MANAGING THE HEALTH OF MUSSEL (*Mytilus* SPP.) SEED FROM NEWFOUNDLAND: THE EFFECTS OF THERMAL STRESSORS, TRANSPORT TIMES, STORAGE CONDITIONS AND ANTI-BIOFOULING TREATMENTS ON THE SHORT-TERM AND LONG-TERM PERFORMANCE OF MUSSEL SEED

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Managing the health of mussel (*Mytilus* spp.) seed from Newfoundland: The effects of thermal stressors, transport times, storage conditions and anti-biofouling treatments on the short-term and long-term performance of mussel seed

by

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Abstract

The application of chemical treatments to rid seed of Aquatic Invasive Species (AIS), in conjunction with temperature shocks and long transport times, has the potential to adversely affect the health and long-term performance of mussel seed from Newfoundland, which are approximately 30-40 mm in length and 1-1.5 yr of age. The Neutral Red Assay and the ability of seed to attach via their byssal threads were used as rapid tests for assessing the short-term performance of mussel seed exposed to temperature shocks, long transport storage times (0, 24 and 48 h), storage conditions (ice or no ice) and 30 s chemical dips (4% hydrated lime, vinegar and 300 ppt brine) either before (rinse or no rinse) or after transport. Short-term performance was correlated with the long-term performance (length, biomass, survival and condition index) of seed following an 8 month *in situ* grow-out. Stress associated with harvesting, storage transport, and socking can be minimized if temperature fluctuations are kept to a minimum and seed is covered during transport. Solutions of 4% hydrated lime or 300 ppt brine can be applied to seed before, with or without a seawater rinse, or after a 24 h transport period, without negatively affecting the long-term performance of seed. The use of vinegar as a defouling agent shows promise as being useful when applied either before transport followed by a seawater rinse or after transport. Although similar AIS management strategies have been investigated in other regions, few studies have investigated the impacts of these strategies on the health of seed, and no prior research of this nature has been performed on seed from Newfoundland.

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1. Introduction

1.1 Canadian and Newfoundland Mussel Industries

In 2006, world production of cultured mussels was approximately 1.8 million tonnes, with a value of US$ 1.07 billion (FAO 2006). Of this, Canadian production contributed only 23,822 tonnes at a value of US$ 37.4 million, with Prince Edward Island (PEI) being the major contributor, producing 17,234 tonnes (FAO 2006). However, the carrying capacities of many bays around PEI have been reached and production has begun to level off. Multiple issues are limiting growth of the mussel industry in Atlantic Canada. First, within the past decade, Aquatic Invasive Species (AIS), such as tunicates have resulted in increased competition for resources (e.g., space, nutrients), as well as an increase in production costs associated with tunicate mitigation. In order for the Atlantic Canadian mussel industry to remain viable more AIS research is required, followed by the development and implementation of AIS management strategies. Second, more reliable and high quality stocks of mussel seed are required for future growth of the industry. This involves the transfer of seed from a collection site to grow-out site. Transferring seed, however, increases the risk of inadvertently transferring aquatic invasive species. Therefore, the development of methods to mitigate mussel seed as an AIS vector, while maintaining a healthy supply of high quality mussel seed, will be critical to the growth and prosperity of the mussel industry in Atlantic Canada.

With an increasing demand for mussels and decreasing supply of available mussel seed, it is anticipated that a large shortfall of mussel product in Europe will occur over the next decade (NAIA 2005; DFO 2006, McKenzie 2006, 2007; McKenzie *et al.* 2008). For the Newfoundland mussel industry, this scenario provides opportunity to extend distribution into European markets. As a result, the Newfoundland mussel industry plans to double mussel production from 3200 tonnes in 2006 to 6500 tonnes by 2011. Currently, more than 90% of blue mussels in Newfoundland are cultivated in the Notre Dame Bay, located on the Northeast coast, which includes Green Bay, Halls Bay, Badger Bay, Seal Bay and the Bay of Exploits. The cold, nutrient-rich waters of Newfoundland, with thousands of bays and fjords, many of which are promising aquaculture areas, provide immense opportunity for growth of the aquaculture industry.

Expansion of the mussel industry in Newfoundland is largely limited by the supply of high quality mussel seed and industry has identified this as their number one research priority (Macneill *et al.* 1999; NAIA 2005; DFO 2006; McKenzie 2006, 2007; McKenzie *et al.* 2008). The Newfoundland Aquaculture Industry Association (NAIA), with the support of Memorial University of Newfoundland and Labrador, as well as the Department of Fisheries and Oceans, Canada with funding from the Aquaculture Collaborative Research and Development Program (ACRDP), is leading a mussel seed supply project which will identify seed collection sites that can best meet the increased demand. Once the best sites/stocks are identified, seed will be collected, transferred and grown-out on farm sites around the province. However, these processes will subject

mussel seed to unique and multiplicative stressors that could compromise the health and subsequent performance of the seed, and therefore the effectiveness of the operation. In order for seed transfers to be viable for industry, transfer time, storage method, and chemical physical treatment methods must adhere to the following guidelines as suggested by Carver *et al.* (2003), Sharp *et al.* (2006), Forrest and Blakemore (2006), and Forrest *et al.* (2007): 1) Cause minimal disruption to operations, 2) Not negatively affect the health of the stock, 3) Be 'environmentally-friendly', 4) Effectively eliminate the viability of any potential invasive species. Adequate knowledge of how mussel seed stocks from Newfoundland are able to endure added stress(e.g., longer transfer times and treatments for the mitigation of invasive species) and how well potential aquatic invasive species can survive such treatments, will be critical to the success of the mussel seed project, and hence the growth, prosperity, and sustainability of the industry.

1.2 Management of Aquatic Invasive Species (AIS)

AIS management requires identifying and analyzing risks, followed by a prioritization of management objectives (Forrest *et al.* 2006; Forrest *et al.* 2007). Forrest *et al.* (2006) categorized risk management into four stages: risk identification, risk assessment, analysis of risk treatment options, and risk evaluation. Risk identification involves the following: 1) Identifying and prioritizing high-value areas (HVAs) (Marine Protection areas, Aquaculture sites) in a defensible way, 2) Developing a target list of

potential high-risk AIS and making predictions about their potential distribution; this often involves comparing an organism's natural tolerances (e.g., salinity, temperature) with the environmental conditions in the area of concern, 3) Identifying the pathways by which potential AIS might be introduced into HVAs. Proper management greatly reduces the risk of AIS becoming established, however, the dynamic interactions of AIS with native species, other AIS and spatiotemporal fluctuations of environmental characteristics can greatly complicate AIS management initiatives. For example, regions more vulnerable to AIS often experience intermittent pulses of resources such as space or nutrients (Forrest *et al.* 2006, 2007). The estuarine environments of PEI are highly developed with regards to agriculture and aquaculture, and are therefore associated with high and fluctuating estuarine nutrient levels (Locke *et al.* 2007). Moreover, many estuaries have a large surface area of artificial substrates (e.g., mussel socks in an area that lacks any significant area of hard substrates) that are kept relatively free of competitors and replaced regularly. The introduction of multiple AIS within a short period of time can lead to unforeseen species interactions and alterations of ecosystem dynamics. For example, the concurrent introductions in PEI of green crabs (*Carcinus maenas* L.) and tunicates (*Styela clava*), may be a contributing factor in the spread of *S. clava*, given that green crab predate on the snail *Istyris lunata*, a known *S. clava* predator (Locke *et al.* 2007). Thus, although risk management of AIS is critical in limiting the introduction and/or spread of AIS, introductions often occur in an unpredictable and uncontrollable manner.

Risk management procedures developed and implemented in PEI can provide guidance in developing a risk management strategy for Newfoundland and Labrador. Over the past decade, the mussel industry in PEI has been struggling with the introduction of multiple AISs. The most problematic of which have been the ascidian species (Subphylum Urochordata), including: the clubbed tunicate (*Styela clava*), the vase tunicate (*Ciona intestinalis*), as well as colonial tunicates including, the golden star tunicate (*Botryllus schlosseri*), the violet tunicate (*Botrylloides violaceus*), and *Didemnum vexillum* (currently at high risk for introduction). Tunicates clump together in high densities resulting in fouled equipment (e.g., ropes, socks, buoys, boat hulls and mussels), and can result in the sloughing of mussel product off of lines, making processing difficult and increasing production costs. In Newfoundland, the recent discovery of several colonial tunicates poses a significant threat to the aquaculture industry. Unlike the solitary clubbed and vase tunicates, which have not yet been found in Newfoundland, colonial tunicates are capable of reproducing sexually as well as through budding or fragmentation, producing new colonies which can rapidly spread among bays and estuaries. Although there has been some question regarding the impact of biofouling on mussel health, preliminary studies show that although mussels continue to grow on socks with heavy infestations of tunicates, in many cases, their condition index is reduced significantly due to reduced exchange of food, oxygen and waste products (Leblanc *et al.* 2007; Denny 2008). This leads to higher production costs and a lower quality product which can significantly impede market competitiveness. Other AIS of concern in Atlantic

Canada include the European green crab (*Carcinus maenus*) and the oyster thief (*Codium fragile*) which have had negative impacts on the aquaculture and fishery industries.

Until recently, very few AIS of concern have been found in the marine environment of Newfoundland and Labrador. However, recent events have highlighted the need for an AIS management plan for Newfoundland, including: the recent establishment of damaging AIS in Maritime marine waters (e.g., PEI, New Brunswick, and Nova Scotia), the discovery of some of these AIS along the south coast of Newfoundland (e.g., golden star tunicate, violet tunicate, and European green crab), increased marine traffic (e.g., Placentia Bay Refinery Project) and the expansion of the aquaculture industry (e.g., salmon aquaculture on the south coast, seed transfer project). AIS in Newfoundland waters are an ever growing concern for the aquaculture and fisheries industries. In Newfoundland, researchers and policy makers have learned from the oversights and successes of dealing with AIS in PEI and elsewhere, and have subsequently expanded on rapid response and risk management plans.

1.3 The Potential for Aquatic Invasive Species (AIS) Transfers in Newfoundland

The Newfoundland mussel industry must balance the need to develop a consistent, reliable, and healthy seed supply with the risk of AIS transfers. Aquatic invasive organisms can have devastating impacts on food production in both the fisheries and aquaculture sectors, as well as on local ecosystems (Baines *et al.* 2006; Forrest and

Blakemore 2006; Callahan *et al.* 2007; Forrest *et al.* 2007). The spread of aquatic invasive species (AIS) can occur via natural dispersal or anthropogenic pathways such as vessel, equipment (e.g., ballast water, hull attachment) and organism transfers (e.g., seed-stock) (Baines *et al.* 2006; Forrest and Blakemore 2006; Callahan *et al.* 2007; Forrest *et al.* 2007). The process of transferring mussel seed is particularly risky. Mussel seed is collected on rope collectors that also provide a habitat suitable for a wide variety of other fouling taxa that may facilitate or inhibit mussel settlement and performance. For example, the conditions that make a long line so suitable for seed collection also make it prime habitat for AIS competitors such as filter feeding organisms (e.g., tunicates, bryozoans) and AIS predators (e.g., green crab). During the initial stages of an AIS infestation, it may not be obvious that an invasive species is present and seed-stock transfers could unintentionally facilitate the spread of larvae propagules to other aquaculture sites. Once an invasive species becomes established, eradication becomes extremely difficult and losses can result from over-settlement and subsequent competition for space and resources with mussels and other native species. Growers in regions that have experienced such occurrences have developed methods to mitigate established AIS and prevent further transfers (chemical and physical treatments). However, the additional cost of AIS management (e.g., defouling, processing) can significantly impede a farmer's ability to compete in the marketplace. The Newfoundland mussel industry is particularly vulnerable, as farmers already have additional transport costs and slower growth rates of mussels due to colder waters (approx. 2 yr in Maritime waters vs. 3 yr in Newfoundland).

1.4 Methods of Aquatic Invasive Species (AIS) Mitigation

Methods of AIS mitigation can be natural, chemical or physical in nature. Most mitigation methods developed to date have been relatively benign chemical applications (e.g., food grade chemicals) administered by dipping or spraying seed-stocks with a sufficient concentration of chemical for a sufficient period of time (Forrest *et al.* 2007). The chemical treatments must satisfy conflicting goals of rendering potential AIS unviable, while minimizing the negative impact on the health of seed. Moreover, the chosen chemical(s) must be delivered in a short pulse with a relatively low cost, low environmental risk, and be safe for workers. Researchers in PEI and Nova Scotia have used a variety of chemical and/or physical treatments for treating tunicates with limited success. Chemicals commonly tested for anti-biofouling purposes, include: saturated brine (300 pt NaCl), sucrose $C_{12}H_{22}O_{11}$, 5% vinegar ($C_2H_4O_2$, vinegar), 4% hydrated lime ($Ca(OH)_2$), 5 % citric acid $C_6H_8O_7.H_2O$), sodium metasilicate (silicic acid, Na_2SiO_3), sodium hydroxide (caustic soda, NaOH), and sodium hypochlorite (bleach, NaClO) (Carver *et al.* 2003; Sharp *et al.* 2006; Forrest and Blakemore 2006; Coutts and Forrest 2007, Forrest *et al.* 2007; Denny 2008). Applications of brine or sucrose result in an osmotic gradient that dehydrates biofouling organisms. Other chemicals used either result in a large increase (e.g., vinegar, citric acid, silicic acid) or decrease (e.g., lime, sodium hydroxide, sodium hypochlorite) in pH, relative to the physiological fluid of the biofouling organisms thus causing mortality.

The efficacy of a chemical treatment on the survivability of an AIS depends on the species being treated, the density of settlement and stage of development (e.g., larval, juvenile, or adult) (Carver *et al.* 2003). Treatments that have been used most successfully to date in the Maritime Provinces of Canada, include, brine for treating algal mats (*Cladophora* sp.) in PEI (Sharp *et al.* 2006); air exposure and vinegar for treating the clubbed tunicate (*Styela clava*) in PEI (LeBlanc *et al.* 2007); and vinegar for treating the vase tunicate (*Ciona intestinalis*) in Nova Scotia (Carver *et al.* 2003). In New Zealand, others have investigated similar strategies for treating *Didemnum vexillum* (Coutts and Forrest 2007; Denny 2008), bryozoans (Forrest *et al.* 2007), and algae (e.g., *Undaria pinnatifida*) (Forrest and Blakemore 2006).

Sharp *et al.* (2006) found that 2-3 applications of brine (15 s dip) over a two month period was effective in significantly reducing *Cladophora* sp., without negatively affecting the health of seed (4-5 mm length). Vinegar treatments (15 s dip), however, resulted in 60% of mussel spat being either unattached or gapping following 24 h of recovery. Other studies have examined similar treatments on larger mussel seed, which have shown a higher tolerance to vinegar. Carver *et al.* (2003) found that exposure of ~20 mm length mussels to vinegar (5% acetic acid), for 30 s, resulted in 95% mortality of tunicates and concluded it to be the most effective strategy for eliminating *C. intestinalis*. Carver *et al.* (2003) also found that spraying and dipping mussel seed were equally as effective. Other chemicals investigated included hydrated lime (8 min exposure), which was 70% effective and saturated brine (8 min exposure), which was 20% effective.

In New Zealand, Forrest *et al.* (2007) have investigated the effects of treating green-lipped mussel seed-stock (*Perna canaliculus*) with vinegar in conjunction with a 24 h air exposure transport period. They found that it is possible to cost-effectively treat mussels to eliminate the majority of problematical biofoulers, (e.g., *Botryllus schlosseri* and *Botrylloides leachi*) without resulting in significant negative health effects to the stock. Forrest *et al.* (2007) suggested that a vinegar treatment be applied, followed by a rinse to remove the vinegar residue before transport, or application of the vinegar treatment at the end of the transport period. Denny (2008) conducted similar trials (vinegar, 24 h transport) on *Didemnum vexillum* and found that vinegar was ineffective at eliminating 100% of *D. vexillum* at concentrations where mussel mortality would be maintained at an acceptable level to mussel farmers. As Denny (2008) pointed out, 100% of problem organisms must be killed. If not, once seed is deployed at the grow-out site, small fragments of *D. vexillum* can survive, bud and start new colonies. Moreover, *D. vexillum* may be have a natural tolerance to vinegar given that many *D. vexillum* colonies have a surface pH of ~ 3 (Pisut and Pawlik 2002; Bullard *et al.* 2007; Denny 2008). Denny (2008) found that a 2 min dip in 0.5% bleach solution was a more effective method of treating *D. vexillum*, however, the long-term effects on mussel performance need further study.

Most of this research has been performed on high densities of organisms, late in their life cycle. In order to maximize the effectiveness of chemical treatments, more

research is needed on the survivability of these species when exposed to such treatments during the larval/propagules, juvenile and adult stage of development.

Physical treatments used on their own, or used in conjunction with chemical treatments, have included air drying, water blasting, encapsulation techniques and fresh- and hot water immersion (Carver *et al.* 2003; Forrest and Blakemore 2006; Forrest *et al.* 2007; Denny 2008). Physical treatments in conjunction with chemical treatments may synergistically increase biofouling mortality. For example, air exposure to seed treated with brine results in the formation of salt crystals that can significantly increase the osmotic stress experienced by a biofouling organism.

A host of physical and chemical treatments have been investigated in the literature, many of which have had limited positive results. For example, treatment methods that were ineffective in rendering *S. clava* unviable include: molasses, mild detergents, citric acid, salt brine, formalin, sodium hydroxide, hydrogen peroxide, ethanol, UV long wave length, short wave length, ultrasound, infra-red light, electricity, pressure washing, salt blast, sugar, puncturing (Davidson *et al.* 2005; LeBlanc *et al.* 2007).

The use of natural predators has the potential to reduce costs and lessen negative environmental impacts. In PEI, growers have experimented with lowering mussel lines onto the benthos, allowing crabs to migrate onto lines so that they would predate on tunicates. These efforts have been met with variable results. Should tunicates become established on mussel farms in Newfoundland, potential predators of tunicates include

snails (*Astyris lunata*), starfish (*Asterias vulgaris*), green crabs (*Carcinus maenas*) (AIS - not recommended), rock crabs (*Cancer irroratus*), and hermit crabs (*Pagurus acadianus*) (Carver *et al.* 2003). The use of predators, however, is limited to treating already established biofouling species and is not a viable option for treating seed to prevent AIS transfers. Some of these species also predate on mussels and could therefore result in a loss of product. In addition, chemical cues released during feeding, both from crab predation and broken mussel conspecifics, have been shown to decrease filtration rates and growth, as well as increase byssal thread production and shell thickness of mussels in the vicinity (Leonard *et al.* 1999).

Regardless of the anti-biofouling treatment used, the ability of an organism (fouling or cultured) to withstand such treatments is going to be species, age, density, and population specific. As a result of this, treating seed as a method of mitigating the risk of inadvertently transferring larvae or propagules of AIS warrants further study.

1.4.1 Current seed transfer procedures in Newfoundland

Currently, mussel farmers in Newfoundland do not treat their seed-stock with chemicals to prevent AIS transfers. However, the process of transferring seed does result in conditions that are unfavorable for biofouling organisms. For example, stripping seed-mussels from the collector line and declumping seed helps to remove biofouling organisms. Storing seed in open-aired containers for transport to the grow-out site

exposes the remaining biofouling organisms to air, thus decreasing their viability. Currently, regulators are not allowing the transfer of mussel seed out of high risk areas (e.g., Placentia Bay) and transfers from low risk regions (e.g., Trinity Bay) have yet to require anti-biofouling treatments.

1.4.2 The effects of chemical treatments on mussel seed health

The negative effect of a chemical treatment on the health of seed primarily depends on the degree of valve gapping, filtering and mantle siphon extension when seed is immersed. High mortalities have occurred when the valves of mussels fail to close prior to, or remain closed during chemical immersions (Forrest *et al.* 2007). Shaking seed prior to immersion is one way of inducing valve closure, thus, reducing the amount of chemical that comes in contact with soft tissue (Forrest *et al.* 2007). *Mytilus edulis* can respond to stressors (e.g., shaking or chemical immersion) by pausing pumping for up to 3 min before re-testing conditions (Sharp *et al.* 2006). Certain conditions may make mussels more prone to re-opening their valves during immersion thus increasing the chances of chemical-soft tissue contact. For example, one factor that influences the degree of valve gapping is the amount of microalgae in the water (Riisgård 2006). Riisgård (2006) found that the critical algal concentration below which mussels close their valves has been identified to be about 700 *Rhodomonas* sp. cells•mL^{-1}, or 0.9 µg•L^{-1} of chlorophyll-*a*. Also, in an *in situ* environment, Riisgård (2006) found that algae

concentrations higher than 1 $\mu g \cdot L^{-1}$ of chlorophyll-*a* stimulated the mussels to keep their valves wide open. Large fluctuations in temperature between the air, water and chemical could further influence the degree of valve gapping (Forrest *et al.* 2007). Also, a variety of anthropogenic or environmentally induced stressors, either before or after treatment, could make mussel seed more vulnerable to stressors associated with the chemical treatment (e.g., stress associated with spawning or high temperatures during transport). For example, Forrest *et al.* (2007) found that warmer conditions during transport may lead to reduced survival of green-lipped mussels. This could be a result of heat stress on mussels during emersion and/or a chemically induced stress as a result of the increased rate of vaporization of vinegar (Forrest *et al.* 2007). Moreover, studies on thermal stress in bivalves have repeatedly shown that exposure to temperature fluctuations, particularly increases in temperature, result in lysosomal destabilization (Hauton *et al.* 2001; Harding *et al.* 2004 a, b) and up-regulation or induction of heat shock proteins, indicating a greater level of stress (Hofmann and Somero 1995).

1.5 Factors that Induce Stress Responses in Mussel Seed

Although there has been extensive research on stress in wild shellfish, mainly as indicators of environmental contamination, there is limited information on how cultured bivalves, mussels in particular, respond to the physical and environmental conditions experienced during cultivation. Studies have looked at the effects of post-harvest

conditions (e.g., stripping, grading, debyssing, and storage condition) on the stress response, loss of flavour, bacterial loads, and shelf life of market sized mussels (60-70 mm length) (Prochazka and Griffiths 1991; Tremblett 2001; Harding *et al.* 2004 a, b). Subsequently, the mussel industry has developed handling practices that maximize operational performance and minimize negative impacts on the health and quality of market mussels (e.g., more precise grading equipment to reduce shell damage and wet storage and icing to reduce heat shock). However, there is limited information on how on-farm husbandry and handling practices influence the stress level and subsequent long-term performance of mussel seed.

