and added to the beakers containing unfertilized eggs. Sperm was added 5 minutes after the repartition of the eggs to represent the established exposure time determined by Bourque *et al.* (2007). Each beaker was then equally divided into three Petri dishes (25 mL) and incubated at 21^0C for 48 hours.

The incubation time of 48 hours was used to maximize the number of larvae reaching metamorphosis. At this temperature, the fertilized egg is expected to transform to a larval stage within 20 hours. The newly hatched larva settles on the

substrate within 2 hours which then triggers metamorphosis which begins with tail resorption (Cirino *et al.*, 2002). A volume of 1 mL of formaldehyde (37 %) was added to the Petri dishes after 48 hours of incubation to kill the propagules. The total number of propagules (eggs, larvae and recruits) within each Petri dish was counted under a dissecting microscope using a Ward Zooplankton counting wheel.

4.3.3 Combined Effects of Turbidity and Water Flow on Egg Fertility

4.3.3.1 Trial Conditions

Turbidity was determined as in the previous trial. The water flow was simulated in an Erlenmeyer flask with a magnetic stir strip. A stir plate was used at the relative speed of 1150 rotations per minute (rpm). The volume of liquid in the flask was 50 mL. While the magnetic strip was rotating, the liquid reached the magnetic strips velocity when little or no turbulence was apparent (Faber, 2004). When this velocity was matched, the flow rate (L/s) can be calculated by multiplying the relative rpm by the volume used. In this case, the water flow was 0.9583 L/s. The experimental design is shown in Table 4.2.

4.3.3.2 Fertilization

The protocol and proportions were the same as used in the turbidity trial, but this trial took account for an additional treatment. Thirty mature *C. intestinalis* were used in each of the repetitions. Eggs were collected from 20 tunicates into a beaker containing 250 mL of ASW. Sperm was gathered from the remaining 10 tunicates into a beaker containing 125 mL of ASW.

The harvested unfertilized eggs were then divided equally by volume (50 mL) into five beakers for the 0 (control, no water flow), 0 (control, with water flow), 300, 600 and 1200 NTU (Table 4.2). The sperm was divided into five equal parts (25 mL) and added to the beakers containing unfertilized eggs after their exposure to water flow. In the case of the control (no water flow), sperm was added 5 minutes after the repartition of the eggs to represent the water flow used in the four other beakers. The remainder of the experiment followed the methods as outlined in the previous trial.

4.3.4 Statistical Analysis

A linear mixed model (Dohoo *et al.*, 2009) was built to determine significant differences between the treatments. Each repetition of the trials was considered as the blocking variable. The models for the trials included a random effect for the beakers (separate treatments). The model assumptions were assessed by standard residuals. Counts for the assessment were distributed by the success of the fertilization. Larvae and recruits were categorized as successful fertilizations while the remaining eggs were categorized as unsuccessful fertilizations. The percentages of successful fertilization were used for the analysis. Data analyses were performed using the statistical package Stata SE 10.0 (Stata Corporation, College Station, Texas).

4.4 Results

4.4.1 Effects of Turbidity on Egg Fertility

One Petri dish in the controls of the third block desiccated and was not counted (missing value). The average proportion (mean ± SE) of successful fertilization was 65.88 ±2.95 % regardless of the treatment, and changes in water turbidity did not impact the fertilization process (P=0.1976). No significant fertility difference was detected between the control (0 NTU) and 300 NTU, 600 NTU and 1200 NTU treatments (Figure 4.1). When looking at the blocks (trial repetitions), the second trial had significantly higher fertilization rates than the first trial (P=0.001) and third trial (P= 0.0007). However, the first and third trials were similar (P=0.928).

4.4.2 Combined Effects of Turbidity and Water Flow on Egg Fertility

Successful fertilization for the turbidity and water flow trials resulted in statistically significant differences between the treatments. The control (no water flow) was significantly different from the 1150 rpm (flow) (P=0.041), the 300 NTU+ flow (P=0.025) and the 1200 NTU (P=0.047) while the 600 NTU+flow (P=0.129) was not (Figure 4.2).