1.5.1 On-farm handling and husbandry induced stress

Harvesting and deployment of mussel seed in Newfoundland usually occurs during late summer, early autumn. Harvesting mussel seed involves bringing collector lines onboard a barge using mechanical devices. Collector lines are then fed through a stripper and declumper, which strip the seed off of lines. Seed is then graded according to size using a mechanical drum grader, and stored in containers for transport to the grow-out location. The grow-out site may be across the bay, in which case seed may only be out of water for a few hours or, seed may be transported over long distances via road, in which seed will be out of water for 24-48 h. During transport, seed may be exposed to variations in temperature, humidity, sunlight, precipitation (snow or rain), wind, and

physical handling conditions. Following arrival at a grow-out site, seed are then redeployed on a long-line in socks. These processes can result in valve gapping, shell damage, loss of mantle fluids and reduced survival (Harding *et al.* 2004a, b). Stripping seed can also result in soft tissue damage to the byssal gland located at the base of the foot (Price 1982). Despite all of the negative effects these processes have on seed health, there is a lack of information on how these processes influence the long-term performance of seed.

1.5.2. Environmental, seasonal, genetic and age variations and susceptibility to stress

Cultured mussels are exposed to a variety of environmental conditions throughout the production cycle. Most estuarine bivalves, such as mussels, are able to endure a wide range of temperature and salinity levels; however, extreme or rapid changes can result in high levels of stress or mortality. Large fluctuations in water temperature, salinity, and turbidity can occur, brought on by tidal energy, up-welling and down-welling caused by storm conditions (e.g., high winds, wave activity). A single stressor event or multiplicative stressors within a short period of time can result in a prolonged recovery period and or mortality of seed (Harding *et al.* 2004a, b).

Extreme seasonal seawater temperatures are one of the most significant factors affecting stress in mussels. Colder temperatures, associated with winter conditions result in mussels maintaining a relatively low level of stress. Ice cover and algal coverage may provide protection from extreme low temperatures (Hatcher *et al.* 1997). Conversely, the

summer months are associated with elevated water temperatures that can become lethal to mussels (Sephton *et al.* 1993).

Blue mussel stocks from Newfoundland consist of *Mytilus edulis*, *Mytilus trossulus*, and hybrids (Toro *et al.* 2004). Pedersen *et al.* (2000) observed temporal genetic heterogeneity among groups of spat, resulting from both variations in the relative proportions of *M. edulis* and *M. trossulus* and genetic heterogeneity within *M. trossulus*. In general, *M. trossulus* mussels dominate the spat and smaller size classes whereas *M. edulis* is more frequent in the larger size-class mussels (Toro *et al.* 2004). Hybrids occur with low frequency for all size classes (Toro *et al.* 2004). This pattern suggests that *M. trossulus* has a greater mortality and slower growth rate compared to *M. edulis* (Toro *et al.* 2004). Thus, seed-stocks with a high *M. edulis* to *M. trossulus* ratio may perform better relative to stocks with a lower ratio. More research is needed to decipher if there are species-specific responses to stressors and hence differences in survival and performance.

With respect to the health of mussels, survivability of mussels exposed to chemical treatments is often size age specific. For example, Carver *et al.* (2003) found that the survivability of oysters and mussels > 20 mm in shell length was relatively unaffected by an vinegar spray dip, but most control mussels < 10 mm in shell length failed to survive a comparative trial. As a result of this, studies that have investigated the effects of chemical treatments on the health of mussels have been done on mussels of different populations and age and size classes, which make meta-analyses difficult. In

PEI, growth rates are faster and seed is therefore socked at the end of the first summer of growth at a size range of 15-30 mm shell length, whereas in Newfoundland, growth rates are slower and seed is socked following one year of growth at a size range of 30-40 mm shell length. Thus, although the chemical trials investigated in Newfoundland are similar to those in the Maritime Provinces, mussel seed from Newfoundland may be more tolerant of such treatments simply due to being treated at a later stage of the life cycle.

1.5.3. Reproduction cycle and susceptibility to stress

Although temperature is itself a stressor to mussels, seasonal seawater temperatures also covary with the reproductive cycle (Thompson 1984; Hatcher *et al.* 1997). This makes mussels most vulnerable to hydrological changes during gametogenesis and spawning, which occur during the spring and summer (Thompson 1984). Blue mussels from Newfoundland have an annual (sometimes semi-biannual) reproduction cycle (Thompson 1984). During reproduction, a number of physiological changes occur which negatively affect the ability of mussels to react to stress. Such physiological changes include: the allocation of energy reserves for gametogenesis, increases in oxygen consumption, ammonia production, and lysosomal activity (Tremblay *et al.* 1998 a, b). Pre-spawning occurs from April to mid-June. During this time energy reserves are allocated into gametogenesis. During and following the spawning event, mussels reallocate energy reserves to gametogenesis, making them vulnerable to the subsequent increase in summer water temperatures and decrease in food

quality and quantity (Carver and Mallet 1991; Tremblay *et al.* 1998a, b). The duration of spawning is also species specific (Toro *et al.* 2002), which likely results in different rates of recovery. Toro *et al.* (2002) found that *M. trossulus* and hybrid mussels from eastern Newfoundland spawned over a prolonged period of 12-15 wk, where as *M. edulis* mussels spawned over a shorter duration of 3 wk in July. *M. trossulus* also reaches maturation at a smaller size relative to *M. edulis* and hybrids, which may be a response to a higher mortality exhibited by *M. trossulus* (Toro *et al.* 2002). Although spawning times differ, this raises the question of whether or not there is a species and or age specific rate of recovery from spawning. Handling mussel seed before it is able to recover from the stressors associated with the spring and summer could potentially prolong the recovery time, resulting in reduced growth and lower survivals.

1.5.4. Tolerance of cultured versus wild mussels

Unlike wild mussels, cultured mussels are not conditioned to the stressors associated with air exposure (e.g., inter-tidal mussels) or mechanical processes such as the breaking of waves in the inter-tidal zone, and are therefore more vulnerable to handling processes including stripping, declumping, grading, transport, storage, and socking, often combined with fluctuations in temperature, salinity, and availability of food. For example, in general, relative to wild mussels, culture mussels exhibit 1) greater gapping when exposed to air (Eertman *et al.* 1993), 2) thinner, weaker shells, (Slabyj

1980), and 3) lower concentrations of heat shock proteins (e.g., Hsp 70) which function as a defense mechanism against protein degradation as a result of thermal stress (Hofmann and Somero 1995). Moreover, some mussel seed stocks from Newfoundland can have a high percentage of *Mytilus trossulus*, which exhibit thinner shells and may have a lower tolerance to temperature and salinity fluctuations (Gardner and Thompson 2001). These differences make cultured mussels and mussel seed in particular, more susceptible to the rigors of on-farm handling procedures.

1.6 Stress and Stress Response

The terminology used in studies examining stress-related issues in organisms has lacked consistency (Harding *et al.* 2004a, b). In the present study, the most relevant definitions of stress and stress response, as they relate to stress in bivalves, have been given by Harding *et al.* (2004a, b). They defined a stress as any factor, externally or internally imposed on an organism, that induces a change at any number of functional levels (subcellular, cellular, or within the whole organisms) required by the organism to maintain homeostasis. Harding *et al.* (2004a, b) defined a stress response as any alteration of a functional property (behavioural, physiological, or biochemical) that is a reaction to stress, and may be reversible or irreversible in their nature. A stress response at the whole animal level is the product of continuing and compounding changes at various functional levels (e.g., subcellular, cellular). Although a stress response can be reversed by

physiological compensation, the process can be metabolically costly and leaves the animal vulnerable to other stressors such as infection. Multiple, prolonged stress responses are reflected in reduced growth, reproduction failure and mortality (Bayne *et al.* 1976; Akberali and Trueman 1985; Koehn and Bayne 1989). A number of biomarkers have been developed for use in monitoring and assessing negative effects in biological systems (Schiedek *et al.* 2006).

The use of multiple biomarkers that assess stress levels at different functional levels provides the most accurate assessment of an organism's health (Schiedek *et al.* 2006). At the sub-cellular level, the neutral red assay is a reliable indicator of stress (Harding *et al.* 2004a, b). At the whole animal level, byssal thread attachment strength is useful in assessing the short-term performance of mussels (Sharp *et al.* 2006; Sullivan and Couturier 2004), whereas condition indices, percent survival and growth rates are useful indicators of the long-term performance of mussels.

1.6.1 Sub-cellular indicators of stress (neutral red assay)

Sedentary filter-feeding molluscs such as mussels and oysters have been used for biomonitoring purposes because of their ability to filter large amounts of water and accumulate chemical contaminants from the seawater and particulate food materials (Bayne *et al.* 1981; Moore and Clarke 1982). Bioassays have been developed to monitor the effect of contaminants on the health of the animal, thus providing indicators of

ecosystem health. One such assay, the neutral red assay (NRA), has been widely used to quantify the stress levels of bivalves exposed to anthropogenic xenobiotics (e.g., heavy metals, organochlorines (PCBs, DDTs), polycyclic aromatic hydrocarbons (PAHs), styrene) (Borenfreund and Puerner 1985; Lowe and Pipe 1994; Lowe *et al.* 1995a,b ; Moore *et al.* 1996; Cheung *et al.* 1998; Fernley *et al.* 2000; Castro *et al.* 2004; Domouhtsidou *et al.* 2004; Mamaca *et al.* 2005). More recently, the NRA has been used for assessing the effects of various stressors on the stress response of bivalves, including: environmental stressors (e.g., fluxes in temperature and salinity) (Hauton *et al.* 1998; Harding *et al.* 2004b; Synard *et al.* 2005), bacterial infections (Hauton *et al.* 2001) and mechanical handling and husbandry stressors (e.g., storage/transport, stripping, declumping, grading, socking) (Harding *et al.* 2004a; Zhang *et al.* 2006).

The NRA measures retention time of neutral red dye in a hemocyte organelle, the lysosome, which can be correlated to the condition of a mussel exposed to a stressor(s). Hemocytes are blood cells found in invertebrates and exist as individual cells within the hemolymph and interstitial spaces of the organism. Structurally, hemocytes appear spherical when observed within tissues, but often appear non-spherical when migrating through epithelial and connective tissues (Fisher and Tamplin 1988). The primary function of hemocytes is in defense; aiding in inflammation, wound repair, encapsulation and phagocytosis (Fisher and Tamplin 1988). They also play a role in digestion and excretion (Fisher and Tamplin 1988). Within the cytosol of hemocytes, lysosomes have many physiological functions, such as host defense, digestion, regulation of secretary

processes, cellular defense mechanisms, apoptosis, protein and organelle turnover, accumulation and sequestration of xenobiotics, and mediation of tissue specific hormones (Moore *et al.* 1979; Chu 1988; Lowe *et al.* 1995a, b). Lysosomes have a structure-linked latency, whereby hydrolytic enzymes are bound within the organelle and are in a state of inactivity during periods of homeostasis. However, membrane stability of lysosomes is lost under varying physiological and pathological conditions resulting in the release of enzymes, such as alkaline phosphatases, non-specific esterases, indoxyl esterase, lipases, lysozyme, β-hemosaminidase and β-glucuronidase (Moore and Clarke 1982). Under normal conditions (homeostasis), lysosomes accumulate and trap weak base substances, such as the cationic probe neutral red (in the unprotonated form), due to protonation by H+ ions (Lowe *et al.* 1995a, b). However, in response to stressful conditions, the membranes of lysosomes destabilize, resulting in failure of the proton pump, allowing for lysosomal contents, including the neutral red dye, to pass into the cytosol.

Under homeostatic conditions, granular hemocytes appear large and irregular in shape and lysosomes stained neutral red appear as red pinpoints within a colourless cytosol (Lowe and Pipe 1994, Lowe *et al.* 1995a, b). Under conditions of stress, hemocytes are rounder and smaller and exhibit enlarged lysosomes. Neutral red leaks from membrane-damaged lysosomes causing the cytosol to stain pink. Following 3 h of incubation cells swell and become vacuolated, enhancing membrane permeability and autophagy (Harding *et al.* 2004a, b). Monitoring the retention and release of neutral red by hemocytes provides an index of stress response. Depending on the level of stress, a

stress response may or may not be reversed. At a lower degree and or shorter duration of stress, the stress response may be quickly reversed and have no effect on future tissue production, shell growth, or mortality of mussels; however, at a higher degree and or longer duration of stress, the stress response may not be reversed, resulting in poor quality and high rates of mortality.

The ability to detect a stress response at the sub-cellular level may provide the opportunity for growers to manipulate conditions and mitigate a stressor, before the stress response is expressed at a higher functional level (e.g., tissue, organ, or whole animal level). Thus, the application of a bioassay, such as the NRA, that works at the subcellular level to identify stressors associated with the processing or on-farm handling of mussels would be advantageous for the mussel industry.

In addition to the NRA, histochemical analysis and protein profiling are reliable indicators of stress in many marine invertebrates, including bivalves such as the Eastern oyster, *Crassostrea virginica* (Brun *et al.* 2000) and the mussels *Mytilus galloprovincialis* (Dailianisa *et al.* 2003) and *M. trossulus* (Hofmann and Somero 1995). For example, Brun *et al.* (2000) found that the electrophoretic profiles of gill mucus proteases of the Eastern oyster becomes altered in response to infection by the turbellarian, *Urastoma cyprinae*. The neutral red assay, however, is a much more rapid, low cost, and reliable method of analyzing stress in bivalves relative to most other sub-cellular indicators of stress.

1.6.2 Whole animal level response (behaviour, condition indices, growth, and survival)

Mussels are relatively immobile, thus any change in the environment or a predatory attack must be accommodated by the organism (Akberali and Trueman 1985). Akberali and Trueman (1985) described the primary mechanism of defense for mussels, which involves isolating their tissue from the external environment by valve closure. When the mussel is initially exposed to a change in salinity, the exhalent siphon closes, which ceases the inflow of water into the mantle cavity via the inhalant siphon. A further decline in salinity will result in closure of the inhalant siphon, followed by complete closure of the valves. Valve closure is a short-term solution, however, as processes such as feeding, reproduction, and gas and metabolite exchange also cease with valve closure. Despite appearing as closed, valves rarely completely close in response to a stressor. *M. edulis* maintain a small gape between valves that allows for some contact between the tissue and the environment, allowing the organism to monitor changes in environmental conditions.

At the whole animal level, condition indices, such as the ratio of shell weight to dry meat weight and changes in length and weight, and behavioral changes are the most common indicators of stress. Condition indices are useful for quantifying the nutritive status of bivalves and their ability to withstand stressors (LeBlanc *et al.* 2007). A low condition index indicates that a significant biological effort has been expended which could be due to high maintenance energy under poor environmental conditions, disease, or gametogenesis and spawning (Beninger and Lucas 1985). The dry tissue weight to dry

shell weight ratio is most widely used as it eliminates any bias associated with variability in pallial fluid volume (Beninger and Lucas 1985).

In response to a significant stressor, such as damage to the byssal thread gland from handling (e.g. declumping or a chemical dip) or poor environmental conditions, mussels that remain unattached are either deceased or significantly stressed. Thus, the number of mussels attached, unattached or gapping can provide an index of stress.

1.7 Objective

In a Network of Centers of Excellence project (AquaNet AP2) Harding *et al.* (2004a, b) evaluated the NRA as a stress response indicator in market sized mussels in relation to seasonal, environmental, handling, harvesting, processing, and post-harvest storage conditions. The NRA was demonstrated to be a reliable test thus providing the opportunity to mitigate whole animal stress responses. The results provided guidance on ameliorating post-harvest losses due to handling of mussels during harvesting, processing and storage. The present study will continue with research using NRA as an indicator of stress, concentrating, however, on the earlier stages of mussel culture. I will assess the NRA as a method of measure stress responses of mussel seed associated with seasonal spatiotemporal and environmental changes (temperature, food supply), handling stressors during husbandry procedures such as harvesting, transport, grading, socking and storage, as well as chemical physical treatments used for mitigating biofouling organisms, such as

invasive species. Moreover, I will assess long-term performances of mussels (growth, mortality rates) following a grow-out period after exposure to various seed handling procedures.

1.8 Hypotheses

1) If feeding mussel seed *in vitro* affects the stress level of seed it will be observed as a significant change in the stress response as measured by the neutral red assay.

2) If fluctuating water temperature affects the stress level of seed an increase or decrease from ambient water temperature will result in a significantly increased stress response as measured by the neutral red assay.

3) If temperature fluctuations and air exposure, as experienced during transport storage, affect the stress level and long-term performance of seed, then longer transport storage times at temperatures above or below ambient water temperature will result in a significantly increased stress response as measured by the neutral red assay and a significant decrease in growth and survival.

4) If chemical treatments (300 ppt brine, 4% hydrated lime, vinegar) in combination with transport affect the stress level and long-term performance of seed, then these treatments will result in a significantly increased stress response as measured by the neutral red assay and a significant decrease in growth and survival.

2. Materials and Methods

2.1 Animals

Experiments were performed using mussel seed (*Mytilus* spp.) 30-40 mm in shell length, which is the size of seed typically socked by the Newfoundland industry. Mussels used in preliminary experiments during the spring of 2007 were collected from Placentia Bay, NL. Subsequent experiments relied on seed from Cap Cove, Trinity Bay, NL. The site of seed collection was changed due to logistical problems, as well as the identification of several aquatic invasive species in Placentia Bay during the summer of 2007, which made obtaining transfer permits problematic. A sample consisted of seed randomly selected seed from collection lines. In order to prevent any additional stress that might have resulted from predatory chemical cues, all visible sea stars were removed from the samples. All other biota associated with seed was not manipulated. Seed was transported on ice to the Aquaculture Facilities at the Marine Institute of Memorial University. Within 24 h, seed was placed in a re-circulation raceway system with aeration and unfiltered sea water and maintained at ambient sea temperature and salinity. Stress levels were assessed daily using the NRA. An acclimation period of 7 d allowed for stress levels to stabilize before experiments were conducted. Water quality parameters, including temperature, dissolved oxygen, salinity, pH, and ammonium were assessed daily using a calibrated oxygen probe, a salinity refractometer, and appropriate test kits. Water was exchanged (approx. 25%) and feces and pseudo-feces were siphoned daily.

2.2 Stress level measured via the Neutral Red Assay (NRA)

The primary means of assessing the short-term performance of mussel seed during experimental trials was to measure stress levels using the Neutral Red Assay. The Neutral Red Assay requires the proper collection and handling of hemolymph, minimal exposure of neutral red dye to light, proper temperature control, and an overall precise and consistent implementation of methodology. The Neutral Red Assay can be divided into 3 main steps: 1) the collection of hymolymph from the animal, 2) the application of neutral red dye to the hymolymph, and 3) the examination of hemocytes by light microscopy.

2.2.1. Hemolymph collection

Tweezers were inserted ventrally between the two valves in order to produce a small gape. Hemolymph was slowly withdrawn from the posterior adductor muscle of mussels using a 1 mL hypodermic needle fitted with a 21-gauge needle. A slow withdrawal speed and a relatively large needle size helped to prevent shear forces that can cause cell damage and subsequent clotting. Hemolymph was drawn into an equal volume of physiological saline (0.1 mL hemolymph, 0.1 mL saline) consisting of 4.77 g HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 25.47 g sodium chloride, 13.06 g magnesium sulphate, 0.75 g potassium chloride, 1.47 g calcium chloride, made up to 1 L with distilled water and adjusted to a pH of 7.36 (Lowe *et al.* 1995a; Harding *et al.*

2004a, b). The needle was then removed and hemolymph was slowly expelled into a siliconised Eppendorf ® microcentrifuge tube (aids in the prevention of cells sticking to tube walls) and placed on ice. Tubes were gently inverted to mix the physiological saline and hemolymph. A 40 µL aliquot of hemolymph : physiological saline solution was then pipetted onto a dry microscope slide, pretreated with a Poly-L-lysine solution (20 µL of poly-L-lysine in 100 µL of distilled water). Slides were placed in a lightproof humidity chamber (cooled with ice) for 15 min to allow for adherence of hemocytes to the slide. Following incubation slides were tipped onto a paper towel in order to remove excess solution.

2.2.2. Application of neutral red dye

Neutral red stock solution was made by dissolving 0.0288 g of refrigerated neutral red dye powder in 1 mL of dimethylsulphoxide (DMSO) in a lightproof vial (NR dye is light sensitive) and kept refrigerated for a maximum duration of 3 wk. Neutral red working solution was prepared by pipetting 20 µL of stock solution into 5 mL of physiological saline and stored in a second lightproof vial. Slides were then treated with 40 µL of neutral red working solution and replaced in the humidity chamber for 15 min to allow dye to penetrate the cellular membrane and accumulate in lysosomes.

2.2.3. NRA light microscopy

A 22 × 22 mm coverslip was placed on the slide and observations made under bright field light microscopy at low level illumination at a magnification of 400×. Slides were observed for a maximum of 1 min before being returned to the humidity chamber. Slides were examined every 15 min for the first 60 min and every 30 min thereafter, up to a maximum of 180 min. Granular hemocyte cells were examined for structural changes (pseudopodia, swelling) and neutral red retention within lysosomes. Twenty five cells were counted and classified as stressed or unstressed (Figure. 1). Once more than 50% of cells were determined to have reached a high level of stress, the preceding observation time was recorded as the Neutral Red Retention (NRR) time and the experiment was terminated.

2.3 Preliminary Experiment

2.3.1 Effect of feeding and extended in vitro holding on stress response of seed

While experiments were performed, mussels were held in a re-circulation raceway system, totally submerged in seawater of ambient salinity (30-31 ppt) and temperature (2-10 °C) for up to 3 wk. Preliminary experiments assessed the effect of long-term holding and feeding on the NRR time of seed. One group of mussels was fed on a daily basis, while the other group remained unfed. A daily ration was supplied at 2-3% of wet body weight and consisted of a mixture of *Isochrysis* sp., *Chaetoceros* sp. and *Tetraselmis* sp.

(Alteen 2002). Mussels (n = 12) were collected for analysis using the Neutral Red Assay (NRA) on the initial day of the experiment and every 7 d thereafter for a period of 3 wk. Samples (n = 30) were also collected on day 0 and day 21 for determining condition indices (dry meat weight dry shell weight, Davenport and Chen 1987). Results showed that the fed group was significantly more stressed. All subsequent experiments were performed on unfed seed.

2.3.2 Effect of temperature shock on stress response of seed

This experiment was performed to assess the NRA as an indicator of stress response for mussel seed exposed to extended water temperature shocks. Exposing seed to temperature shocks was meant to mimic temperature differences that can occur as a result of transferring seed from a collection site to grow-out site and or deploying or retrieving seed through a thermocline. Following the initial temperature shock stress levels were monitored for a recovery period of 24 h. Two groups of mussels were taken from raceways (10 °C) and placed in aerated aquariums, one group at a water temperature of 19 °C and the other at 1 °C. Mussels were exposed to these temperatures for 4 h before being placed back into the raceways (10 °C) for a 20 h recovery period. Samples (n 6) were taken for analysis using the NRA, at times 0, 2, 4, 6, 9, 12, and 24 h. Controls from the raceways were sampled at h 0 and 24 for each treatment. Experiments were

performed on mussels in the spring and autumn of 2007. Samples (n = 30) of mussel seed were also collected in the spring and autumn for analysis of condition indices.

2.3.3 Effect of storage condition on stress response of seed

This experiment evaluated the effect of storage conditions on NRR time over a 3 day period as occurs during transport from a collection site to a grow-out site. One batch of seed was stored on ice (1-2 °C), while another batch was stored at 8 °C. Samples for each storage condition were taken on days 1, 2, and 3 for NRA analysis. Additionally, samples were taken from each group and placed back into the raceway (10 °C) for a 24 h recovery period, after which an additional sample was taken for NRA analysis. Experiments were performed on mussels in the spring and autumn of 2007.

2.4 Effects of Storage Condition and Anti-biofouling Treatments on the Short and Long-term Performances of Mussel Seed

2.4.1 Short-term performance

The short-term performance of seed was assessed via the neutral red assay as well as behaviorally, by the ability of seed to attach via their byssal threads 24 h post treatment.

2.4.1.1. Neutral Red Assay (NRA)

See section 2.2 for the NRA procedure.

2.4.1.2. Byssal attachment 24-h-post treatment

The purpose of this experiment was to assess the effects of a 30 s exposure and 30 s rinse (if applicable) to 300 ppt brine, 4% lime, or vinegar on the short-term performance of seed. This experiment was conducted in June during pre-spawning, a stage in the reproductive cycle in which mussels are stressed (Harding *et al.* 2004a, b). The Neutral Red Assay was therefore not a useful tool during this period given the high level of stress exhibited by the mussel seed (e.g., base level NRR times were too low). Thus, the ability of mussel seed to reattach via their byssal threads was used as an alternative index of stress. The effects of antifouling treatments on the short-term performance of seed was measured via survivorship and the ability of seed to reattach via their byssal threads 24-h-post treatment; with high survivorship and high number of seed attached via their byssal threads 24-h-post treatment indicating a relatively lower level of stress.

Following each 30 s dip, 3 replicates of 20 mussels were placed in 5 L buckets and randomly submerged in an aerated, re-circulation raceway system for 24 h (Forrest and Blackmore, 2006). Following 24 h of recovery time, mussels were classified as a) reattached via their byssal threads (either attached to the side of the container or other mussels), b) unattached, but still alive, and c) gaping and unattached (mortalities). Morbidity was determined to have occurred when the mussels' valves failed to remain

shut after being held closed for 5 s (Harding *et al.* 2004a, b). Preliminary experiments assessed attachment post-24, 48, and 72 h. Results for each of these times did not significantly differ, suggesting that if mussels were able to recover from a treatment, they did so within the first 24 h. Therefore, all subsequent experiments only assessed attachment post-24 h.

2.5 Long-term Performance

The long-term effects of antifouling treatments and storage conditions were determined by assessing growth, physiological condition and survivorship following an 8 month *in situ* grow-out in Cap Cove, Trinity Bay. Initial measurements of total biomass, shell length (subsample n = 25, 30-40 mm length), and condition index (n = 10, dry meat weight/dry shell weight, 80 °C for 24 h) were performed.

Batches of treated seed (n = 50 × 3 replicates) were placed in pearl nets (5 mm mesh size) and deployed on a long line at a depth of 5-7 m. Nets were deployed on October 12, 2007 and retrieved May 22, 2008. Following retrieval, pearl nets were transported back to the lab where mussels were removed and total biomasses recorded. Seed was then placed in bags for freezing. Batches of seed were then randomly sampled for length, survivorship and condition index measurements.

2.5.1 Chemical treatments

During transport, seed-stock can remain out of water anywhere from a few hours to more than 24 h. During this time, handling, temperature and humidity can vary greatly depending on equipment, weather and storage condition (e.g., ice or no ice). Rinsing seed with filtered seawater, prior to transport may help to increase survivability and reduce stress; however, not rinsing would theoretically increase the potency of the treatment and decrease the viability of any biofouling organisms. Thus, experiments were designed to evaluate the effects of chemical treatments combined with storage transport condition and saltwater rinsing.

Seed was stripped from the collection line, declumped, and stored in tote pans; mimicking industry handling practices as close as possible. Declumping of mussel seed insured that the complete surface area of seed was exposed to the chemical. Seed was collected and deployed within the same location, Cap Cove, Trinity Bay, and storage transport was simulated on shore. Batches of mussel seed (30-40 mm length) were stored at an air temperature of 4 °C and 100% humidity, either on ice or not on ice, and subjected to the following antifouling treatments, either before (rinse or no rinse), or after a 24 h simulated storage transport period: 300 ppt brine, 4% hydrated lime, or vinegar. Chemicals, concentrations and dip times were based on literature and current industry practices (see Forest and Blackmore 2006; Sharp *et al.* 2006; Forest *et al.* 2007).

Mussels that were treated after storage/transport, where deployed within an hour of being treated thus no manual rinsing was performed. Mussels were placed in a plastic sieve and manually shaken for 10 s in order to induce valve closure. Mussels were then immediately dipped into the chemical solution for a 30 s period. Mussels were then removed, given a 30 s rinse treatment (rinse or no rinse), and placed into pearl nets. Pearl/lantern nets were then placed in fish pans for storage/transport before being deployed on a long line. References consisted of 1) Control - no dip treatments, no transport, no rinsing, 2) no dip treatment, storage on ice, no rinsing, 3) no dip treatment, storage at ambient air temperature, no rinsing, 4) dip treatment, no storage/transport, immediately deployed (rinsing defaulted).

2.5.2 Correcting for mantle fluid loss in biomass measurements

Mussels treated on the second day of field work had biomasses that were markedly reduced relative to those treated on the first day. This drop in biomass was believed to be a result of drip loss as a result of seed not being stored on ice. In order to verify this hypotheses a drip loss experiment was conducted in the lab. Two batches of seed (500 g each) were held in perforated containers inside a refrigerator; one batch on ice, one at ambient air temperature (8 °C at 100% humidity). Drip loss was assessed by weighing the seed batches at 0, 24 and 48 h. Biomass estimates were corrected by adding

the percentage of drip loss, in grams, to the biomass measurements performed following 24 h.

2.5.3 Physical and environmental measurements

Mussel shell size (mean length ± 0.01 mm) was measured using a Traceable Digital Caliper (Fisher Scientific, Nippon, ON). Weights (± 0.1 g) of mussels were determined using a Mettler AE 100 analytical balance (Mettler Instruments AG, Zurich, Switzerland). Seawater temperature, salinity, dissolved oxygen as well as chlorophyll-*a* levels were continuously measured during the 8 month *in situ* grow-out using a YSI Sonde 6-Series Multi-parameter Water Quality Monitor, Model number 6600EDS.

2.6 Data Analysis

All data collected were analyzed using Minitab software (version 15). Figures were constructed using Microsoft Office Excel 2007. Statistical tests performed include: descriptive statistics, General Linear Model (GLM) one-way ANOVA, GLM two-way ANOVA, post-hoc tests (Tukey's b) and Pearson's correlation analysis (r – coefficient). Assumptions of normality were tested by comparing a histogram of residuals to a normal probability curve. Homogeneity of variance was analyzed by examination of residual vs. predicted values for each response variable. If violations occurred data were log-transformed (log (x+1)). The level of significance was set at α 0.05.

3. Results

3.1 Preliminary Lab Experiments

3.1.1 Influence of feeding and extended re-circulation raceway

After one week in seawater at a temperature of 2 °C, seed that was fed had a neutral red retention time that differed significantly from the control (one-way ANOVA, $F_{6,77} = 7.73$, $p < 0.001$) (Figure 2). Seed that remained unfed had Neutral Red Retention (NRR) times that did not differ from the control for the entire 3 wk duration. The NRR times decreased from 155 min for the control to 120 min and 95 min, for unfed and fed groups, respectively. The seawater temperature was then increased from 2-10 °C, by 1 °C daily. Once the seawater reached 10 °C, seed was allowed to acclimate for a further 10 days. Feeding was then continued for another 3 wk. Seed that was fed remained significantly more stressed relative to seed that was not fed and the NRR times decreased steadily for the 3 wk period. The NRR times decreased from 118 min to 63 min and 78 min to 34 min, for unfed and fed groups, respectively. Following long-term holding in lab raceways the condition indices of fed and unfed seed increased significantly relative to the control (one-way ANOVA, $F_{2,87} = 5.84$, $p = 0.004$) (Figure 3). However, following the lab holding period, condition indices of seed that was fed did not differ significantly from seed that was not fed ($t_{1,58} = 0.2076$, $p = 0.8363$). Also, no correlation was observed between lengths and condition indices of seed ($r^2 = 0.125$, $p = 0.510$).

3.1.2 Temperature shock and recovery

Condition indices of seed collected in the spring (mean = 0.288, ± 0.015 S.E.) did not significantly differ from those collected in the autumn (mean = 0.283, ± 0.015 S.E.) ($t_{2,58}$ = 0.20, p = 0.844).

3.1.2.1 Spring seed

The NRR times of mussels exposed to an increase or decrease in temperature for 4 h and allowed to recover for 20 h showed significant differences among the control and experimental treatments (10-19 °C, one-way ANOVA, $F_{7,40}$ = 18.54, p < 0.001; 10-1 °C one-way ANOVA, $F_{7,40}$ = 12.42, p < 0.001). An increase in temperature from 10-19 °C for 4 h resulted in a significant decrease in NRR time from 75 min at 0 min of exposure to 2 min at 2 h of exposure (Figure 4). A decrease in temperature from 10-1 °C resulted in a significant decrease in NRR time from 72.5 min at 0 min of exposure to 32.5 min at 2 h of exposure. Following a 24 h recovery period the NRR time of seed exposed to an increase or decrease in temperature had increased to 72.5 min and 67.5 min, respectively, reaching a NRR time equivalent to 24 h prior. Thus, within a relatively short period of time (24 h) pre-spawning seed was able to tolerate and recover from, both a large decrease and increase in water temperature.

3.1.2.2 Autumn seed

The NRR times of mussels exposed to an increase or decrease in temperature for 4 h and allowed to recover for 20 h, showed significant differences among the control and experimental treatments (10-20 °C, one-way ANOVA, $F_{3,40}$ = 18.54, p < 0.001; 10-2 °C one-way ANOVA, $F_{3,40}$ = 12.42, p < 0.001). An increase in temperature from 10-19 °C for 4 h resulted in a decrease in NRR time from 64 min at 0 min of exposure to 0 min at 4 h of exposure (Figure 5). A decrease in temperature from 10-1 °C resulted in a decrease in NRR time from 66 min at 0 min of exposure to 35 min at 4 h of exposure. The NRR time of mussels exposed to a decrease in temperature, did not significantly differ from the control until 6 h into the experiment. Following a 24 h recovery period the NRR time of seed exposed to a decrease in temperature had increased to 45 min, reaching a NRR time equivalent to 24 h prior; whereas, the NRR time of seed exposed to an increase in temperature had increased to only 15 min. Thus, seed was able to tolerate, and recover from, a large decrease in temperature; however, seed was unable to recover from a large increase in temperature within the 24 h recovery period.

3.1.3 Extended air exposure and recovery

3.1.3.1 Spring seed

The NRR time of seed decreased over time for both storage conditions and significant differences were observed (Dry storage, one-way ANOVA, $F_{6,--}$ = 21.17, p <

0.001; Storage on Ice, one-way ANOVA, $F_{6,-}$ = 22.34, p < 0.001). After 3 days of storage on ice, seed had NRR times that differed significantly from the control. Also, seed allowed to recover after 2 d of storage had significantly lower NRR times (Figure 6). Seed stored at 4 °C, 100% humidity (dry storage) had significantly lower NRR times after 24 h and was unable to recover within 24 h.

3.1.3.2 Autumn seed

The NRR times of seed decreased over time for both storage conditions. Seed stored on ice had NRR times that were significantly reduced after 24 h, whereas seed stored at ambient air temperature had NRR times that were significantly reduced after 48 h (one-way ANOVA, $F_{4,55}$ = 4.04, p = 0.006) (Figure 7). Placing seed back into seawater resulted in a further decrease in NRR times. Seed stored on ice or at ambient air temperature was unable to recover to the NRR times of the control. Regardless of storage condition, after 48 h the NRR times stabilized at approximately 20 min.

3.1.4 Effects of storage condition and anti-biofouling treatments on the short-term performance of mussel seed

Seed treated with 300 ppt brine solution had a mean number of seed attached that did not differ significantly from the control when measured at 24 h (Figure 8). 48 h

(Figure 9), or 72 h (Figure 10). Similarly, lime (fresh and seawater solvents) treatments did not differ significantly from the control, except for the *treat (no rinse) - storage transport* treatment (one-way ANOVA, $p < 0.001$ for both solvents, post-72 h). Without rinsing seed, following 72 h of recovery, ~ 25% of mussels were gapping (mortality), 25% unattached and 50% attachment, for both solvents.

Vinegar was the most potent treatment with, *treat (no rinse) - storage/transport*, resulting in ~ 75% gapping (mortality), 23% unattached and 2% attachment, followed by, *treat (rinse) - storage/transport*, resulting in ~ 33% gapping (mortality), 18% unattached and 49% attachment following 24 h of recovery (Figure 8). Seed exposed to *15 h air exposure - treat*, however, was not significantly different from the control (one-way ANOVA, $p > 0.05$).

3.1.5 Tolerance of seed exposed to extended 300 ppt brine and 4 % lime solutions

The mean number of seed attached following a 1 min exposure to 300 ppt brine decreased to 75%, but was not significantly different from the control (Figure 11A). After 2 min of exposure to 300 ppt brine the mean number of seed attached was significantly different from the control.

Similarly, the mean number of seed attached following exposure to 4% lime for 1 min was reduced to 75% (Figure 11B). However, it took 3 min of exposure with 4% lime to reach a mean number of attached seed that was significantly different from the control.

3.2 Effects of Storage Condition and Anti-biofouling Treatments on the Short and Long-term Performances of Mussel Seed

3.2.1 Correcting for mantle water loss in biomass measurements

Mussels treated on the second day of field work had biomasses that were markedly lower relative to those treated on the first day. This decrease in biomass was most likely caused by drip loss as a result of seed not being stored on ice. Seed stored on ice showed no significant drip loss over the first 24 h. Since all experiments were done within this time period, no corrections were made with respect to biomass measurements for seed stored on ice. Seed stored at ambient air temperature showed a drip loss of 4.5% of total biomass following 24 h of storage and an additional 5% following 48 h of storage. As a result of these findings, all biomasses of seed batches measured on day 2 were corrected for the 4.5% drip loss.

3.2.2 Short and long-term performance of seed exposed to brine treatments

Seed exposed to 300 ppt brine for a 30 s period displayed significant differences in NRR times (one-way ANOVA, $F_{9,110} = 6.46$, $p < 0.001$), with lower NRR times indicating a higher degree of stress response (Figure 12A). Seed not stored on ice during transport (NI) had NRR times that were significantly lower than seed stored on ice (I), indicating a higher level of stress. However, treatments did not differ significantly from the control.

Byssal attachment did not differ among any of the treatments (one-way ANOVA, $F_{9,20} = 1.2$, $p = 0.350$), indicating a low level of stress. Survival was greater than 90% for all treatments (Figure 12B).

Significant correlations occurred between condition index and length ($r = 0.427$, p 0.019), biomass and length ($r = 0.554$, $p = 0.001$), and biomass and survival ($r = 0.575$, $p = 0.001$) (Table 1A).

Following an 8 month *in situ* grow-out, survival and growth (as measured by the change in biomass and length of seed), did not differ significantly among the treatments (one-way ANOVA $_{\text{Biomass}}$, $F_{9,20} = 2.37$, p 0.052; one-way ANOVA $_{\text{Length}}$, $F_{9,20} = 2.39$, p = 0.051; one-way ANOVA $_{\text{Survival}}$, $F_{9,20} = 0.96$, $p = 0.502$;) (Figures 13A, B, and C, respectively). Similarly, the condition indices of seed did not significantly differ among brine treatments suggesting that the ratio of shell to tissue was not negatively affected by the brine treatments (one-way ANOVA $_{\text{CI}}$, $F_{9,20} = 1.00$, $p = 0.472$) (Figure 13D). Means and standard deviations of initial and final lengths and biomasses are listed in Table 2.

3.2.3 Short and long-term performance of seed exposed to lime treatments

The NRR times of seed exposed to the lime treatments were significantly different (one-way ANOVA, $F_{9,110} = 8.25$, $p < 0.001$). Seed that was not stored on ice (N1) had NRR times that were significantly lower than the control, indicating a greater level of stress (Figure 14A). Additionally, NRR times for seed that was not rinsed prior to

transport and stored on ice during transport (NR, I) were significantly lower than the control.

Seed that was not rinsed prior to transport and not stored on ice during transport (NR, NI) showed a significant reduction in byssal thread production, indicating a greater level of stress, relative to the control (one-way ANOVA, $F_{9,20} = 5.63$, $p = 0.001$)(Figure 14B). Thus, for the lime treatments, storage condition (NI/I) seemed to be the main determining factor influencing NRR time and byssal attachment of seed, followed by whether or not seed was rinsed (R, NR), and transport/storage time.

Following an 8 month *in situ* grow-out, seed biomass was not significantly lower than the control. However, biomass of seed exposed to 24 h transport, no lime treatment, and not stored on ice (24 h transport - NI) and seed not stored on ice, but treated after transport (24 h - L (NI)), were significantly greater than the control (one-way ANOVA, $F_{9,20} = 4.15$, $p = 0.004$) (Figure 15A). Lengths and condition indices of seed exposed to lime treatments were not significantly different from the control (one-way ANOVA, $F_{9,20} = 2.37$, $p = 0.052$ and one-way ANOVA, $F_{9,20} = 0.86$, $p = 0.571$, respectively) (Figure 15B and 15D). Seed that was treated with lime and then immediately put back into the water was the only treatment to have a significantly reduced survival of 84% (Figure 15C).

Significant correlations occurred between attachment and biomass ($r = 0.397$, $p = 0.030$, attachment and survival ($r = 0.534$, $p = 0.002$), biomass and length ($r = 0.371$, $p =$

0.043), and survival and biomass ($r = 0.377$, $p = 0.377$) (Table 1B). Means and standard deviations of initial and final lengths and biomasses are listed in Table 3.

3.2.4 Short and long-term performance of seed exposed to vinegar treatments

Vinegar was the most potent chemical treatment tested. NRR times of seed were significantly reduced for seed exposed to vinegar and then immediately put back into ambient seawater, as well as seed that was not rinsed prior to transport (L-24 h (NRNI), (NRI)) (one-way ANOVA, $F_{9,110} = 6.22$, $p < 0.001$) (Figure 16A). Byssal attachment post-24 h was significantly reduced in all treatments, with the exception of seed that was rinsed prior to transport and not stored on ice during transport (one-way ANOVA, $F_{9,20} = 21.24$, $p < 0.001$) (Figure 16B).

Biomasses of seed exposed to vinegar treatments did not differ significantly from the control; however, there were significant differences observed with respect to the other 3 reference treatments (24 h transport (NI); 24 h transport (I); vinegar (no transport)) (one-way ANOVA, $F_{9,20} = 6.48$, $p < 0.001$). Mussel seed that was not rinsed prior to transport and not stored on ice had increases in biomass that were significantly reduced (18.5 g) and was the most toxic treatment overall (Figure 17A). Mussel seed that was not rinsed prior to transport and stored on ice during transport (NR, I) resulted in the second lowest increase in biomass of seed (40.5 g). Rinsing of seed prior to transport resulted in an increase in biomass of 81.5 g and 72.0 g when not stored/transported on ice (R, NI)

and stored transported on ice (R, I), respectively. Seed that was treated after transport had the greatest biomass, with seed stored on ice having an increase in biomass of 108 g and seed not stored on ice having an increase in biomass of 112 g.

Relative to the control and other treatments, the lengths of seed were significantly reduced for seed not rinsed prior to transport (one-way ANOVA, $F_{9,20} = 5.97$, $p < 0.001$) (Figure 17B).

Survival of seed differed significantly among the vinegar treatments (one-way ANOVA, $F_{9,20} = 3.49$, $p = 0.009$). The only treatments to result in a significant level of mortality of seed was that which was not rinsed prior to transport and not stored on ice during storage/transport (NR, NI) (survival = 64.6%) (Figure 17C). Seed that was not rinsed prior to transport and stored on ice during storage/transport had the next lowest survival of 66.3%. Survival was greater than 90% in all other treatments.

Condition indices of seed did not differ for any of the seed batches treated (one-way ANOVA, $F_{9,20} = 0.60$, $p = 0.779$) (Figure 17D).

A distinct qualitative observation made was that nearly all seed not rinsed prior to transport exhibited blistering of the periostracum, which became increasingly more pronounced in the posterior regions of the valves (Figure 18).