The water flow with 0 NTU was marginally significant from the 300 NTU+flow (P=0.0463) and non-significant with the 600 NTU+flow treatments (P=0.1047) and the 1200 NTU+flow (P=0.0665). The turbidity treatments were not significantly different from each other. When looking at the blocks (trial

repetitions), the second trial had significantly lower fertilization rates than the first trial (P=0.045) and the third trial (P= 0.0109). The first and third trials were similar (P= 0.350).

4.5 Discussion

C. intestinalis eggs display a tolerance to variations in environmental conditions. Morgan (1945) showed that delaying the addition of sperm to unfertilized eggs for up to 3 hours does not affect normal larval development. It was also reported that a reduction in the volume of seawater, for the same amount of gametes, does not affect normal development. *C. intestinalis* egg and larval development is tolerant to low temperature (~8^{0}C) (Ramsay *et al.*, 2008; Joly *et al.*, 2007; Dybern, 1965). Dybern (1967) and Bourque *et al.* (2007) also reported the ability of *C. intestinalis* to tolerate lower salinity (12 ‰) for short time periods. The current objective was to assess the fertility of *C. intestinalis* eggs using environmental conditions similar to the effluent water of mussel processing plants in a controlled laboratory setting.

In our experiments, the treatment levels of turbidity, low to high, did not alter the success of fertilization. Observations made throughout the trials showed that the eggs that did not develop had lost their vitelline coat. The number of eggs impaired by the loss of the vitelline coat increased accordingly with greater water turbidity. The silt added to the water to increase the turbidity likely contributed to the vitelline coat decomposition. This only occurred later during the incubation period since the fertilization rates in the treatments were not different from the

controls. The same observation was made during the combined turbidity and water flow trials. The control was shown to have a higher fertilization rate over the treatments. Although the control was not significantly different with 600 NTU+flow, it had a ~10 % higher difference in total fertilization rate. But, when comparing the treatments together, the percentages where similar. This suggests that flow, even 600 NTU+flow, decreases the likelihood of fertilization.

C. intestinalis eggs in the current trials were subjected to silt concentration of 1.75 g/L to 7 g/L without effects on fertilization. This is opposite to what was found for the eggs of *Mercenaria mercenaria*, the Northern quahog, as they didn't develop into larvae in silt concentrations over 2 g/L (Davis, 1960). Similarly, fertilization success in coho and sockeye salmon was reduced to <80 % when turbidity levels were ~10 g/L (Galbraith *et al.,* 2006). This suggests that *C. intestinalis* eggs are more tolerant to turbidity, which increases their success at fertilization.

The flow rate used in the trials was constant at 0.9583 L/s. This contributed to the lower fertilization success but this only matches the lower end flow rates that *C. intestinalis* propagules would encounter in mussel processing plant effluent water (0.05 L/s to 67.7 L/s). Reproducing the higher water flow rates in a laboratory setting would be difficult. The effect of the higher flow rates in effluent water within the processing plant system is not well known. The eggs could lose their vitelline coat, and therefore lose their follicle cells, which would compromise the fertilization process (Rosati and De Santis, 1978; Kawamura *et al.*, 1988; Miller, 1975; Villa and Patricolo, 1993; Yoshida *et al.*, 1993).

In the purple sea urchin, *Strongylocentrotus purpuratus*, a fraction (2 %) of the fertilized eggs exposed to turbulent conditions were shown to develop into normal blastulae indicating that turbulence stress affects reproductive success. Although low levels of turbulence can aid in mixing of gametes and aid fertilization, high turbulence levels can have severe mechanical effects that hinder development (Mead and Denny, 1995). Tung *et al.* (1941) reported that stronger flow rates might halt the development by displacing the mitochondria, the hyaloplasm, and the yolk from their normal position in *C. intestinalis* eggs.

In the present study, fertilization of *C. intestinalis* eggs was 48 % or more in the treatments (Figure 4.1 and Figure 4.2). This fertilization success should ensure a continuation of the colonization of bay waters surrounding processing plants by *C. intestinalis.* However, regardless of this high settlement possibility, some mussel processing plants around PEI that routinely encounter AIS infested aquaculture material, are not experiencing repercussions to the surrounding water bodies.

For example, a New London area processing plant has discharged wastewater from processed mussel socks carrying *S. clava.* Although there is extensive aquaculture and agricultural runoff, there were no further indications of *S. clava* establishing in that bay/estuary (Locke *et al.*, 2007). Since this mussel processing plant uses wellwater which exposes the gametes, early life stages and mature animals to freshwater at 8^0C temperature as part of the effluent water, this may kill the tunicates. The numbers of intact eggs present in the effluent was found to be lower and they had an abnormal appearance (loss of vitelline coat,

discolouration) (D. Bourque pers. comm.). Therefore, freshwater within the effluent would lower the salinity that the tunicate eggs would be exposed and likely reduces their viability.

Another bay is currently being studied in a research project with the Department of Fisheries and Oceans in collaboration with the Université de Moncton. A processing plant near Orwell Bay deals with *C. intestinalis* infested product without the ensuing establishment of this tunicate. The study in progress is looking at the conditions surrounding this processing plant and comparing it with a bay that is highly infested (watershed structure, land use, hydrological patterns, meteorological data, bathymetry, topography and water quality). This processing plant also uses well water, but only uses drum filters (710 μm). Despite multiple introduction opportunities into the aquatic ecosystem, *C. intestinalis* has not established. A high amount of suspended inorganic matter is suspected of interfering in *C. intestinalis'* life cycle and reproduction.

Further investigations into the effect of the effluent water on the gametes of *C. intestinalis* are needed. The laboratory trials provided a controlled setting for the exposure of eggs to the potential external factors (turbidity and water flow rate) that may impact tunicate development. However, these controlled experiments cannot replicate all variables found within the effluent of processing plants. Morgan (1945) found that contamination, likely bacterial or organic in origin, can affect later stages of the tunicate eggs development. These same factors could be found at the exit of the effluent water into the surrounding water bodies.

Although tunicate sperm are activated by saltwater, the added effects of the treatments on the sperm might be more damaging then what was seen on the eggs. The flow and added water from the washes and surrounding water bodies likely decreases the sperm concentration, resulting in a lower fertilization rate (Levitan *et al.*, 1992). Hence, the eggs would also be dispersed in the receiving water body. The fertilization would then depend on the local concentrations of sperm, which would decrease (Levitan *et al.,* 1991). The effects of the effluent needs to be evaluated on the sperm of *C. intestinalis* and on the eggs and sperm simultaneously in a laboratory setting to provide a more comprehensive understanding of the fertilization process of this tunicate. Ultimately, a larger scale trial conducted *in situ* (processing plant effluent) would give a more realistic view of the viability of *C. intestinalis* gametes after exposure to environmental factors found in mussel processing plants.

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Table 4.1 Treatment conditions for the turbidity trials on unfertilized *C. intestinalis* eggs.

Treatment #	Turbidity (NTU)	Silt (g)
1	0	-
2	300	0.0875
3	600	0.1750
4	1200	0. 3500

Table 4.2 Treatment variations for the turbidity and water flow trials on unfertilized *C. intestinalis* eggs.

Treatment #	Turbidity (NTU)	Silt (g)	Turbulence
1	0	-	No
2	0	0.0000	Yes
3	300	0.0875	Yes
4	600	0.1750	Yes
5	1200	0.3500	Yes

Table 4.3 Proportions of successful fertilization and egg count for *C. intestinalis* by Petri dish and average grouped within repetition and treatments.