Significant correlations occurred between NRA and attachment ($r = 0.545$, $p = 0.002$), CI ($r = 0.459$, $p = 0.011$), survival ($r = 0.612$, $p < 0.001$), length ($r = 0.404$, $p = 0.027$), and biomass ($r = 0.665$, $p < 0.001$) (Table 1C). All other variables correlated with

each other, with the exception of no significant correlation between attachment and condition index ($r = 0.323$, $p = 0.081$) or survival and condition index ($r = 0.342$, $p = 0.064$) (Table 1C). Means and standard deviations of initial and final lengths and biomasses are listed in Table 4.

3.2.5 Short and long-term performance of seed exposed to long-term storage

In the short-term, relative to the control, storage condition and time had no significant effect on the NRR time or byssal attachment (Figures 19A and 19B, respectively). However, among all treatments, seed stored on ice for 24 h had NRR times that were significantly greater relative to seed stored for 48 h (ice or no ice) (one-way ANOVA, $F_{4,55} = 4.04$, $p = 0.006$). There were no significant differences among treatments with respect to biomass (one-way ANOVA, $F_{4,10} = 3.08$, $p = 0.068$), survival (one-way ANOVA, $F_{4,10} = 0.18$, $p = 0.946$) or condition index (one-way ANOVA, $F_{4,10} = 0.26$, $p = 0.898$) (Figures 20A, C, and D, respectively). Lengths of seed were significantly greater for seed not stored on ice for 48 h, relative to seed stored on ice for 24 h (Figure 20B).

There were no significant correlations observed between any of the five variables (NRR time, byssal attachment, length, biomass, condition index, survival) (Pearson's correlation analysis, $p > 0.05$). Means and standard deviations of initial and final, lengths and biomasses are listed in Table 5.

3.3 Environmental Data (chlorophyll-*a*, dissolved oxygen, temperature, salinity)

A summary of the environmental data for Cap Cove, Newfoundland for the entire duration of the grow-out period (e.g., October, 2007 to May, 2008) can be found in Figure 21. Chlorophyll-*a* levels in Cap Cove remained between 1.0 and 1.5 μgL^{-1} from October until April when levels increased to 3.4 μgL^{-1}. Dissolved oxygen remained between 90 and 100% until April when it increased to slightly greater than 100% saturation. Temperatures decreased steadily from a high of 8 °C in October to a low of -1.2 °C in March before rising once again in April and May. Salinity remained relatively constant over the grow-out period rising from 32 ppt in October to 33.4 ppt in April before decreasing in May.

4. Discussion

4.1 Preliminary Lab Experiments

4.1.1 Influence of feeding and extended holding in re-circulation raceways

Seed was held in the lab at 2 °C, for a period of 3 wk, followed by 10 °C for another 3 wk, during which one batch of seed was fed, and one batch remained unfed. Seed that was fed showed a significant stress response after only one week, whereas the unfed group showed no significant stress response during the 3 wk of holding. The reason for the significant stress response shown by the fed seed is not definitively known. The most likely factor is the method of feeding. For example, the daily feeding of seed involved turning the re-circulation water off and adding the algal mixture as one large dosage. During the feeding period, the re-circulation system remained shut off for a 6-8 h period each day, until feeding concluded as indicated by the turbidity of the water returning to normal. This intermittent method of feeding likely resulted in food being applied in excess, resulting in the reallocation of energy into digestion and pseudofeces and initiating an increased stress response. Consequently, all NRA experiments were conducted on seed that was unfed and held in re-circulation systems for a maximum of 3 wk. Similarly, Bayne and Thompson (1970) showed that holding mussels for a few weeks in the laboratory also resulted in a significant change in condition indices. For the duration of the present experiment, the seawater temperature was increased to 10 °C. The NRR time of seed continued to decrease, indicating that seed continued to become more

stressed the longer it was held in the lab. Moreover, seed that was fed continued to show higher levels of stress relative to unfed seed. The results of this experiment therefore support the hypothesis that feeding mussel seed *in vitro* affects the stress level of seed as observed using the NRA.

4.1.2 Temperature shock and recovery

The results of this experiment are similar to those of Harding *et al.* (2004a, b), in which the lysosomes of hemocyte cells of market-sized mussels showed a definitive response to temperature shocks. Destabilization of lysosomes in response to thermal stress has also been observed in *Ostrea edulis* (Hauton *et al.* 1998; Hauton *et al.* 2001), *Crassostrea gigas* (Hauton *et al.* 2001; Gagnaire *et al.* 2006; Zhang *et al.* 2006), *Crassostrea virginica* (Synard *et al.* 2005), and *Placopecten magellanicus* (Synard *et al.* 2005). Other changes at the sub-cellular level in response to thermal stress in mussels and oysters include the upregulation or induction of molecule chaperones (e.g., heat shock proteins and ubiquitin) that help in the prevention of protein denaturing and aggregation (Hofmann and Somero 1995; Chapple *et al.* 1998; Buckley *et al.* 2001; Halpin *et al.* 2004).

Similar to Harding *et al.* (2004a, b), the NRA results for seed exposed to an increase in temperature showed that thermal stress can induce a rapid stress response in mussels at the subcellular level. The present study, however, suggests that seed may be

more sensitive to temperature shocks relative to market sized mussels. The NRR times of seed exposed to an increase in temperature of 10-19 °C, showed a greater stress response, reaching a NRR time of 0 min after 4 h, compared to market sized mussels exposed to a similar increase in temperature of 5-15 °C, which reached a minimum NRR time of 30 min after 9 h before recovering to basal stress response levels. Both experiments were conducted on mussels that were in the pre-spawning stage of reproduction (early spring). However, the difference in the baseline temperatures of the two experiments makes comparing the results difficult. Moreover, the market mussels used by Harding *et al.* (2004a, b) and the seed used in the present study were from different populations, which could have different ratios of *Mytilus edulis* to *Mytilus trossulus* and levels of intra-species heterozygosity and therefore varying tolerances to thermal stress (Myrand and Gaudreault 1995, Sullivan and Couturier 2005). Seed mussels may be more vulnerable to temperature shocks for several reasons. Seed has a higher surface area to volume ratio, which allows for a more rapid heat exchange at the whole organism level, whereas relatively larger mussels have a greater thermal inertia, which buffers them against rapid changes in environmental conditions (Helmuth 1998). Batches of seed may also have a higher level of homozygosity and therefore contain individuals that are less capable of enduring and recovering from stressors (Myrand and Gaudreault 1995). More research is needed to determine if there is a definitive difference in the ability of seed and market sized mussels to cope with thermal stress.

In the spring, the NRR times of seed exposed to an increase in temperature returned to that of the control following 24 h. In the autumn, however, the NRR times remained significantly less than the control following 24 h of recovery, indicating that seed had not fully recovered from the thermal shock. The ability of seed to withstand and recover from thermal stress is, in part, related to their basal stress level, which is higher in early spring than to the autumn. For example, overwintering and pre-spawning mussels exhibit the less stress than at other times of the year (Harding *et al.* 2004a, b). However, during gametogenesis, spawning and post-spawning, mussels exhibit high levels of stress as shown by lysosomal destabilization, higher metabolic rate, lower survival, reduction in feeding, reallocation of energy, lower $\dot{O}_2$ consumption, and changes in glycogen and byssal thread production (Bayne and Thompson 1970; Bayne 1973; Thompson *et al.* 1978; Worrall and Widdows 1984; Young 1985; Eertman *et al.* 1993; Myrand and Gaudreault 1995; Tremblay *et al.* 1998b; Harding *et al.* 2004a, b). Throughout the summer and into the autumn, mussels show an increase in basal stress response levels as they begin to recover from compounding stressors of spawning, high water temperatures and decreased food quality (Harding *et al.* 2004a, b). The basal stress response may be lowered again if environmental conditions favour secondary spawning; however, lower water temperatures help to reduce the compounding effects that occur in the spring. Moreover, seed (~1-year-old) is less likely to undergo secondary spawning and put the same reproductive effort as more mature mussels (2+ years old) (Bayne *et al.* 1983). Condition indices of seed collected in the spring were not significantly different from

mussel seed collected in the autumn, despite being in different stages of the reproductive cycle. In the spring, seed was the pre-spawning stage of reproduction and therefore had a high level of glycogen reserves in gametes and vesicular connective tissue cells. In the autumn, seed likely had high levels of stored glycogen reserves in preparation for winter. Thus, seed may be vulnerable to thermal stress associated with harvesting, transport/storage, and socking if performed in the early summer, before seed has recovered from the main spawning event.

Seed exposed to a decrease in temperature of 9 °C (10-1 °C), showed less of a stress response than seed experiencing an increase in temperature. Following a 24 h recovery period the NRR time of seed exposed to a decrease in temperature had increased to 45 min, reaching a NRR time equivalent to 24 h prior; whereas, the NRR time of seed exposed to an increase in temperature had increased to only 15 min. Thus, seed was able to tolerate, and recover from, a large decrease in temperature, but seed was unable to recover from a large increase in temperature within the 24 h experiment. These results suggest that temperature differentials between collection sites and grow-out sites should be considered when transferring seed. For example, transferring seed to a region with relatively high water temperatures will induce a stress response that could compromise the long-term performance of seed and also compromise the ability of seed to become attached via their byssal threads, thus increasing the potential for seed loss following socking (Moeser and Carrington 1996). The results of these experiments support the

hypothesis that an increase or decrease from ambient water temperature will result in an increased stress response as observed using the NRA.

4.1.3 Extended air exposure and recovery

This experiment was designed to simulate the extended air exposure conditions, such as those occurring during the transport of seed between collection sites and socking sites. Long-term exposure to air results in desiccation and reduces the metabolic rate of mussels, resulting in eventual death (Eertman *et al.* 1993). Moreover, in wild mussels air exposure causes physiological changes in which lysosomal stability is altered, ammonia accumulates in the plasma, and the number of hemocytes in the hemolymph increases (Thompson *et al.* 1978; Tremblay and Pellerin-Massicotte 1997). Extended air exposure during transport is not unusual in Newfoundland, given its relatively large area.

Seed can spend two hours to several days out of water between harvesting and socking. The purpose of this experiment was to determine the impact of two storage conditions, ice and no ice (ambient air temperature), on the daily stress level of seed for a total of 3 d of storage and also to assess the ability of seed to recover within 24 h of re-immersion in seawater. As noted by Harding *et al.* (2004a, b), air temperatures equivalent to seasonal ambient water temperatures are least stressful to market sized mussels, followed by air temperatures below ambient water temperatures (but still above subzero levels) or within 5 °C of ambient water temperature. The results of the present experiment

are in agreement with Thompson *et al.* (1978), Tremblay and Pellerin-Massicotte (1997), and Harding *et al.* (2004a, b), and suggest that seed stored at ambient air temperature exhibited a significant stress response after 24 h, whereas seed stored on ice at ambient air temperature did not exhibit a significant stress response until after 48 h. In the spring, 24 h of recovery resulted in no significant change in the stress levels of seed. However, in the autumn, seed showed a greater stress response following 24 h of recovery. For example, for both storage conditions, seed re-immersed in seawater for a 24 h recovery period failed to recover to the stress levels of 24 h before, and on some days there was a slight increase in stress response following recovery. Also, seed placed on ice showed less recovery when placed back into seawater, relative to seed stored at ambient air temperature. The recovery period has the potential to induce a stress response greater than if the seed had been exposed to air exposure alone. Placing seed on ice results in a thermal stress caused by a lowering of internal temperature, followed by an increase in relative temperature following socking deployment. The recovery time required for seed to return to basal stress levels needs further study.

4.1.4 Effects of storage condition and anti-biofouling treatments on the short-term performances of mussel seed

As a preliminary experiment, the effects of 300 ppt brine, 4% lime, or vinegar on the short-term performance of seed was tested. This experiment was conducted in late

June, just prior to spawning - a stage in the reproductive cycle in which mussels are prone to stressors (Harding *et al.* 2004a, b). The short-term performance of seed treated with brine, either before (with or without a rinse) or after the simulated 15 h transport period, was not impeded. Similarly, lime (fresh and seawater solvents) treatments did not differ significantly from the control, except for the *treat (no rinse) - storage/ transport*. Not rinsing the lime off of seed prior to transport resulted in a mortality of 25%, a level that would not be acceptable for industry. The short-term performance of seed did not differ between the two types of solvent used to make up the lime solution (freshwater versus saltwater). Therefore, the use of freshwater as a solvent in lime solutions could lead to greater osmotic stress and be of greater potency to marine biofoulers, without negatively affecting seed health. Vinegar was the most toxic treatment with, *treat (no rinse) - storage/transport*, resulting in ~ 75% gapping (mortality), followed by, *treat (rinse) - storage/transport*, resulting in ~ 3% gapping (mortality). The short-term performance of seed exposed to *15 h air exposure - treat*, however, was not negatively affected. Forrest *et al.* (2007) also concluded that green-lipped mussel seed should be treated after transport in order to reduce mortality. Treating seed after transport, however, will require strict biosecurity measures at the site of destination (e.g. land based treatments, sterilization of treatment effluent).

Byssal attachment did not significantly differ between 24, 48 and 72 h of recovery. Sharp *et al.* (2006) found that exposure to 5% vinegar resulted in similar byssal attachment results for 24 and 48 h of recovery. This suggests that if seed is capable of

recovering, it likely does so within the first 24 h. It should be noted that interspecies differences in byssal production are a potential source of error in experiments related to byssal attachment. For example, *Mytilus trossulus* has higher attachment rates, a larger surface area of attachment and a faster crawling rate than *Mytilus edulis* (Sullivan and Couturier 2005). Moreover, *M. trossulus* has been shown to have the highest daily byssal production at 20 ppt salinity while *M. edulis* produces the most byssal threads at 30 ppt (Sullivan and Couturier 2005). This suggests that *M. edulis* is more tolerant of high salinities than *M. trossulus*, and therefore may be more tolerant of a brine dip (Gardner and Thompson 2001, Sullivan and Couturier 2005). Alternatively, *M. trossulus* may be more capable of sensing the high salinity of brine, therefore maintaining tighter valve closure than *M. edulis*, reducing soft tissue - brine contact. More research regarding species-specific tolerances to anti-fouling treatments is required. The results of this experiment also helped to direct field experiments in this study, which involved a longer transport time and the use of seawater as the solvent for the lime solution.

4.1.5 Tolerance of seed exposed to an extended period of 300 ppt brine or 4 % lime solutions

Initial trials suggested that seed was capable of withstanding exposure times to 300 ppt brine and 4% lime of greater than 30 seconds. The longer seed can be treated without resulting in significant stress or mortality, the greater the chemical's

effectiveness as a defouling agent. In this experiment, seed was treated with increasingly longer exposure times (e.g., 1, 2 and 3 min dip times) of 4% lime or 300 ppt brine. Seed exposed to a 1 min dip in 300 ppt brine or 4% lime, reduced the mean number of seed attached post-24 h of recovery to 75%. Thus, exposing seed to dip times of greater than 1 min, in combination with other compounding stressors, would likely result in a loss of seed that exceeds levels considered to be acceptable by industry (Denny 2008). The mean number of seed attached via their byssal threads was not significantly different among treatments (1, 2, and 3 min of exposure). This suggests that the exposure of the chemicals to soft tissue likely occurs within the first minute of the application. Once exposed to unfavourable environment (e.g., variation in salinity or pH) most mussels maintained valve closure for the duration of the dip. Similarly, Sharp *et al.* (2006) concluded that mussels respond to stressors by pausing pumping for up to 3 min, before re-testing conditions.

4.2 Effects of Storage Condition and Anti-biofouling Treatments on the Short and Long-term Performances of Mussel Seed

4.2.1 Short and long-term performance of seed exposed to brine treatments

The use of brine as an anti-biofouling chemical has many advantages over other chemicals; however, brining alone has limited effectiveness in treating established epibionts.

Mussels often live in estuarine environments and are therefore able to respond slight changes in salinity (Sharp *et al.* 2006). For example, within an estuarine environment mussels may routinely experience variations in salinity from 0 - 35 ppt. Mussels therefore respond quickly to a high osmotic stress (e.g., 300 ppt brine) by stimulating the induction of valve closure, and therefore minimizing soft tissue - brine contact. Unlike lime and vinegar, which work by providing a large change in pH, the toxicity of brine to biofoulers is based on osmotic stress which causes water to leave cells of the biofouling organisms. Brine is also relatively cheap, easy to work with and non-damaging to the environment. Brining alone, however, has a limited effect on some tunicate species. For example, Carver *et al.* (2003) found that exposure of the tunicate *Ciona intestinalis* to brine for 8 min resulted in 24% mortality. Brine is an effective treatment for treating some plant species, such as *Cladophora* spp. (Sharp *et al.* 2006), in which the difference in salt concentration between the cell contents and the bath of brine is significant. The water in the algal cells moves across the cell membranes into the brine solution, causing the remaining contents of the cells to shrink and separate away from the cell wall (Sharp *et al.* 2006). The short-term stress response of the mussel seed was minimal and the long-term performance of seed was unaffected by all brine treatments tested. Without the transport phase, and for the dip time tested, it is unlikely that brine would be potent enough to kill established biofouling communities. However, a long period of air exposure following the brine dip may result in gradual evaporation in low humidity environments (increasing the osmotic gradient and causing salt crystals) thereby

increasing the potency of the treatment without negatively affecting the health of the seed.

The data do not support the hypothesis that a 300 ppt brine treatment negatively affects the short or long-term health of seed. The immediate stress response was minimal and insufficient enough to result in any reduction in the long-term performance of seed, suggesting that under the conditions tested seed is able to tolerate the combined stressors of transport and brining.

4.2.2 Short and long-term performance of seed exposed to lime treatments

Similar to the brine treatments, the negative effects of lime were minimal in the short-term and negligible in the long-term. Seed not stored on ice and not rinsed prior to transport showed greater levels of stress and significantly decreased attachment 24 h post treatment. The long-term performance of seed, however, was not negatively affected with respect to survival, biomass, length or condition indices. In fact, the biomasses of seed exposed to 24 h transport (no ice, no lime) and seed not stored on ice, but treated after transport were significantly greater than the control. However, smaller mussels grow faster than larger mussels, thus the differences observed could be a result of these seed batches having a slightly lower initial biomass than the other batches of seed treated. Similar to brine, a short exposure to lime (30 s) has a minimal affect on the survival of some tunicate species. Seed that was treated with lime and then immediately put back

into the water was the only treatment to reduced survival significantly. This reduced survival is likely attributable to the intake of lime into the mantle cavity, before the lime has had a chance to adequately dilute following the re-immersion of treated seed in seawater. Carver *et al.* (2003) found that exposure of *C. intestinalis* to lime for 8 min resulted in only 70% mortality. However, when combined with a long transport phase, the efficacy of a lime treatment may be significantly greater for the same reason given above. Lim may be more useful in preventing tunicate settlement on collection lines. Tunicates can inhibit seed collection in the summer months, when the spawning periods of tunicates and mussels overlap (Gill *et al.* 2007). Gill *et al.* (2007) found that when 4 % hydrated lime was sprayed onto collection lines, they had a mean (±SE) mussel abundance of 104.6 ± 26.9 per 30 cm, compared with 38.7 ± 13.1 for vinegar and 0 for the controls, clubbed tunicate (*Styela clava*) abundances showing a reverse trend. Thus, treating seed with lime could potentially render larvae of *S. clava* unviable and/or incapable of settlement.

The data support the hypothesis that under certain circumstances (e.g., liming seed and placing it immediately back into seawater) 4% hydrated lime causes a significant stress response as measured by the neutral red assay and significant decrease in the growth and survival of mussel seed. The immediate stress response was far more pronounced than any reduction in the long-term performance of seed, suggesting that under most of the conditions tested, seed is able to recover from stress at the sub-cellular level.

4.2.3 Short and long-term performance of seed exposed to vinegar treatments

The use of vinegar as an anti-biofouling treatment has been studied in the Canadian Maritime Provinces and New Zealand (Carver *et al.* 2003; Forrest *et al.* 2007; Denny 2008). Relative to lime and brine, vinegar is a potent treatment that has potential as an anti-biofouling treatment in Newfoundland, particularly in high risk areas. The stress levels of seed were significant for seed exposed to vinegar and then immediately put back into ambient seawater (Vinegar (no transport)), as well as for seed that was not rinsed prior to transport (V-24 h-(NRNI), (NRI)). Byssal attachment post-24 h was significantly reduced for all treatments, with the exception of seed that was rinsed prior to transport and not stored on ice during transport. Similar results between the NRA and byssal attachment support the hypotheses that seed should not be treated and placed immediately into seawater, nor should seed be left un-rinsed during transport. Similarly, Forest *et al.* (2007) found that seed mortality could be minimized if the vinegar treatment was applied either before (with a rinse) or after a 24 h transport period.

Placing seed immediately back into seawater following vinegar treatment is particularly detrimental to the health and performance of seed for several reasons. First, the negative effect of a chemical treatment on the health of seed primarily depends on the degree of valve gapping, filtering and mantle siphon extension when seed is immersed in the chemical. For example, Forrest *et al.* (2007) observed high mortalities of seed when the valves of mussels failed to close or remained closed during chemical immersion. Once a significant stressor is detected (e.g., high salinity), *Mytilus edulis* often responds

by pausing pumping for up to 3 min, before re-testing conditions (Sharp *et al.* 2006). However, the toxicity of vinegar to mussels may be related to the inability of the mussel to react quickly enough to prevent the chemical from entering the mantle cavity. This is in contrast to change in salinity caused by brine, to which mussels likely react more quickly (Sharp *et al.* 2006). Moreover, without adequate rinsing, residual amounts of vinegar remain on the periostracum and therefore likely affect the pH of the surrounding seawater within the first min or so of deployment re-immersion.

Environmental conditions experienced prior to harvest may also affect the degree of valve gapping and pumping that occurs when chemical treatments are applied. For example, Riisgård (2006) found that valve opening and closing responses are strongly influenced by the preceding feeding conditions. When seed is harvested, the concentration of algae in the water column influences the basal level of valve gapping, which may affect the likelihood of mussels closing their valves during a chemical dip. Riisgård (2006) found that the critical algal concentration below which mussels close their valves is 700 *Rhodomonas* sp. cells•ml^{-1}, or 0.9 µg•L^{-1} of chlorophyll-*a*. Also, in an *in situ* environment Rissgard (2006) found that algal concentrations higher than 1 µg•L^{-1} chlorophyll-*a* stimulated the mussels to keep their valves wide open. In October, chlorophyll-*a* in Cap Cove, NL averaged 1.33 µg•L^{-1} and slowly declined until March (1.1 µg•L^{-1}), before rising again in April and May (3.32 µg•L^{-1}) during the annual spring bloom. In the present study, mussels likely exhibited an average level of valve gapping prior to harvest and chemical immersion. The effect of water temperature on the degree

of valve gapping is less pronounced (Kittner and Riisgård 2005). Further research is needed to determine the effects of environmental characteristics on valve gapping among harvesting, chemical immersion and socking of seed.

The duration of valve closure is likely reduced the less time mussels have been out of water. Immediate re-emersion of mussels following application of the vinegar could result in mussels pumping seawater before the residual vinegar has been diluted to a pH that can be tolerated by the seed. Second, the rate of dilution of the vinegar will be reduced in low energy environments, resulting in an increased duration of exposure to seawater of low pH. Third, large fluctuations in temperature (e.g., a large difference between air and water temperature and/or ascending or descending through a thermocline) could also affect the duration of value closure following re-immersion in seawater.

Treating seed prior to transport without the application of a seawater rinse also resulted in high levels of physiological stress and eventual mortality (V-24 h-(NRNI), (NRI)). Mussels held out of water for a long period of time exhibit valve gapping, which aids in gas exchange. However, excessive valve gapping in relatively low humidity, high temperature environments with vinegar vapours resulted in mussels becoming stressed. Storing seed on ice has been shown to aid in the prevention of mantle water loss, as well as reduce metabolic activity, valve gapping and physiological stress (Harding *et al.* 2004a, b). However, if the melting of ice during storage transport becomes excessive, this likely increases the contact between residual vinegar and soft tissue. Thus, transporting

seed treated with vinegar, without rinsing prior to transport, is not a viable option for industry. Rinsing seed is a relatively straight forward procedure for most operators, but poses the risk of re-inoculating treated seed with larvae or propagules of invasive species. As noted by Forrest *et al.* (2007), application of the treatment after the transport phase would be a better alternative, but would require stricter biosecurity procedures at the site of deployment.

The vinegar treatments also affected the long-term performance of seed with respect to survival, biomass and length. The higher biomass of seed treated after transport is likely a result of several factors. First, mussels that have been exposed to air are more stressed and therefore more likely to have a more rapid and longer valve closure response to shaking before being immersed in vinegar, reducing vinegar - soft tissue contact. Second, vinegar may also aid in the cleaning of seed, reducing the amount of mud, silt and fouling organisms (e.g., sea stars) (Warwick 1984). Fouling organisms can include filter feeders that compete with mussels for food, and predators, some of which have been shown to release predator-prey chemical cues which can induce a stress response and induce predator avoidance responses in mussels. Thus, the combination of a more pronounced valve closure response before immersion, in conjunction with the removal of fouling material, likely resulted in seed performing better relative to seed exposed to the other vinegar treatments.

Condition indices of seed did not differ among the seed batches treated. This suggests that the physiology of the seed that survived the long-term grow-out was not affected by the vinegar treatments.

Relative to the control and other treatments, the growth of seed was significantly reduced for seed not rinsed prior to transport. Blistering of the periostracum was also observed on the majority of seed that was not rinsed prior to storage/transport. The periostracum is the proteinaceous pigmented layer of the shell, which is secreted by the mantle. Ventral to the periostracum lays the prismatic shell layer, which is composed of calcium carbonate crystals oriented at a right angle to the horizontal plane of the shell (Checa 2000). The function of the periostracum in freshwater bivalves is to prevent acid erosion of the prismatic and nacreous layers. In *Mytilus* spp. acid erosion is less of an issue because of the high buffering capacity of seawater. However, when exposing *Mytilus* spp. to a vinegar dip, the periostracum still functions as a barrier between the acidic solution (vinegar) and the prismatic layer. The blistering observed suggests that the acidity of the vinegar exceeded the protective capabilities of the periostracum. Blistering was most pronounced along the posterior regions of the valves where the periostracum layer is thinner, and therefore less protective, relative to the thicker regions more proximal to the umbo. Stripping, declumping, and grading of seed could cause abrasions to the periostracum, further compromising its protective function (Dare 1974). Some of the mussels that survived the 8 month *in situ* grow-out exhibited new shell growth extending beyond the blistered area, whereas others showed no additional growth. In the

former group, damage to the mantle edge was likely kept to a minimum, whereas mussels of the latter group likely had some damage to the mantle edge that inhibited further shell growth. Similar experiments on green lipped mussels *Perna canaliculus* (26-56 mm shell length) did not show blistering of the periostracum as a result of not rinsing vinegar residue from seed prior to transport (Forrest and Blakemore, 2006). Experiments in which vinegar has been applied to *Mytilus* sp. have been carried out on larger mussels (50+ mm shell length) without a long transport period, and have also not mentioned any shell abnormalities (LeBlanc *et al.* 2007). This is likely the first time that damage to the outer shell of mussels has been observed as a result of a chemical treatment for the purpose of mitigating AIS.

Survival of seed differed significantly among the vinegar treatments. Treating seed with vinegar and immediately re-immersing it in seawater induced a significant stress response in the short-term, but survival exceeded 90%. This is in contrast to experiments on seed from Prince Edward Island (PEI), in which exposure to 5% vinegar for 30 s resulted in 60% of mussel spat being unattached and/or gaping (Sharp *et al.* 2006) and a reduction in biomass to 67% (relative to the control), following a 7 month *in situ* grow-out (LeBlanc *et al.* 2007). These results are comparable to the performance of seed exposed to vinegar in combination with 24 h storage transport.

The only treatment to result in a significant level of mortality was seed that which was not rinsed prior to transport and not stored on ice during storage transport (NR, NI) (survival = 64.6%). Seed that was not rinsed prior to transport and stored on ice during

storage transport had the next lowest survival (66.3%). In the present study seed ranged in shell length from 30-40 mm, however, the experiments performed by Sharp *et al.* (2006) and LeBlanc *et al.* (2007) were of different size ranges of 4-5 mm shell length and ~13 mm shell length, respectively. Therefore, even with the additional stressors associated with a long transport time, seed from Newfoundland performed similarly to seed from the Maritimes when treated with vinegar. This further supports the hypothesis that the larger size range of seed from Newfoundland is better able to cope with the combined affects of a vinegar treatment and a long transport time. Unfortunately, these survival percentages, in combination with the potential for blistering of the periostracum, would not be acceptable to industry. Moreover, higher temperatures could result in a synergistic effect, making the vinegar more potent and/or resulting in a greater heat shock, leading to a further decrease in survival (Marsden and Weatherhead 1998; Breidt *et al.* 2004 and Forrest *et al.* 2007). All other vinegar treatments tested had a survival of greater than 90%, which would likely be acceptable based on industry standards (Carver *et al.* 2003; Forrest *et al.* 2007). As Carver *et al.* (2003) suggested, these treatments could be a viable option for preventing the spread of the vase tunicate *C. intestinalis*. Carver *et al.* (2003) found that exposure to 5% vinegar for 30 s resulted in 95% mortality of established *C. intestinalis* and concluded that this treatment is an effective strategy for eliminating *C. intestinalis*. Moreover, Carver *et al.* (2003) found that mussels ~20 mm in length were able to withstand the treatment. Similarly, in New Zealand, Forrest *et al.* (2007) found that treatment with vinegar in conjunction with a 24 h air exposure transport

period was a cost-effective method of eliminating the majority of problematical biofoulers (e.g., *Botryllus schlosseri* and *Botrylloides leachi*) without resulting in significant negative health effects to the mussel stock. Similar to the results here in, Forrest *et al.* (2007) found that treatment followed by a rinse to remove the vinegar residue before transport, or application of the 4% treatment at the end of the transport period was most effective in reducing stress on the seed.

Although there has been much research regarding the effectiveness of chemical treatments on solitary tunicates in other regions (e.g., New Zealand and the Canadian Maritime provinces) the effectiveness of such treatments on colonial tunicates, adapted to the sub-arctic conditions of Newfoundland remains unclear. The recent discovery of the colonial tunicates *Botryllus schlosseri* and *Botrylloides violaceus* in Newfoundland waters reinforces the need for further research. Moreover, the need for chemical treatments specific to each tunicate species requires additional attention. Although vinegar seems to be a viable method of treatment for industry, it may not be an option for treating some AIS that are of great concern. The colonial tunicate *Didemnum vexillum*, which has been found in high abundances on the Grand Banks (south of Newfoundland), poses a potential threat to the fisheries and aquaculture industries of Atlantic Canada. Denny (2008) conducted similar trials (vinegar, 24 h transport) on *D. vexillum*, from New Zealand and found that vinegar was ineffective at eliminating 100% of *D. vexillum* at concentrations of acid which allowed mussel seed (*Perna canaliculus*) mortality to be maintained at a level acceptable to mussel farmers. Denny (2008) suggested that a 2 min

dip in 0.5% bleach solution was a more effective method of treating *D. vexillum*. However, the effect of this treatment on the stress and performance of mussel seed from Newfoundland requires study.

These results support the hypothesis that treating seed with vinegar in combination with a long transport time causes a significant stress response as measured by the neutral red assay and significant decrease in the growth and survival of mussel seed.

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4.2.4 Short and long-term performance of seed exposed to long-term storage

The transfer of mussel seed from collection site to grow-out site is a common practice in the Newfoundland mussel industry. Moreover, given the large geographic area of Newfoundland, transfer times can range anywhere from a few hours to a couple of days. Despite this, the effects of long-term air exposure on the short and long-term performances of seed have yet to be fully investigated. The purpose of this experiment was to determine how storage condition and storage time affect the stress response of seed in the short-term, and subsequent performances in the long-term. In the short-term, seed stored on ice for 24 h, exhibited stress levels that were significantly lower, relative to seed stored for 48 h (ice or no ice). This indicates that the longer seed is exposed to air, the greater the stress response and storing seed on ice during transport reduces this stress response. This is in agreement with studies on market sized mussels, in which storing

seed on ice after harvest significantly reduced stress levels and increased shelf life (Harding *et al.* 2004a, b). However, the process of re-deploying (e.g., socking) seed that was stored on ice during transport exposes it to an additional temperature shock (e.g., 8 °C water - 1 °C storage - 8 °C water). Covering seed during storage at ambient air temperature, however, resulted in a much more consistent temperature exposure (8 °C water - 8 °C air - 8 °C water); this despite an air temperature fluctuation of nearly 10 °C. Thus, the fact that seed failed to recover to basal NRR times within 24 h of being re-immersed in ambient seawater suggests that the process of re-deploying seed is an additional stressor for seed. Moreover, the greater the fluctuation in temperature of the air and/or water, the greater the stress imposed on the seed. The long-term performance results give some support to this, lengths of seed being significantly greater for seed not stored on ice for 48 h than in seed stored on ice for 24 h. However, the other long-term performance variables were not significantly affected by transport storage, suggesting that the stressors associated with seed transfer were not prolonged or intensive enough to induce a whole animal level response (Harding *et al.* 2004a, b). This is further supported by the fact that no significant correlations were observed between any of the 5 variables (NRR time, byssal attachment, length, biomass, condition index, survival) (Pearson's correlation, $p > 0.05$). In conclusion, mussel seed can be removed from water for up to 48 h without compromising long-term performance, as long as temperature fluctuations between harvesting, transport/storage and socking are kept to a minimum.

These results support the hypothesis that if transport conditions affect the health of mussel seed, then longer transport times, at temperatures higher or lower than ambient water temperature, will result in an immediate stress response. However, the hypothesis that such conditions result in a decrease in the long-term performance of seed was not adequately supported.

5. Conclusions and Recommendations

Associated with the transport of mussel seed around the island of Newfoundland is the risk of inadvertently transferring the larvae or propagules of Aquatic Invasive Species (AIS). The application of anti-biofouling chemical treatments has the potential to dramatically decrease this risk, but when combined with temperature shocks and long transport times, may adversely affect the health and long-term performance of mussel seed.

The NRA and byssal thread attachment show promise as rapid tests for assessing the short-term performance of mussel seed. Additionally, correlating short and long-term performance proved to be useful and could aid in the development of models to predict the long-term performance of seed, based on a short-term stress responses. These findings are especially important because few studies on bivalves of commercial interest have investigated the effects of early handling and husbandry on long-term performance. The ability to predict how mussel seed will perform would be invaluable for stress mitigation and therefore the production of an optimum product.

In the pre-spawning period (spring) mussels were better able to cope with temperature shocks than they were in the autumn. The earlier in the summer seed is harvested and socked, the greater the chance of inducing a significant stress response due to compounding stressors associated with recovery from spawning, elevated water temperature, and decreased food quality. Stress associated with harvesting,

storage transport, and socking can be minimized if temperature fluctuations are kept to a minimum and seed is covered during transport.

The 300 ppt brine solution can be applied to seed before (rinse or no rinse) or after a 24 h transport period, without affecting the long-term performance of seed. This offers a great deal of flexibility with respect to the logistics involved in transferring seed. Brine alone is of low toxicity to most established tunicate species. More research is required to determine whether brining in conjunction with a 24 h transport period is of greater toxicity to the larvae or propagules of AIS. Liming also shows promise as a method of treating mussel seed for transport. However, treating seed with lime and then immediately re-immersing in seawater likely resulted in the intake of seawater of high pH, resulting in a significant stress response and poor long-term performance. Application of vinegar as a defouling agent could be useful when carried out either before (with a rinse) or after transport. Failing to rinse seed of vinegar prior to transport resulted in a significant stress response and reduced survival. This practice should therefore be avoided by industry.

Future research should address the impact of longer exposure times of lime and brine on the long-term performance of seed. Moreover, with the recent discovery of several AIS on the south coast of Newfoundland, the effect of defouling chemicals on their survival will be critical for the Newfoundland aquaculture and fishery industries.

Storing and transporting seed for up to 48 h was possible without affecting the long-term performance of seed. However, seed transported earlier in the season (August and September) likely experience higher air temperatures compared to seed treated in October. The effects of greater temperature differentials between air and water, on the long-term performance of seed need further study.

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Tables

Table 1. Pearson's correlation analysis of short and long-term measures of performance of seed exposed to A) 300 ppt brine, B) 4 % hydrated lime, and C) vinegar. Numbers indicate correlation coefficient (upper) and p-value (lower). Bold numbers denote a significant correlation between performance measures ($p < 0.05$).

A)					
	Neutral Red Retention	Byssal Attachment	Biomass	Length	Survival
Byssal Attachment	0.269				
	0.15				
Biomass	-0.071	**0.397**			
	0.711	**0.03**			
Length	-0.12	0.117	**0.371**		
	0.529	0.538	**0.043**		
Survival	0.151	**0.534**	**0.377**	0.146	
	0.424	**0.002**	**0.04**	0.441	
Condition Index	-0.181	0.209	0.3	0.181	0.138
	0.339	0.267	0.107	0.34	0.468
B)					
	Neutral Red Retention	Byssal Attachment	Biomass	Length	Survival
Byssal Attachment	0.269				
	0.15				
Biomass	-0.071	**0.397**			
	0.711	**0.03**			
Length	-0.12	0.117	**0.371**		
	0.529	0.538	**0.043**		
Survival	0.151	**0.534**	**0.377**	0.146	
	0.424	**0.002**	**0.04**	0.441	
Condition Index	-0.181	0.209	0.3	0.181	0.138
	0.339	0.267	0.107	0.34	0.468
C)					
	Neutral Red Retention	Byssal Attachment	Biomass	Length	Survival
Byssal Attachment	**0.665**				
	< 0.001				
Biomass	**0.545**	**0.472**			
	0.002	**0.008**			
Length	**0.459**	**0.554**	**0.821**		
	0.011	**0.002**	**< 0.001**		
Survival	**0.612**	**0.61**	**0.804**	**0.771**	
	< 0.001	**< 0.001**	**< 0.001**	**< 0.001**	
Condition Index	**0.404**	0.323	**0.465**	**0.453**	0.342
	0.027	0.081	**0.01**	**0.012**	0.064

Table 2. Means (upper) and standard deviations (lower) of initial and final lengths (± 0.01 mm) (n = 25) and biomasses (± 0.01 g) (n = 50) of seed exposed to 300 ppt brine treatments. B - brine, NR - no rinse, R - rinse, I - stored on ice, NI - not stored on ice.

	Initial shell length (mm)	**Final shell length (mm)**	**Initial biomass (g)**	**Final biomass (g)**
Control	34.51	42.03	148.40	223.07
	0.44	0.40	8.98	30.62
24-h - Ice	36.03	42.32	174.00	270.93
	0.41	0.27	4.58	9.74
24-h - No Ice	35.12	42.85	157.38	266.77
	0.92	1.39	0.60	24.00
Brine - (no 24-h transport)	34.78	41.88	153.70	229.20
	0.10	0.41	8.31	36.30
B - 24-h - NR NI	34.55	42.92	154.00	270.33
	0.50	0.32	5.57	8.77
B - 24-h - R NI	34.94	43.23	154.33	271.13
	0.55	0.65	4.73	21.83
B - 24-h - NR I	35.05	42.65	163.33	276.77
	0.72	0.69	5.51	16.78
B - 24-h - R I	36.63	43.80	173.67	285.23
	0.64	0.99	0.58	6.07
24-h - B - I	35.29	43.04	164.33	273.13
	0.09	0.77	5.13	25.96
24-h - B - NI	34.43	42.35	152.16	263.17
	0.96	0.80	5.75	11.74

Table 3. Means (upper) and standard deviations (lower) of initial and final lengths (± 0.01 mm) (n = 25) and biomasses (± 0.01 g) (n = 50) of seed exposed to 4% hydrated lime. L - lime, NR - no rinse, R - rinse, I - stored on ice, NI - not stored on ice.

	Initial shell length (mm)	**Final shell length (mm)**	**Initial biomass (g)**	**Final biomass (g)**
Control	34.51	42.03	148.40	223.07
	0.44	0.40	8.98	30.62
24-h - Ice	36.03	42.32	174.00	270.93
	0.41	0.27	4.58	9.74
24-h - No Ice	35.12	42.85	157.38	266.77
	0.92	1.39	0.60	24.00
Lime - (no 24-h transport)	34.16	41.39	149.00	222.53
	1.33	1.47	20.66	19.21
L - 24-h - NR NI	35.22	43.35	157.33	261.53
	0.16	0.80	9.87	6.35
L - 24-h - R NI	34.78	42.43	162.67	274.83
	0.62	0.14	5.69	9.30
L - 24-h - NR I	34.93	42.42	166.33	254.80
	0.08	0.24	1.15	5.89
L - 24-h - R I	35.21	43.58	166.33	274.83
	0.49	0.36	2.52	10.12
24-h - L - I	35.70	43.31	167.00	273.57
	0.30	0.80	0.00	14.71
24-h - L - NI	35.37	43.18	151.81	264.93
	0.64	0.40	4.71	9.12

Table 4. Means (upper) and standard deviations (lower) of initial and final lengths (± 0.01 mm) (n = 25) and biomasses (± 0.01 g) (n = 50) of seed exposed to vinegar (4% acetic acid). V - vinegar, NR - no rinse, R - rinse, I - stored on ice, NI - not stored on ice.

	Initial shell length (mm)	**Final shell length (mm)**	**Initial biomass (g)**	**Final biomass (g)**
Control	34.51	42.03	148.40	223.07
	0.44	0.40	8.98	30.62
24-h - Ice	36.03	42.32	174.00	270.93
	0.41	0.27	4.58	9.74
24-h - No Ice	35.12	42.85	157.38	266.77
	0.92	1.39	0.60	24.00
Vinegar - (no 24-h transport)	34.28	41.89	155.27	235.40
	0.23	1.13	4.82	3.93
V - 24-h - NR NI	36.53	39.83	161.67	180.13
	0.31	0.29	4.04	14.08
V - 24-h - R NI	35.69	42.30	161.67	243.17
	0.21	0.43	9.24	11.94
V - 24-h - NR I	35.48	39.93	164.67	205.20
	0.33	2.53	5.13	60.81
V - 24-h - R I	35.50	41.68	161.00	233.07
	1.12	0.82	6.08	8.96
24-h - V - I	35.82	42.93	163.00	271.00
	0.11	0.31	6.93	8.80
24-h - V - NI	34.71	41.83	148.68	261.10
	0.31	0.93	8.11	7.41

Table 5. Means (upper) and standard deviations (lower) of initial and final lengths (± 0.01 mm) (n = 25) and biomasses (± 0.01 g) (n = 50) of seed exposed to zero (control), 24 h, or 48 h of air exposure, stored on ice or not stored on ice, in temperatures ranging from 1-10 °C.