Rep.	Turbidity	Petri 1		Petri 2		Petri3		Mean (%)
		F (%)	Egg count	F (%)	Egg count	F (%)	Egg count	
1	0NTU(control)	60.8	965	66.2	910	67.2	955	64.7
	300NTU	54.5	963	55.9	993	51.0	1117	53.8
	600NTU	64.3	1024	64.4	1057	71.3	893	66.7
	1200NTU	68.5	897	68.9	938	74.1	1008	70.5
2	0NTU(control)	69.1	1131	63.9	1266	71.3	1186	68.1
	300NTU	73.7	1450	73.6	1371	77.4	1243	74.9
	600NTU	77.5	1118	79.0	1182	79.7	1220	78.7
	1200NTU	79.2	970	81.8	1038	81.7	1098	80.9
3	0NTU(control)	missing		65.4	1078	68.3	1086	44.6
	300NTU	64.4	1035	58.3	888	59.1	865	60.6
	600NTU	63.5	1050	64.4	1094	69.3	1017	65.7
	1200NTU	60.0	754	61.7	725	62.7	751	61.4

Table 4.4 Percentage of successful fertilization by Petri dish and average for *C. intestinalis* grouped within blocks by treatments and flow.

Rep	Turbidity	Water flow (1150rpm)	Petri 1		Petri 2		Petri 3		Mean (%)
			F (%)	Egg count	F (%)	Egg count	F (%)	Egg count	
1	0NTU(control)	No	62.7	1100	59.7	1216	58.2	1079	60.2
	0NTU	Yes	58.8	962	55.0	1021	46.0	1467	53.3
	300NTU	Yes	55.4	1127	54.9	1012	54.3	867	54.9
	600NTU	Yes	51.5	915	54.6	980	49.9	1016	52.0
	1200NTU	Yes	51.0	1243	52.5	904	53.3	921	52.3
2	0NTU(control)	No	60.3	982	62.0	892	63.4	746	61.9
	0NTU	Yes	42.0	975	41.5	549	39.3	478	40.9
	300NTU	Yes	34.2	672	35.1	658	28.6	655	32.6
	600NTU	Yes	55.0	825	53.5	817	55.7	668	54.7
	1200NTU	Yes	34.6	956	33.0	860	30.1	505	32.6
3	0NTU(control)	No	71.4	871	71.2	897	55.7	607	66.1
	0NTU	Yes	57.0	869	56.1	866	50.8	709	54.6
	300NTU	Yes	53.4	890	56.8	722	63.0	548	57.7
	600NTU	Yes	47.4	770	53.4	481	56.0	414	52.3
	1200NTU	Yes	65.5	667	63.6	826	66.3	719	65.1

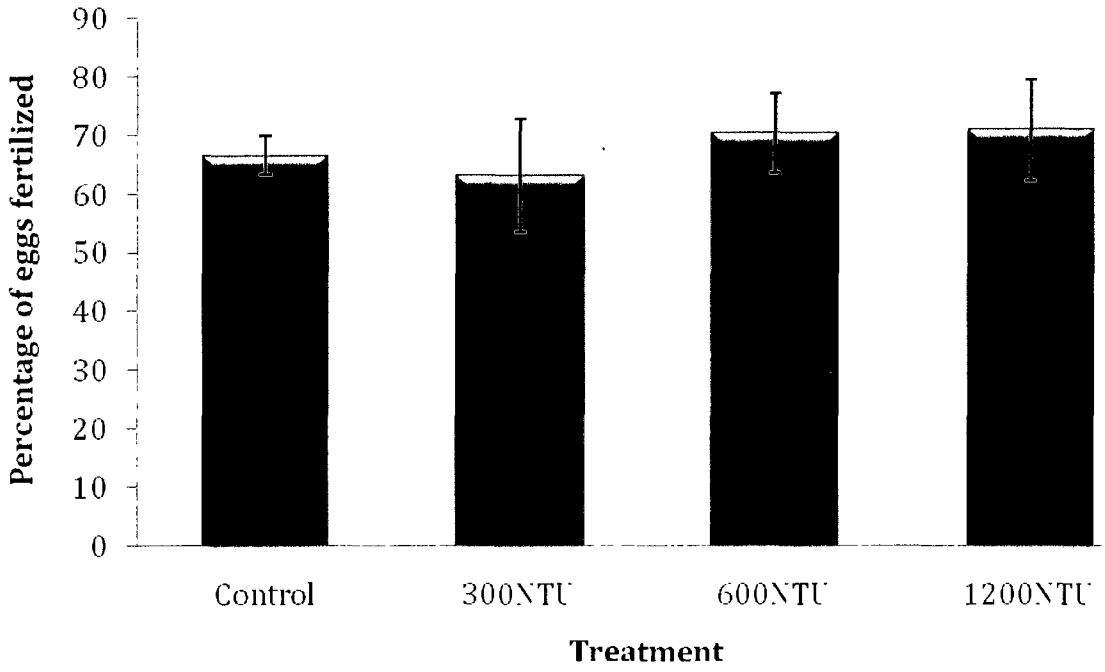


Figure 4.1 Percentage of *C. intestinalis* eggs successfully fertilized in each treatment (Control, 300 NTU, 600 NTU and 1200 NTU) with standard errors bars.