	Initial shell length (mm)	**Final shell length (mm)**	**Initial biomass (g)**	**Final biomass (g)**
Control	34.51	42.03	148.40	223.07
	0.44	0.40	8.98	30.62
24-h - Ice	36.03	42.32	174.00	270.93
	0.41	0.27	4.58	9.74
24-h - No Ice	35.12	42.85	157.38	266.77
	0.92	1.39	0.60	24.00
48-h - Ice	35.54	43.00	165.33	273.23
	0.30	0.86	3.79	11.10
48-h - No Ice	34.77	43.57	166.43	284.63
	0.50	0.61	6.96	10.90

Figures

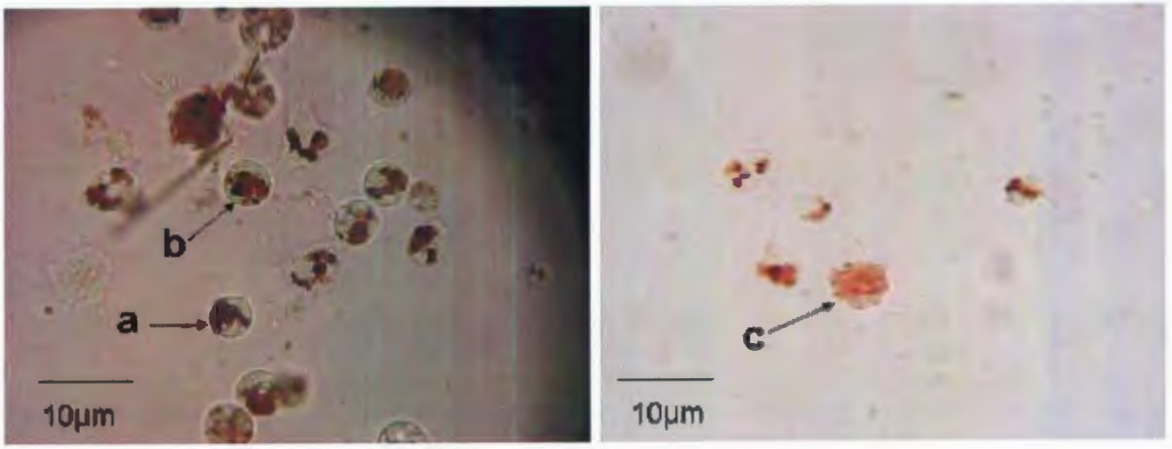

Figure 1. Hemocytes of mussel seed of varying levels of stress stained with Neutral Red dye, under 400× light microscopy. A) Slightly stressed hemocyte - characterized by dark red dots, which are lysosomes containing neutral red dye particles. Lysosomes are swollen and beginning to fuse, but still intact. B) Moderately stressed cell - lysosomes are swollen and beginning to rupture, releasing hydrolytic enzymes and neutral red dye into surrounding cytosol. C) Highly stressed cell - lysosomes are completely ruptured, cytosol is tinged pink due to leakage of the neutral red dye into cytosol and pseudopodia are well defined.

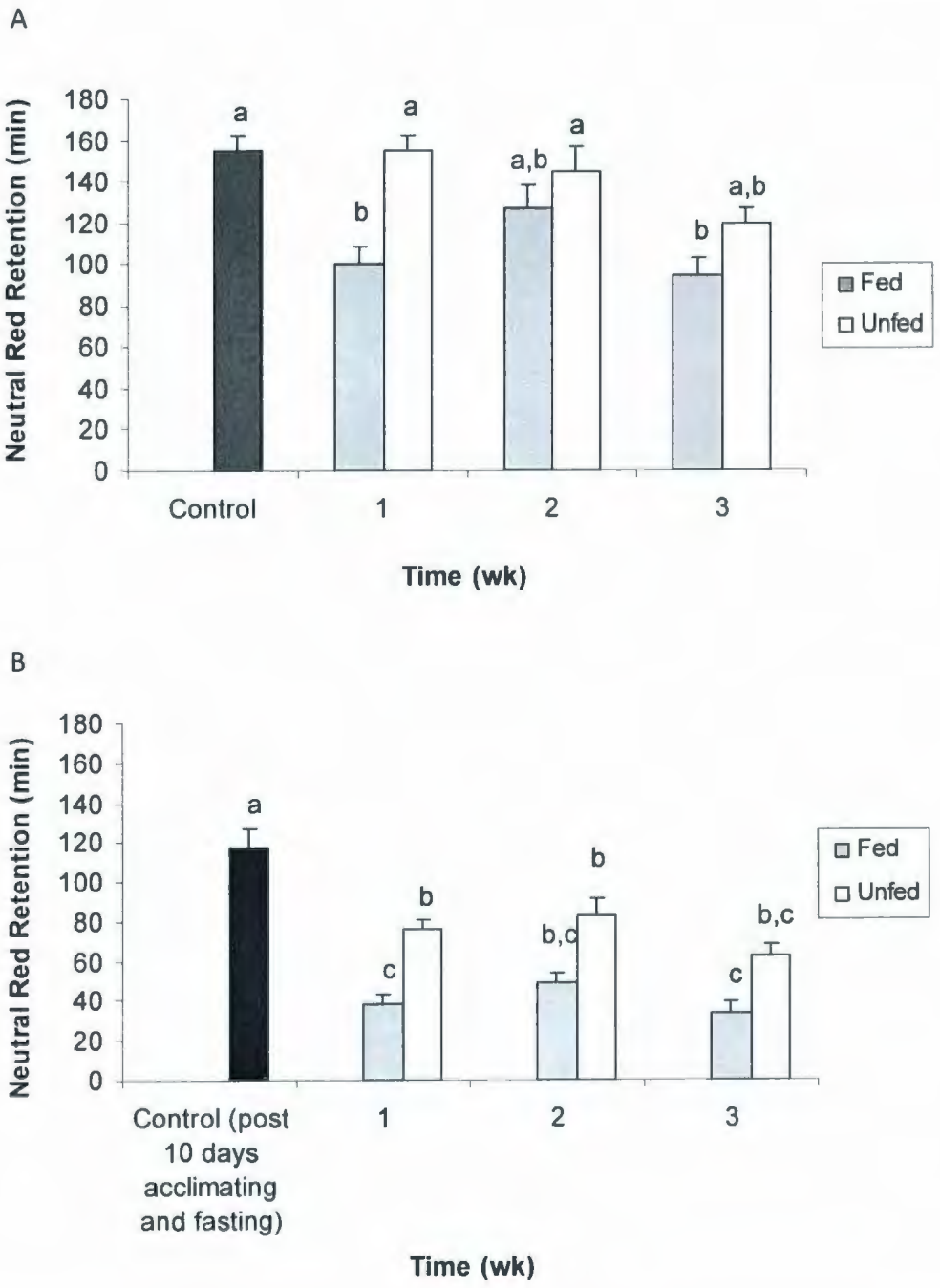

Figure 2. Effects of long-term holding and feeding on NRR times in lysosomes of mussel seed held in re-circulation raceways at a temperature of A) 2 °C and B) 10 °C. Bars express the mean + S.E., n =12 mussel seed. Common letters indicate no significant difference among treatments (Tukey's b, $p>0.05$).

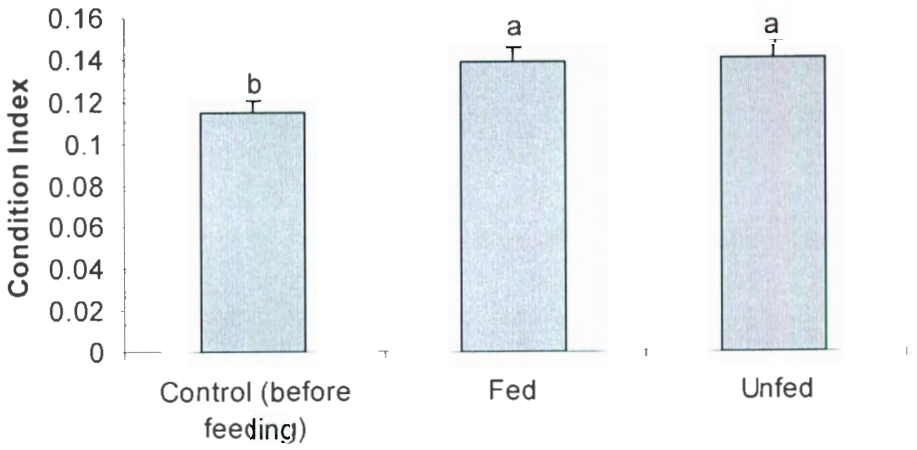

Figure 3. Effects of long-term holding and feeding on condition indices (dry tissue weight/dry shell weight) of mussel seed held in re-circulation raceways for a period of 10 wk. Bars express the mean + S.E., n = 12 mussel seed. Common letters indicate no significant difference among treatments (Tukey's b, $p>0.05$).

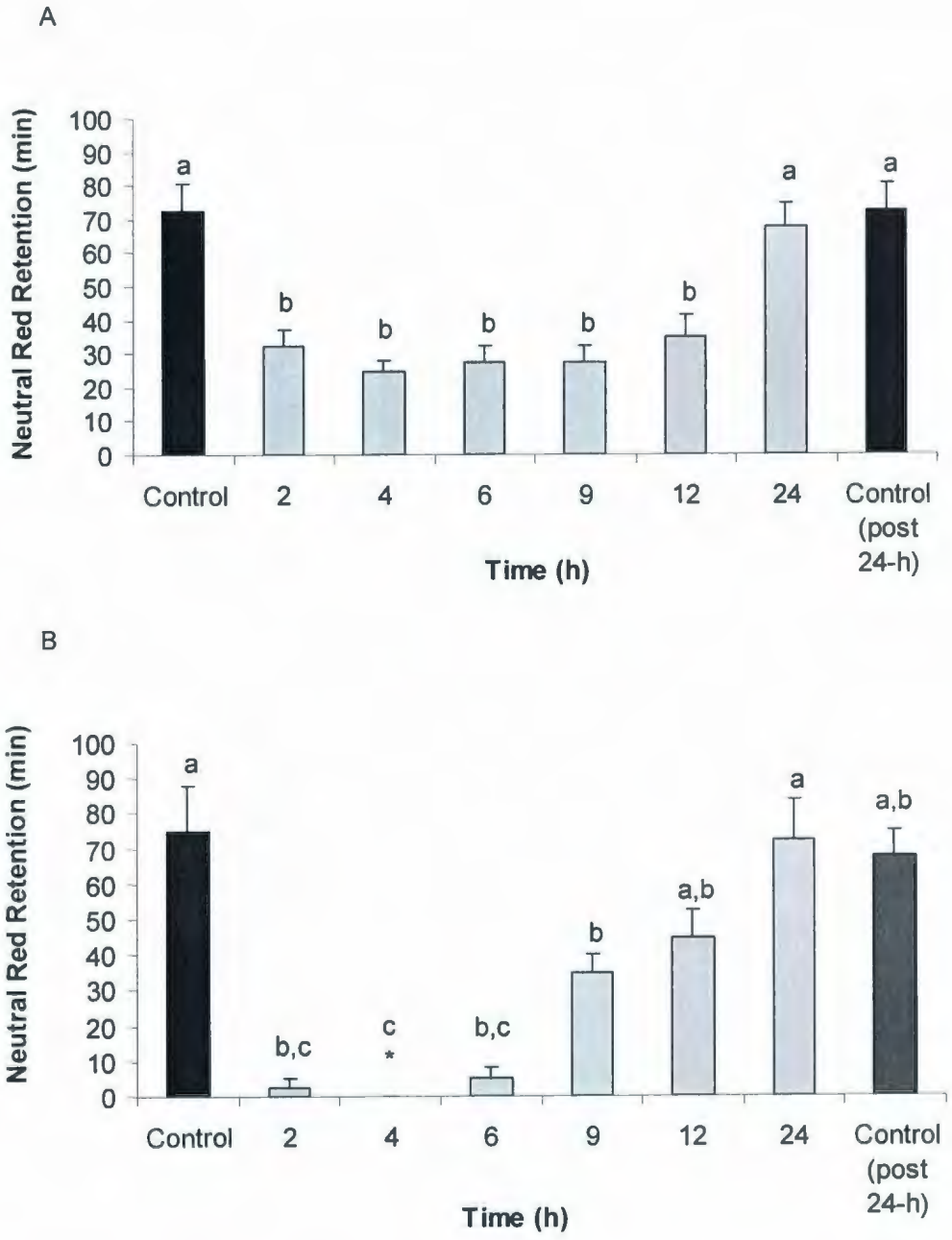

Figure 4. Effects of a 4 h temperature shock A) 10-19 °C and B) 10-1 °C, and 20 h recovery time, on NRR times in lysosomes of mussel seed collected in the **spring** and held in re-circulation raceways. Bars express the mean + S.E., $n = 12$ mussel seed. Common letters indicate no significant difference among treatments (Tukey's b, $p>0.05$). * denotes a value of zero.

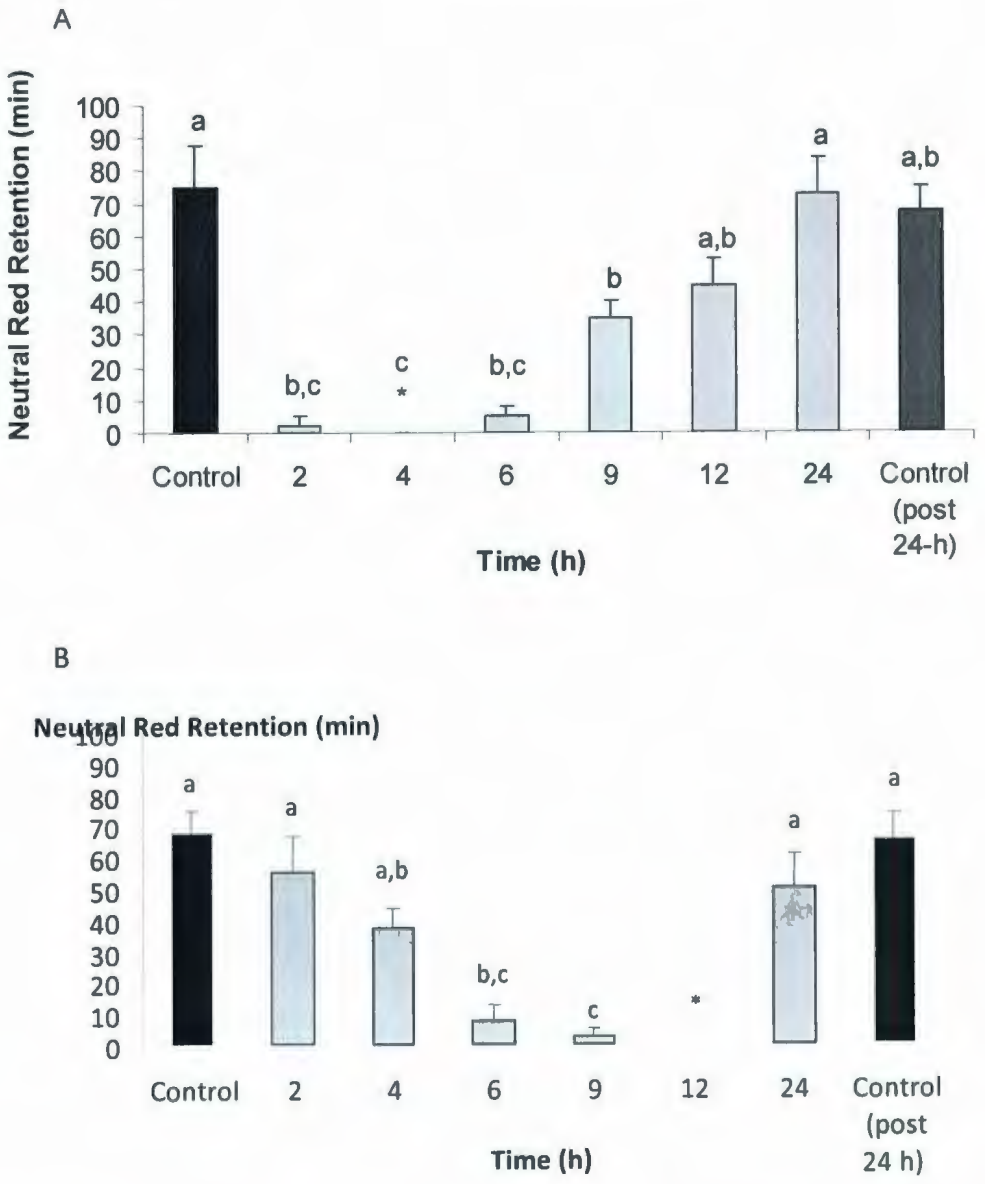

Figure 5. Effects of a 4 h temperature shock A) 10-19 °C and B) 10-1 °C, and 20 h recovery time, on NRR times in lysosomes of mussel seed collected in the **autumn** and held in re-circulation raceways. Bars express the mean + S.E., n = 12 mussel seed. Common letters indicate no significant difference among treatments (Tukey's b, $p>0.05$). * denotes a value of zero.

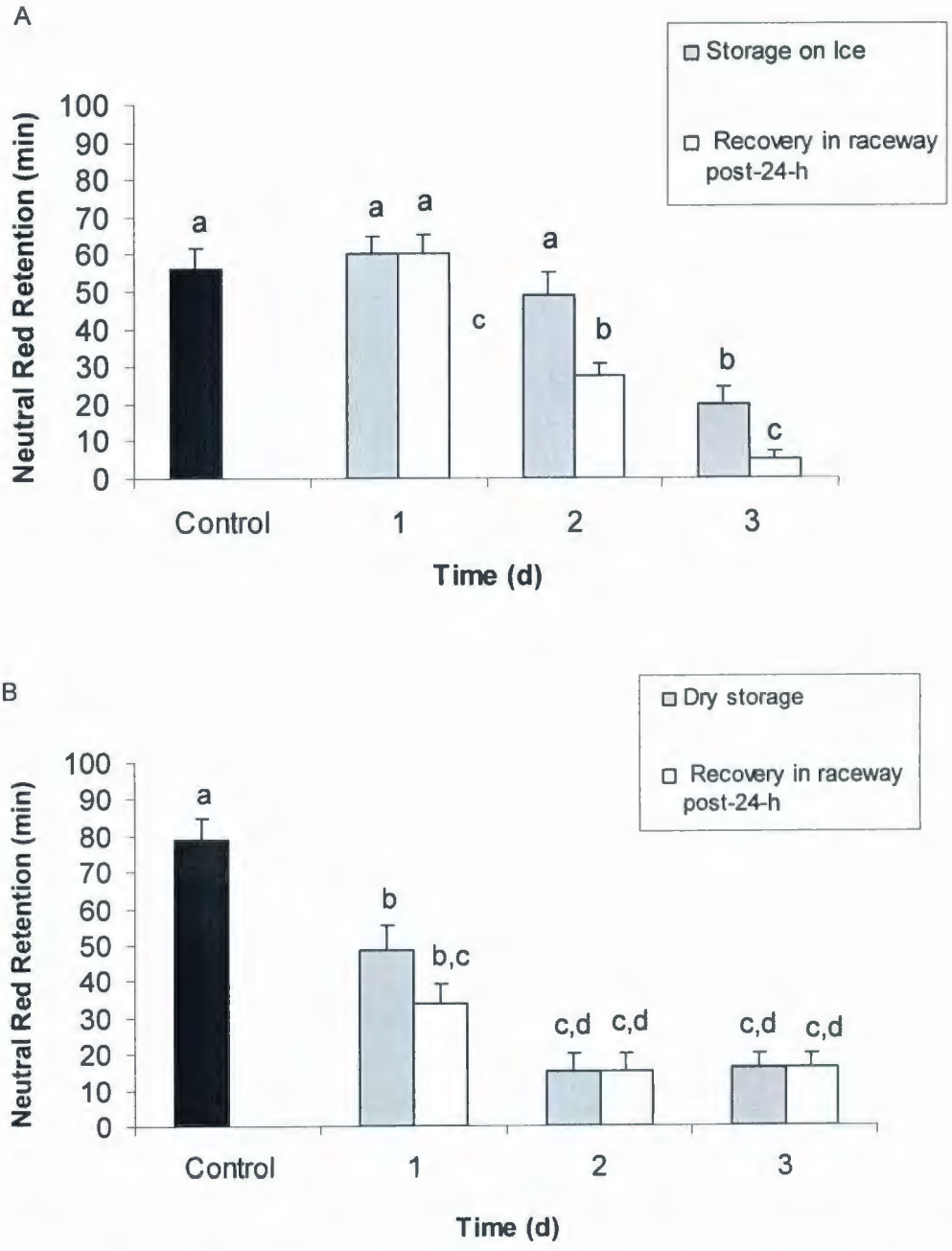

Figure 6. Effects of storage condition A) storage on ice and B) dry storage at 4 °C (100% humidity) and 24 h of recovery, on NRR times in lysosomes of mussel seed collected in the **spring** and held in re-circulation raceways. Bars express the mean + S.E., $n = 12$ mussel seed. Common letters indicate no significant difference among treatments (Tukey's b, $p > 0.05$).

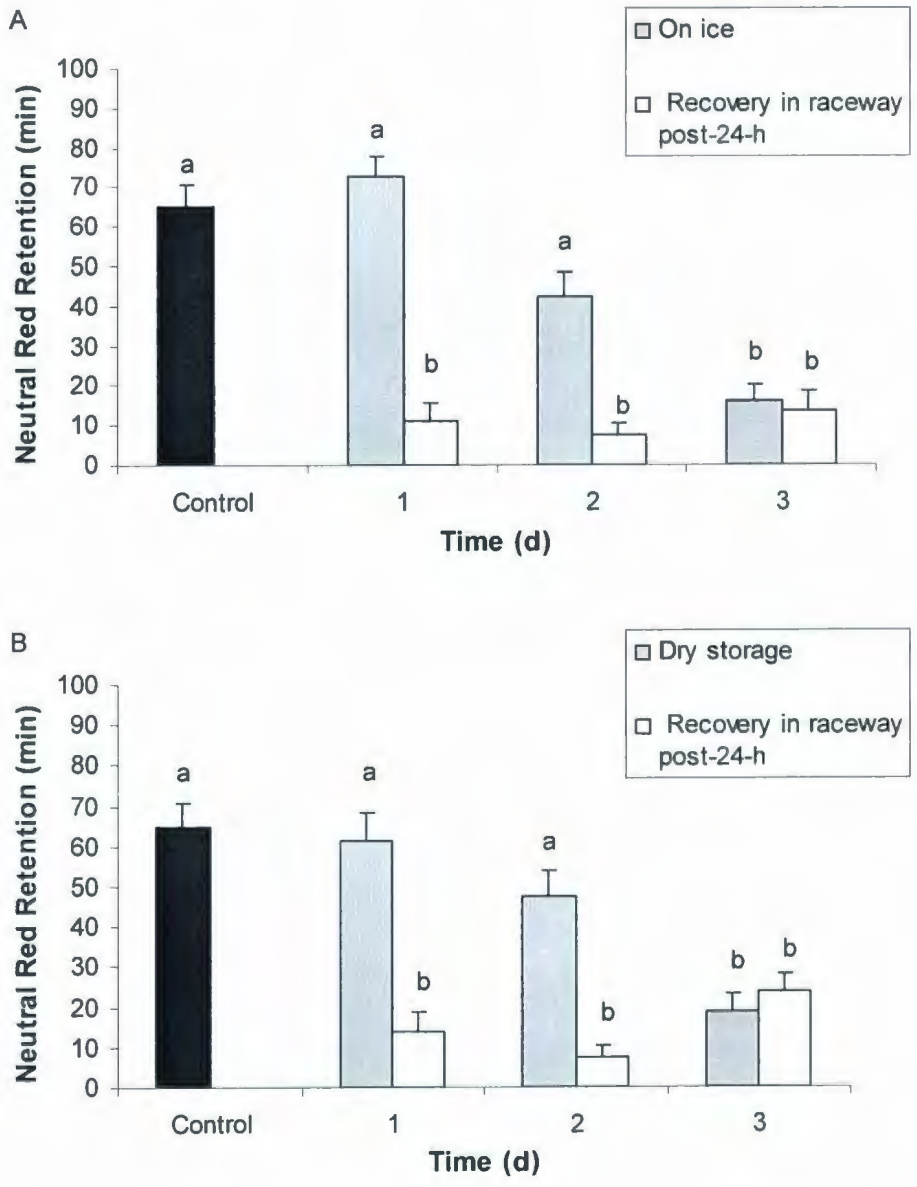

Figure 7. Effects of storage condition A) storage on ice and B) dry storage at 4 °C (100% humidity) and 24 h of recovery, on NRR times in lysosomes of mussel seed collected in the **autumn** and held in re-circulation raceways. Bars express the mean + S.E., $n = 12$ mussel seed. Common letters indicate no significant difference among treatments (Tukey's b, $p > 0.05$).