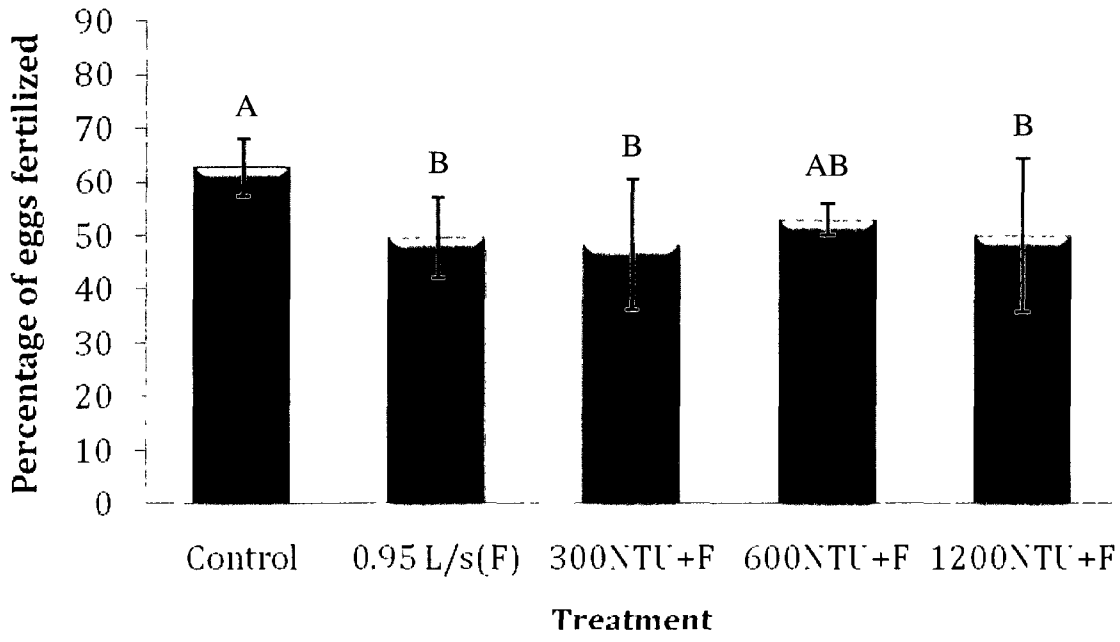
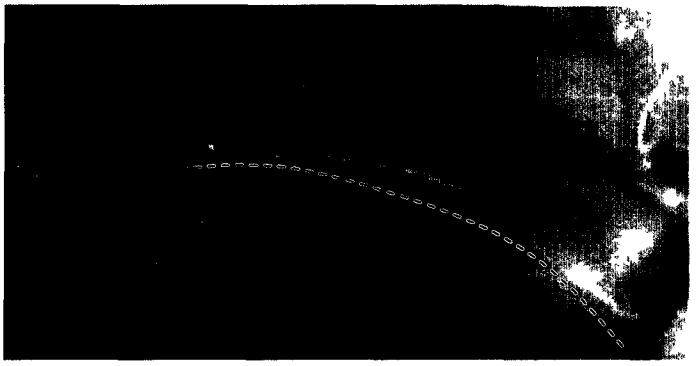


Figure 4.2 Percentage of *C. intestinalis* eggs successfully fertilized in each treatment (Control, 0.95 L/s (F), 300 NTU+F, 600 NTU+F and 1200 NTU+F) with standard errors bars. Treatments with the same letter are not significantly different at the 5% level.

Appendix A:

S. Clark 08/27/2007- Producing *Ciona* larvae in the lab

Without removing the tunic, make an incision alongside the sperm duct and oviduct from just anterior of the ovary to the anterior end of the tunicate between the siphons. Be careful not to pierce either of the ducts with this incision. Ensure that the perm and oviduct are accessible, removing any membranous material or tissue surrounding the ducts. (If this tissue is not removed, when the ducts are pierced, gametes will be released inside the membranous layers instead of externally where they can be collected).



Gently massaging the oviduct, push as many of the eggs as possible to one region of the oviduct. At this region, pierce the oviduct with a 25 gauge syringe needle. Immediately insert a 100 ul pipette into the pierced region and collect eggs as they escape. If possible, insert the pipette tip into the pierced opening of the oviduct and suck any remaining eggs from the oviduct.

Pipette eggs into a glass beaker containing 150 ml of filtered sea water. Keep adding eggs to the beaker as they are collected.

Be careful not to pierce the sperm ducts while removing eggs. If this occurs, discard the specimen without using any of the sperm or eggs. Once all of the eggs have been removed, set tunicate aside for later sperm collection and proceed to collect eggs from the next specimen. I recommend harvesting eggs from nine tunicates and sperm from 6 tunicates for each experiment.

Once all of the eggs have been collected out of the tunicates, proceed to harvest sperm from each of the previously dissected tunicates. To harvest sperm, pierce the sperm duct with a 25 gauge syringe needle. Place a 100 ul pipette into the pierced region and collect sperm. Pipette sperm into a beaker filled with 50ml of filtered sea water.

Sperm should not be left for long periods of time in this beaker. As soon all of the sperm has been collected, pour the contents of the sperm beaker into the beaker containing the eggs. Gently swirl the beaker to facilitate gamete mixing. Pour contents of beaker into Petri dishes and let sit on a lab bench until larval collection occurs. About 16 hours post mixture of sperm and eggs free swimming larvae will be present.

Chapter 5

Summary and Conclusion

Aquatic invasive species (AIS) are introduced into coastal regions of the world at a high frequency (Carlton, 1989). More than 3000 species are potentially in movement around the world in large vessels resulting in more invasions (Carlton and Geller, 1993). Other vectors are found regionally, contributing to secondary spread of AIS (Lodge *et al.*, 1998). Anthropogenic transportation has been the main cause of successful spread of AIS (Lambert, 2005). Among regional vectors (i.e. movement of recreational boats, fishing industries), the aquaculture industry is a vector not to be overlooked. In Prince Edward Island (PEI) and British Columbia (BC), there is a concern that vessels, gear and stock are moved between estuaries since leases sites and processing plants are usually in more than one location. The research presented serves as part of preliminary assessment of the aquaculture industry with respect to AIS.

5.1 *Didemnum vexillum* in the Pacific Oyster Aquaculture Practices

Aquaculture transfers are regarded by experts to be a vector of high importance (Herborg *et al.*, 2009). Although similar culture and transportation methods were used since the early 1950s (Quayle, 1969), *Didemnum vexillum* has still not been reported in the proximity of processing plants on the East coast of Vancouver, Island.

The observational study, demonstrated a significant loss of *D. vexillum* coverage on *Crassostrea gigas* clusters from Post-Harvest to Post-Shucking for both sites. Although receiving the largest reduction, the additional hours of transport and the water mode of transport was not likely the determining factor in this reduction

of coverage in Okeover Inlet. Being able to track the loss of *D. vexillum* highlighted that the major loss of tunicate in transit represents a possible spread between the two geographical locations. The difference between the two sites (Lemmens and Okeover Inlet) was linked to the added manipulation by the grower in Lemmens Inlet. The higher manipulations easily regulated the amount of biofouling that was transferred to the next steps in the process. This process demonstrated that the clusters maintained a stable level of coverage from Post-Harvest to Post-Shucking reducing the chance of spread along the way.

The significant decrease in tunicate coverage on the clusters of Pacific oyster still leaves a substantial amount to be disposed of in the intertidal zones. Although not investigated, reasons for the absence of *D. vexillum* in these waters are unknown. Further investigations on colonial tunicate viability were conducted in Chapter 3.