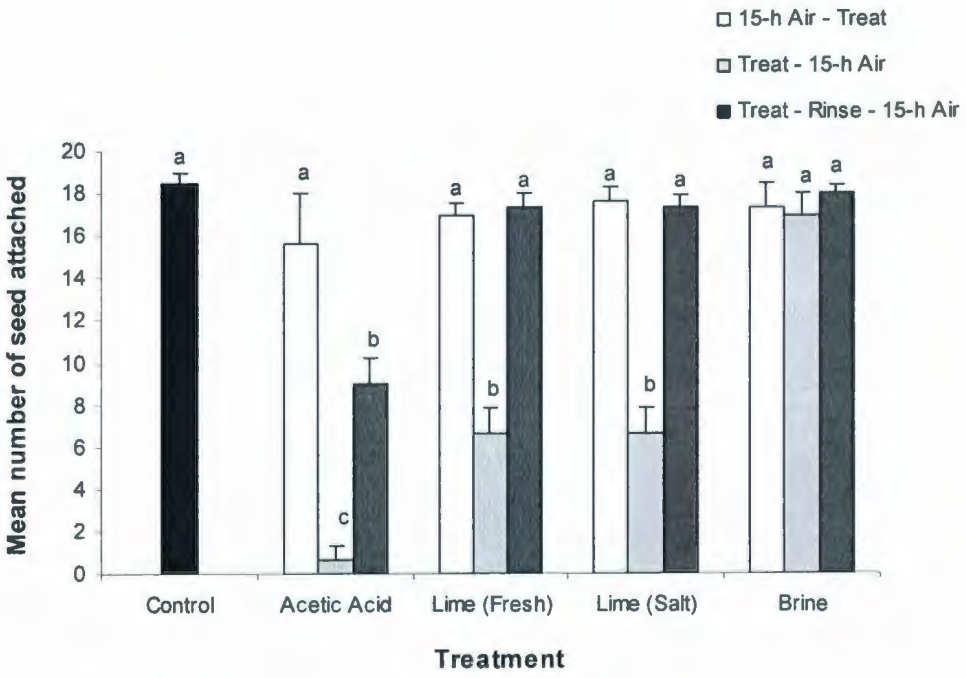

Figure 8. Effects of vinegar, 4% lime (fresh or salt water solvent), and 300 ppt brine, applied before (rinse and no rinse) or after a simulated 15 h transport/storage period at an air temperature of 4 °C (100% humidity) on mean number of mussel seed attached via their byssal threads 24 h post treatment. Seed collected in the spring and held in re-circulation raceways prior to the experiment. Bars express the mean + S.E., $n = 3$ replicates of 20 mussel seed. Common letters indicate no significant difference among treatments (Tukey's b, $p > 0.05$).

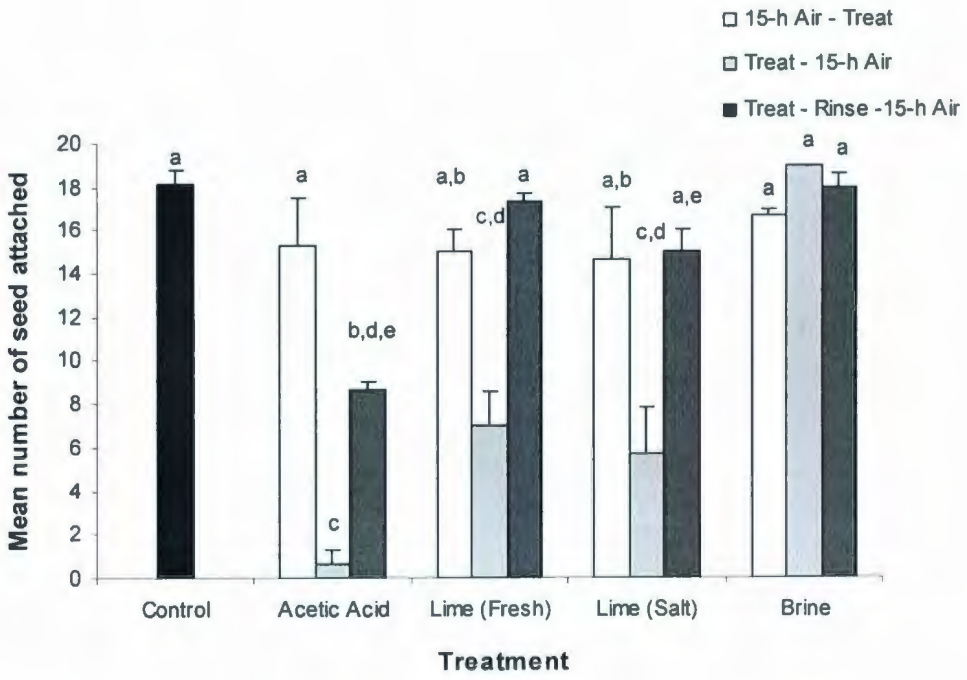

Figure 9. Effects of vinegar, 4% lime (fresh or salt water solvent), and 300 ppt brine, applied before (rinse and no rinse) or after a simulated 15 h transport/storage period at an air temperature of 4 °C (100% humidity) on mean number of mussel seed attached via their byssal threads 48 h post treatment. Seed collected in the spring and held in re-circulation raceways prior to the experiment. Bars express the mean + S.E., $n = 3$ replicates of 20 mussel seed. Common letters indicate no significant difference among treatments (Tukey's b, $p > 0.05$).

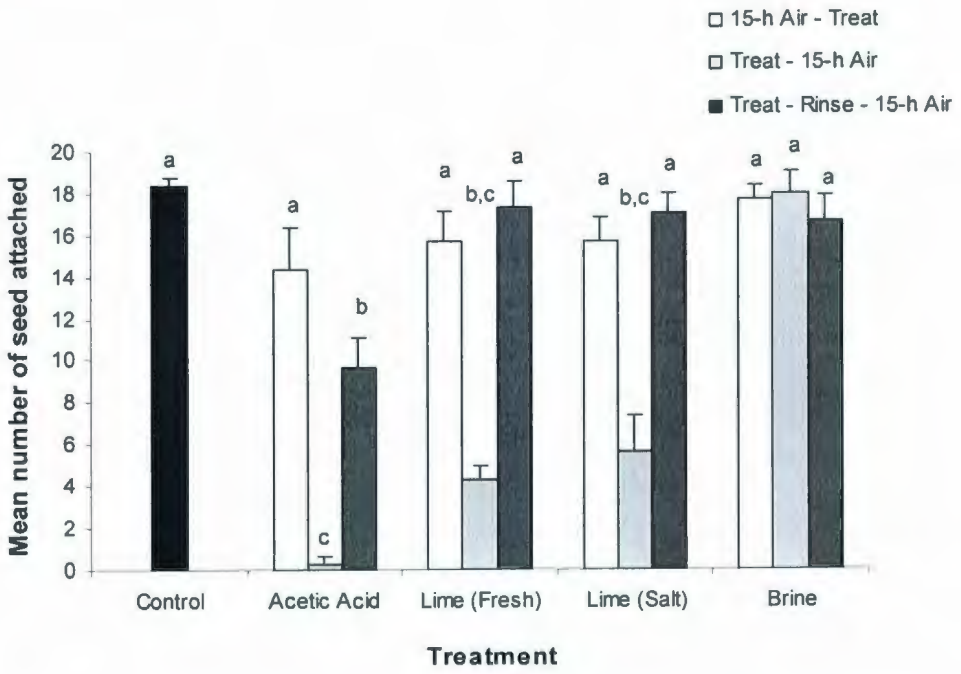

Figure 10. Effects of vinegar, 4% lime (fresh or salt water solvent), and 300 ppt brine, applied before (rinse and no rinse) or after a simulated 15 h transport/storage period at an air temperature of 4 °C (100% humidity) on mean number of mussel seed attached via their byssal threads 72 h post treatment. Seed collected in the spring and held in re-circulation raceways prior to the experiment. Bars express the mean + S.E., $n = 3$ replicates of 20 mussel seed. Common letters indicate no significant difference among treatments (Tukey's b, $p > 0.05$).

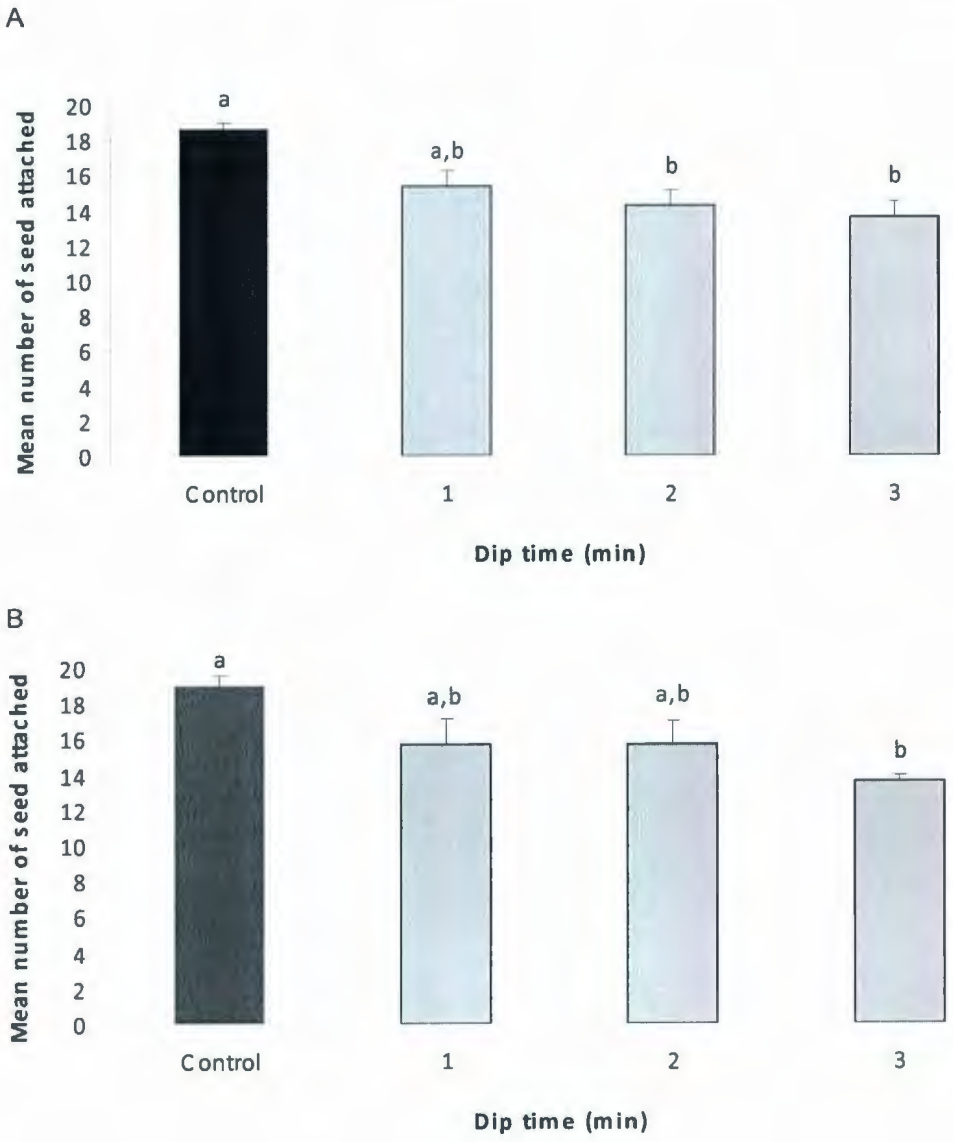

Figure 11. Effects of extended dip times of A) 300 ppt brine and B) 4% lime, on the mean number of mussel seed attached via their byssal threads after 24 h of recovery. Bars express the mean + S.E., $n = 3$ replicates of 20 mussel seed. Common letters indicate no significant difference among treatments (Tukey's b, $p > 0.05$).

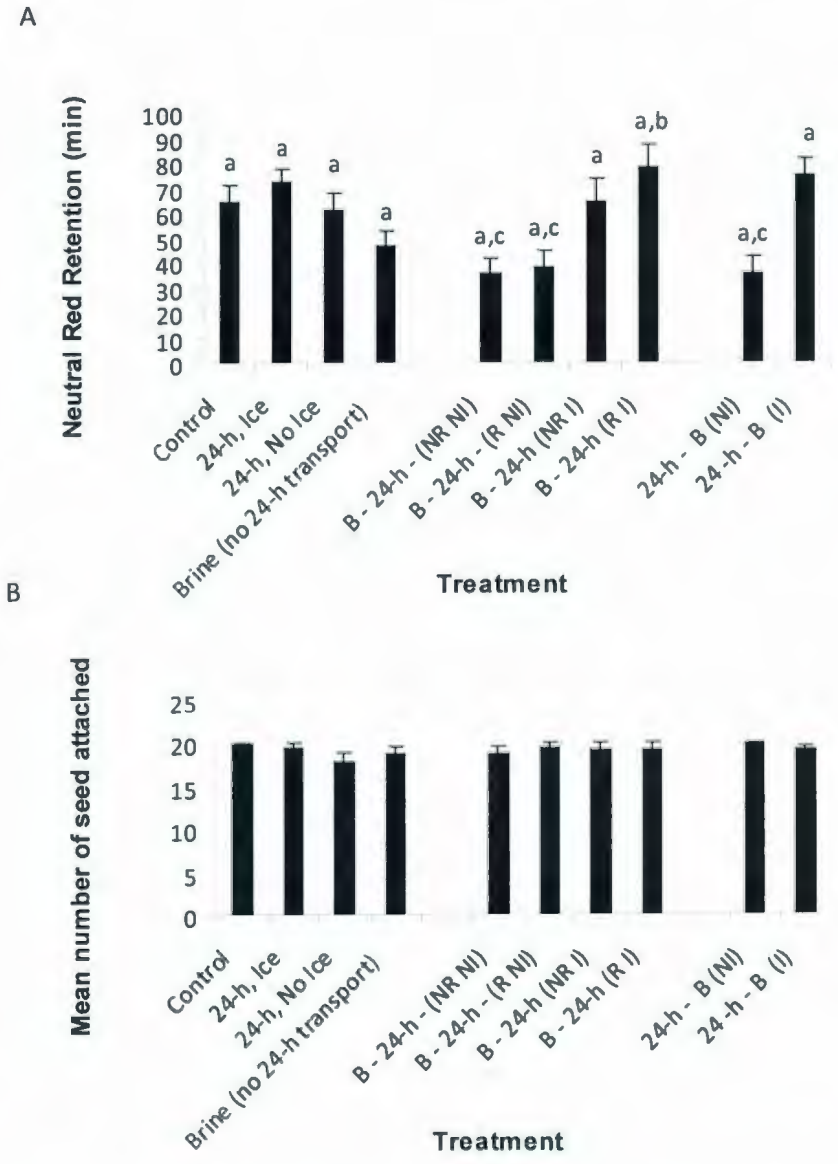

Figure 12. Effects of a 30 s, **300 ppt brine** dip before (rinse, no rinse) or after a 24 h simulated storage/transport period, on the short-term performance of mussel seed A) Neutral Red Retention time (n = 12), B) mean number of seed attached via their byssal threads 24 h post treatment (n = 3 replicates of 20 mussel seed). Bars express the mean + S.E. Common letters indicate no significant difference among treatments (Tukey's b, $p > 0.05$).

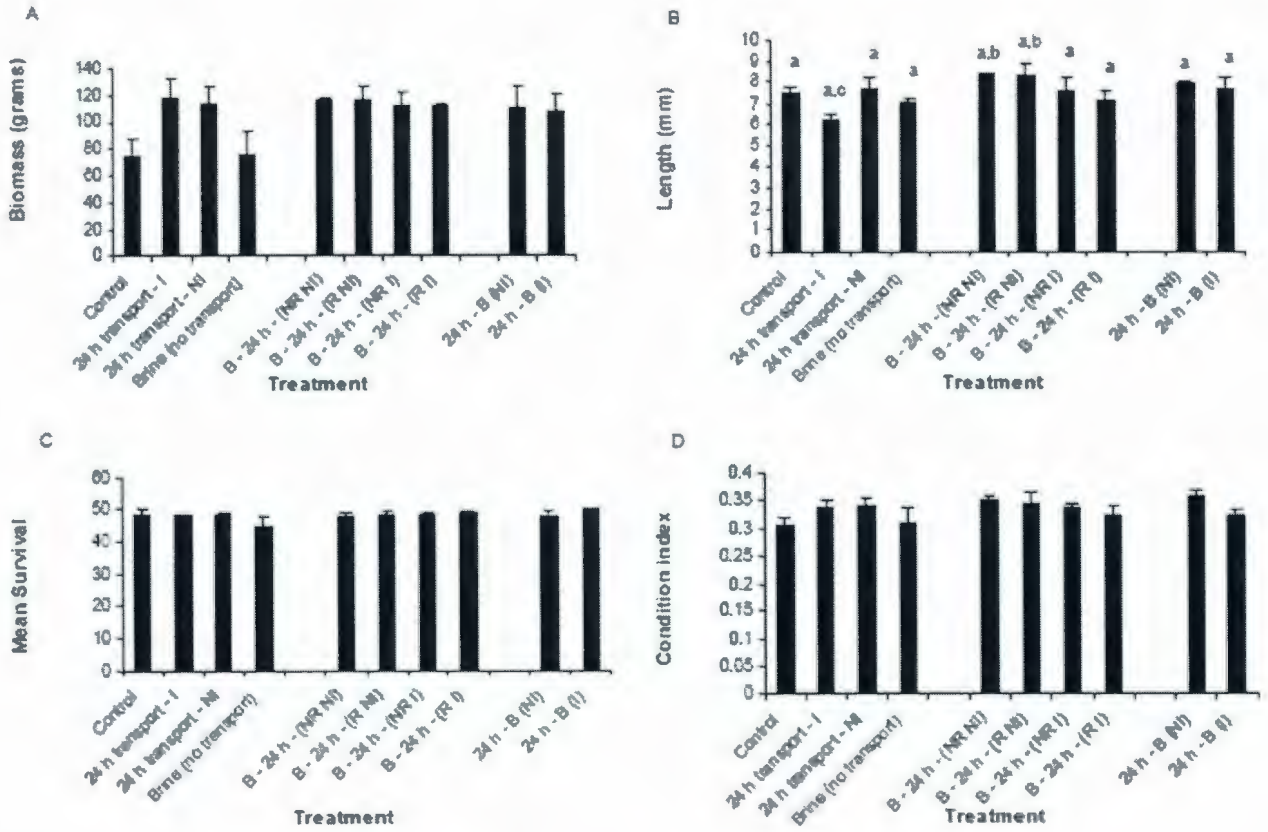

Figure 13. Effects of a 30 s, **300 ppt brine** dip before (rinse, no rinse) or after a 24 h simulated storage/transport period, on the **long-term performance** of mussel seed A) biomass. B) length. (n = 25), C) survival, and D) condition index. (n = 30). Bars express the mean + S.E. Common letters indicate no significant difference among treatments (Tukey's b, $p>0.05$).