5.2 *Didemnum vexillum* Viability Assessment

As mentioned previously, transfers of Pacific oysters colonised with *D. vexillum* to processing plants in non-infested areas were observed on Vancouver Island, BC. Assessing the viability of *D. vexillum* related to husbandry activities, particularly air exposure from transportation and processing activities is a critical factor in determining the threat of further invasions.

This part of the study assessed different methods to determine viability in *D. vexillum* under controlled conditions that simulated the air exposure during transfer

from harvest sites to the processing plant. Validation experiment on *Botrylloides violaceus* using clinical viability markers (filtration and movement of the zooids and/or movement of particles in the water with or without tactile stimulus) and Neutral Red (NR) vital stain in assessing tunicate viability proved to be successful. However, determining viability of *D. vexillum* using these techniques was inconclusive.

The biology of each tunicate species might explain these contrasting results. The zooids in *D. vexillum* (1-2 mm) are smaller in comparison with *B. violaceus* (2-4 mm) and *D. vexillum* has been shown to have higher tolerance to environmental changes in comparison with other colonial tunicates. For example, a 4% acetic acid immersion killed the colonial ascidians *Botryllus schlosseri* and *Botrylloides leachii*, which are morphologically and functionally similar to *D. vexillum* (Forrest *et al.*, 2007). Similarly, 10 minutes of fresh water immersion had no effect on the survival of *D. vexillum* (Denny, 2008). *D. vexillum* is suspected to induce and maintain dormancy as long as conditions are unfavourable (Cáceres, 1997). Researchers have also encountered difficulties in keeping *D. vexillum* alive in a closed circuit aquarium system and problems have arisen in accurately determining death in this colonial tunicate (Forrest BM, pers. comm., Valentine PC, pers. comm., Therriault TW, pers. comm.).

5.3 *Ciona intestinalis* Eggs in Processing Plant Effluents

A laboratory based trial was performed to assess the ability of *C. intestinalis* eggs to be fertilized after exposure to two effluent water parameters, water

turbidity and flow. The experimental parameters approximated what occurs within processing plants, but temperature and salinity were controlled to obtain optimal fertilization rates.

The levels of turbidity evaluated did not alter the fertilization success, but higher turbidity contributed to decomposition/decapsulation of the unfertilized egg vitelline coat. The added flow parameter demonstrated a significant effect on fertilization. The flow rate (0.9583 L/s) contributed to the lower fertilization success but this only matched the lower flow rates encountered in mussel processing plant (0.05 L/s to 67.7 L/s). It is possible that the eggs would lose their vitelline coat which would compromise the fertilization process (Rosati and De Santis, 1978; Kawamura *et al.*, 1988; Miller, 1975; Villa and Patricolo, 1993; Yoshida *et al.*, 1993).

The higher level of fertilization (48 % or more) could result in new or continued colonization of waters surrounding processing plants. The laboratory trials provided a controlled setting for the exposure of eggs to turbidity and water flow rate and did not replicate other conditions found within the effluent of processing plants. This could possibly explain why some mussel processing plants around PEI that routinely encounter AIS infested aquaculture material, are not experiencing repercussions to the surrounding water bodies. These trials provide a clear indication of the potential propagule pressure of processing plants with results providing additional information to all parties for future pest management strategies.

5.4 Conclusion

Overall, tunicate AIS appear to have a detrimental impact ecologically and economically. The ever changing global conditions and the increasing number and speed of vectors will certainly contribute to the spread of AIS of tunicates. We have seen in this thesis the importance of ongoing surveys in regards to the locations of AIS, and the importance of having a strong knowledge of biological factors of AIS. These parameters and future ones will help in the modeling, integrated management, and the understanding of the capacity and strengths that exotic species have in unfamiliar grounds. With the knowledge of their strengths, we will also understand and exploit their weaknesses. I believe that continued monitoring, education and communication to minimize, if not stop, the ongoing spread is a necessity, especially for shellfish growers in PEI and BC who are already combating tunicate invaders.

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