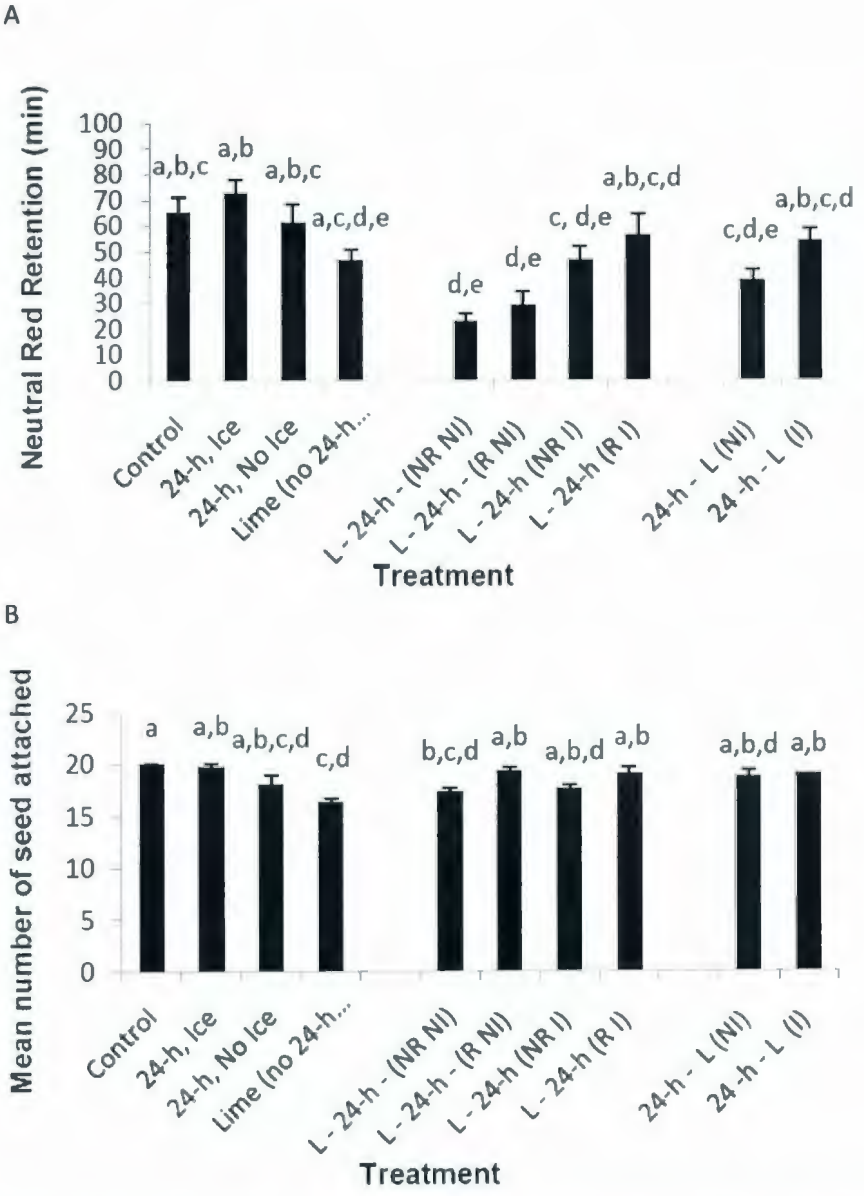

Figure 14. Effects of a 30 s, **4% hydrated lime** dip before (rinse, no rinse) or after a 24 h simulated storage/transport period, on the short-term performance of mussel seed A) Neutral Red Retention time (n = 12), B) mean number of seed attached via their byssal threads 24 h post treatment (n = 3 replicates of 20 mussel seed). Bars express the mean + S.E. Common letters indicate no significant difference among treatments (Tukey's b, $p > 0.05$).

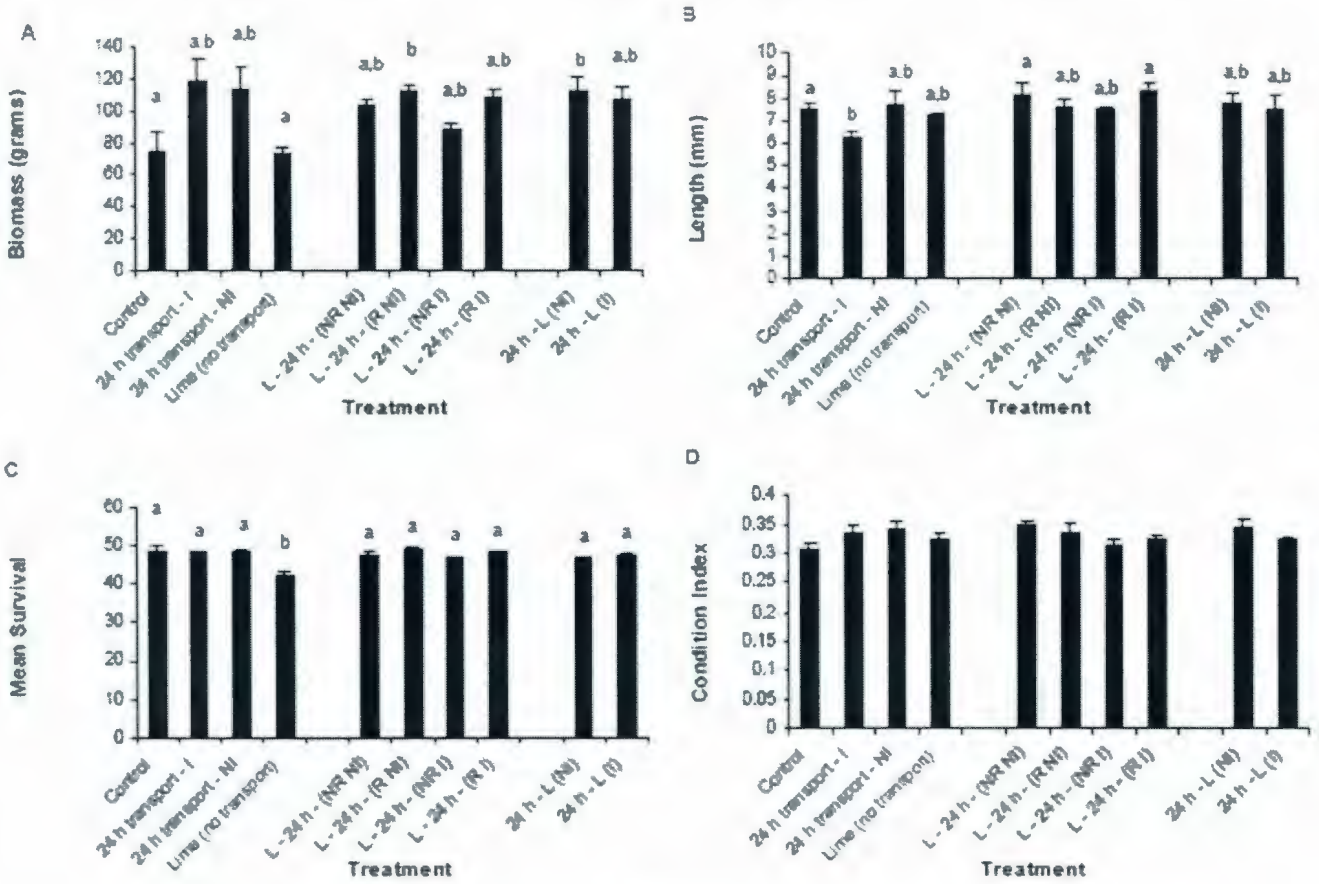

Figure 15. Effects of a 30 s, **4% hydrated lime** dip before (rinse, no rinse) or after a 24 h simulated storage/transport period, on the **long-term performance** of mussel seed A) biomass, B) length, (n = 25), C) survival, and D) condition index, (n = 30). Bars express the mean + S.E. Common letters indicate no significant difference among treatments (Tukey's b, $p>0.05$).

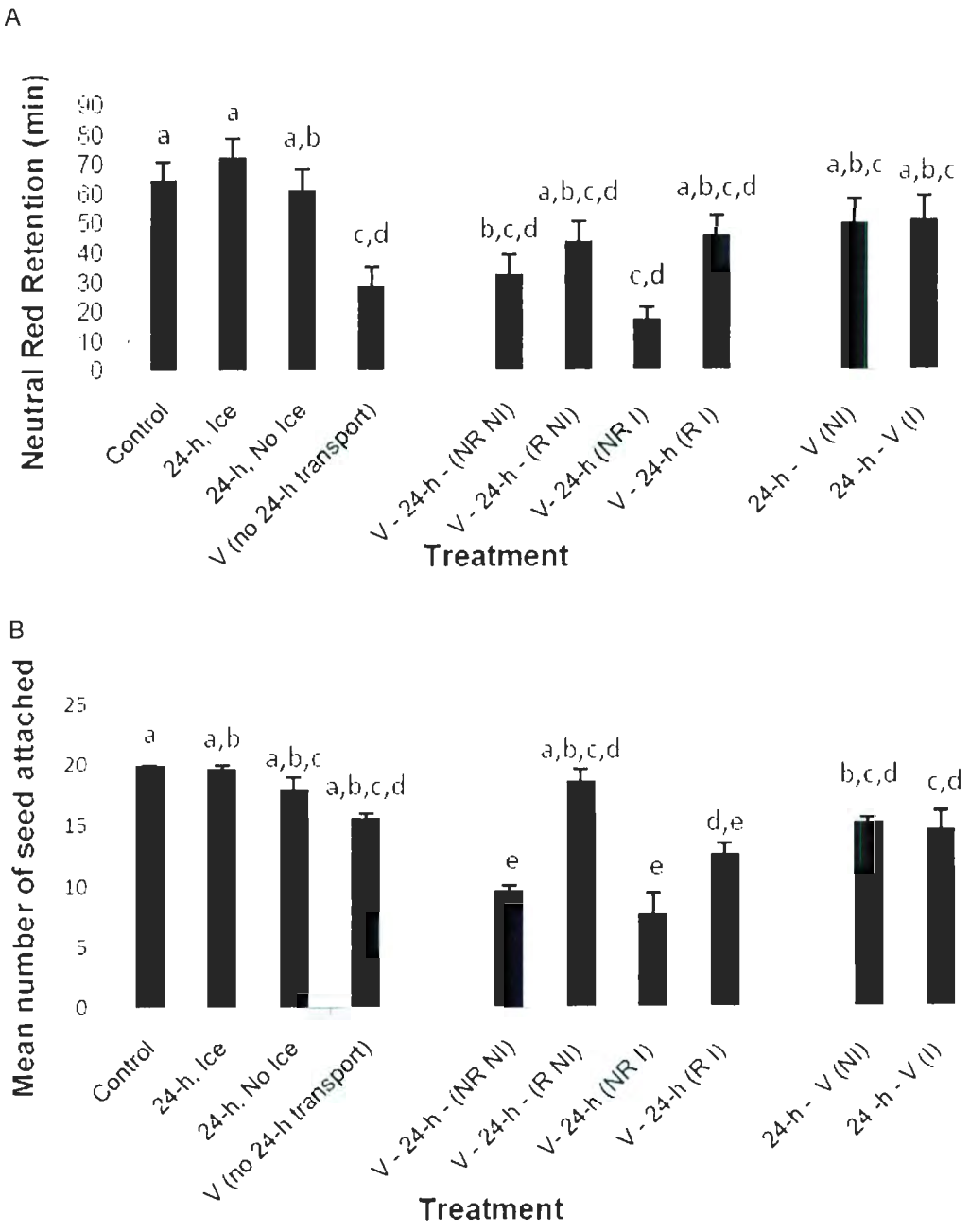

Figure 16. Effects of a 30 s, **vinegar** (4% acetic acid) dip before (rinse, no rinse) or after a 24 h simulated storage/transport period, on the short-term of mussel seed A) Neutral Red Retention time (n = 12), B) mean number of seed attached via their byssal threads 24 h post treatment (n = 3 replicates of 20 mussel seed). Bars express the mean + S.E. Common letters indicate no significant difference among treatments (Tukey's b, $p > 0.05$).

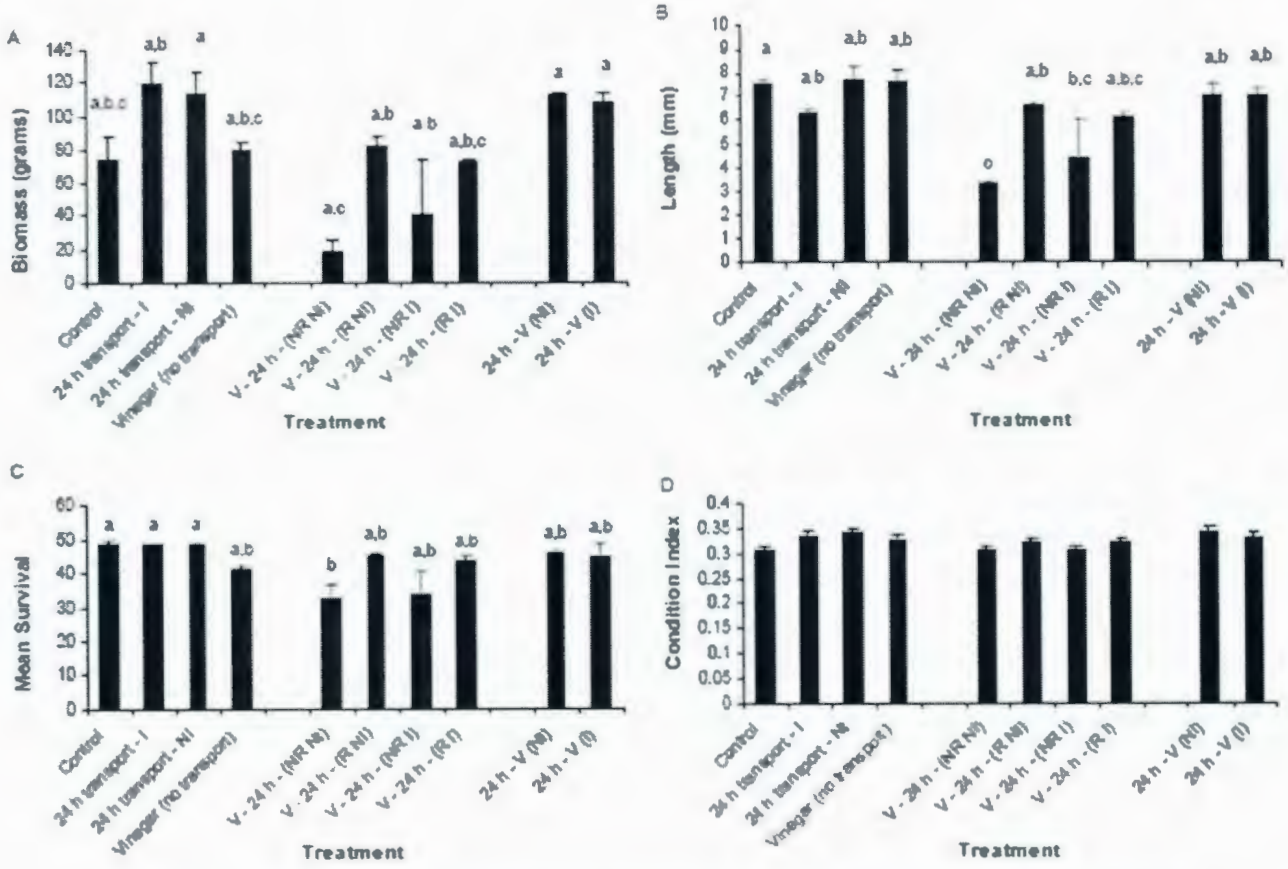

Figure 17. Effects of a 30 s, vinegar (4% acetic acid) dip before (rinse, no rinse) or after a 24 h simulated storage transport period, on the long-term performance of mussel seed A) biomass, B) length, (n = 25), C) survival, and D) condition index, (n = 30). Bars express the mean + S.E. Common letters indicate no significant difference among treatments (Tukey's b, $p>0.05$).

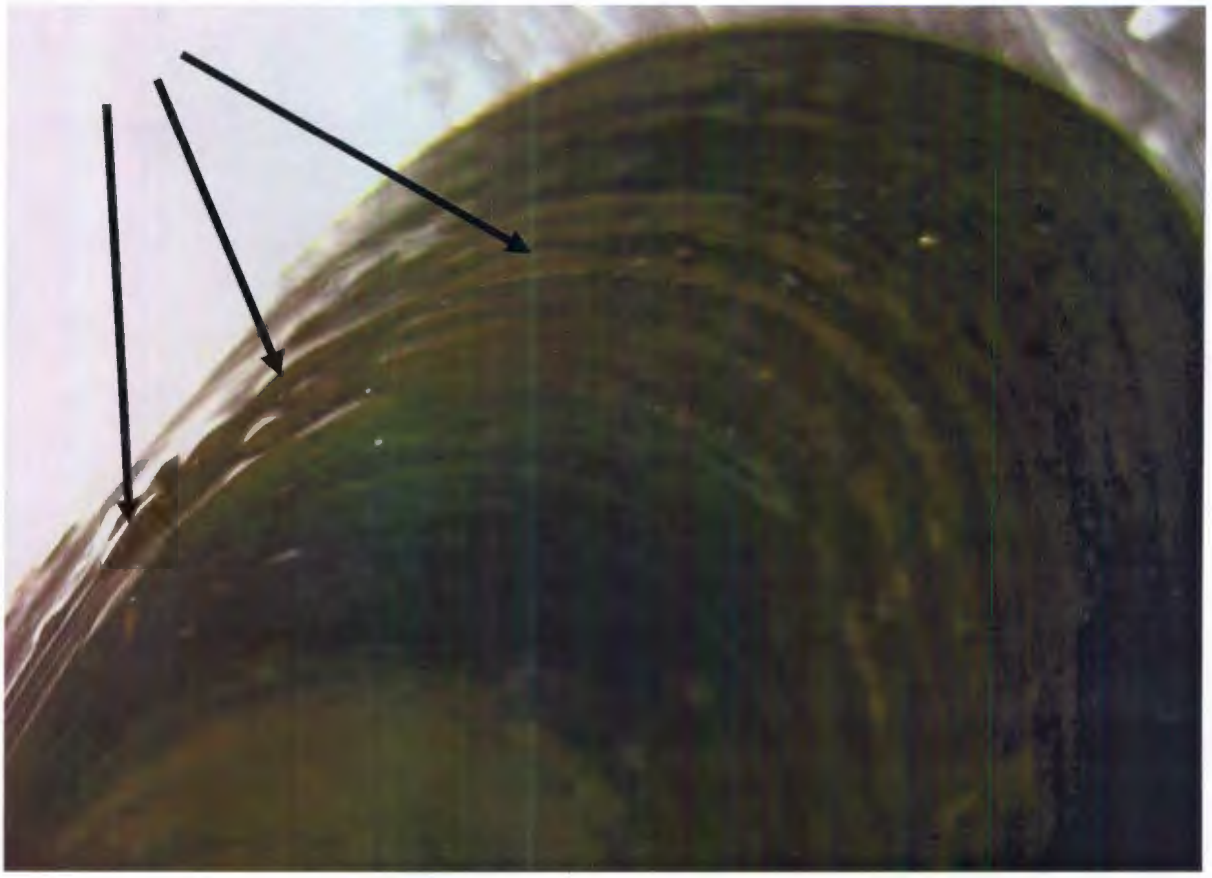

Figure 18. Photo a mussel (approx. 35 mm length) exhibiting blistering of the periostracum as a result of not rinsing vinegar residue from seed prior to a 24-h storage/transport period. Arrows indicate blistered regions of the periostracum.

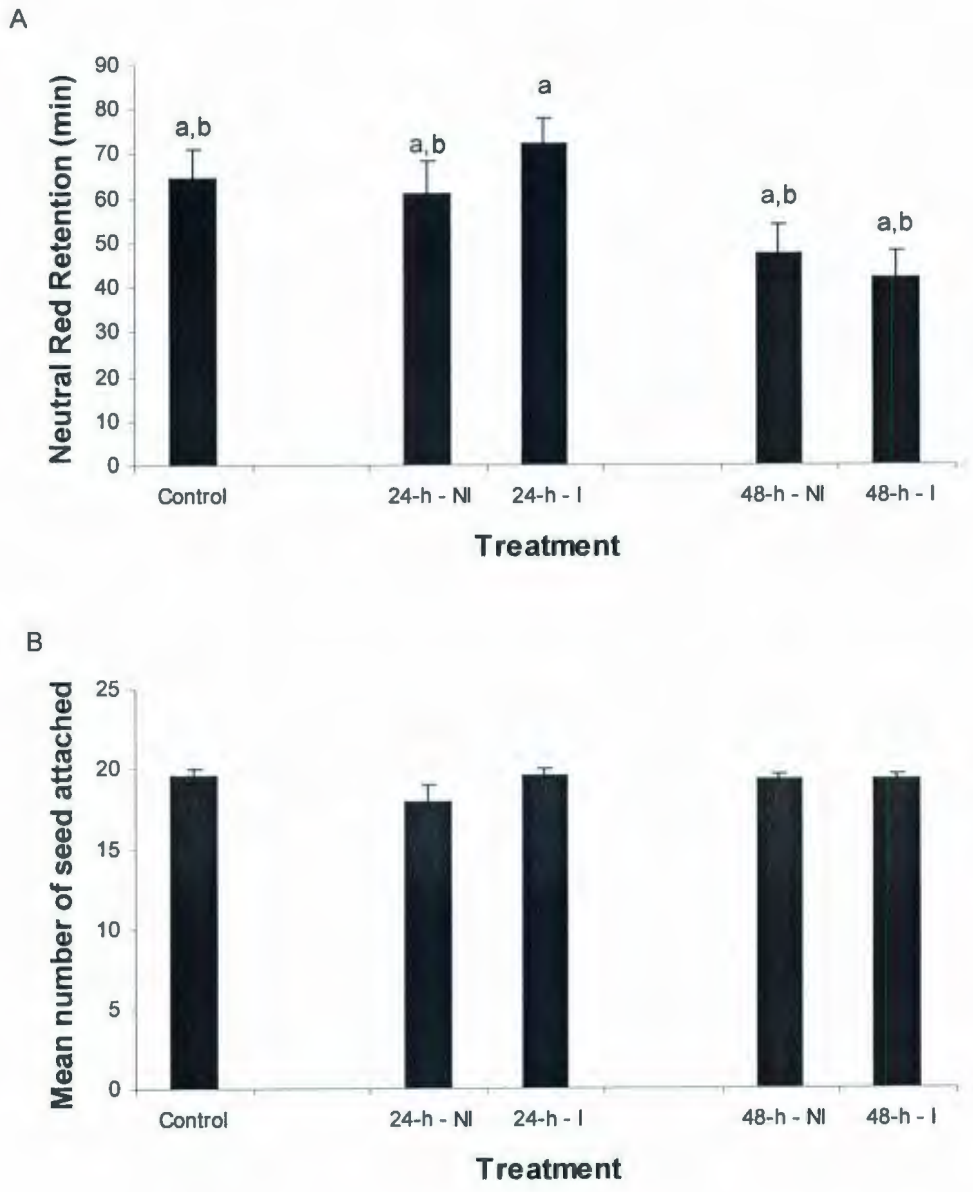

Figure 19. Effects of storage condition (on ice or ambient air temperature, 4 °C, 100% humidity) and storage time (0, 24, and 48 h) on the short-term performance of mussel seed A) Neutral Red Retention time (n = 12), B) mean number of seed attached via their byssal threads 24 h post treatment (n = 3 replicates of 20 mussel seed). Bars express the mean + S.E. Common letters indicate no significant difference among treatments (Tukey's b, $p > 0.05$).

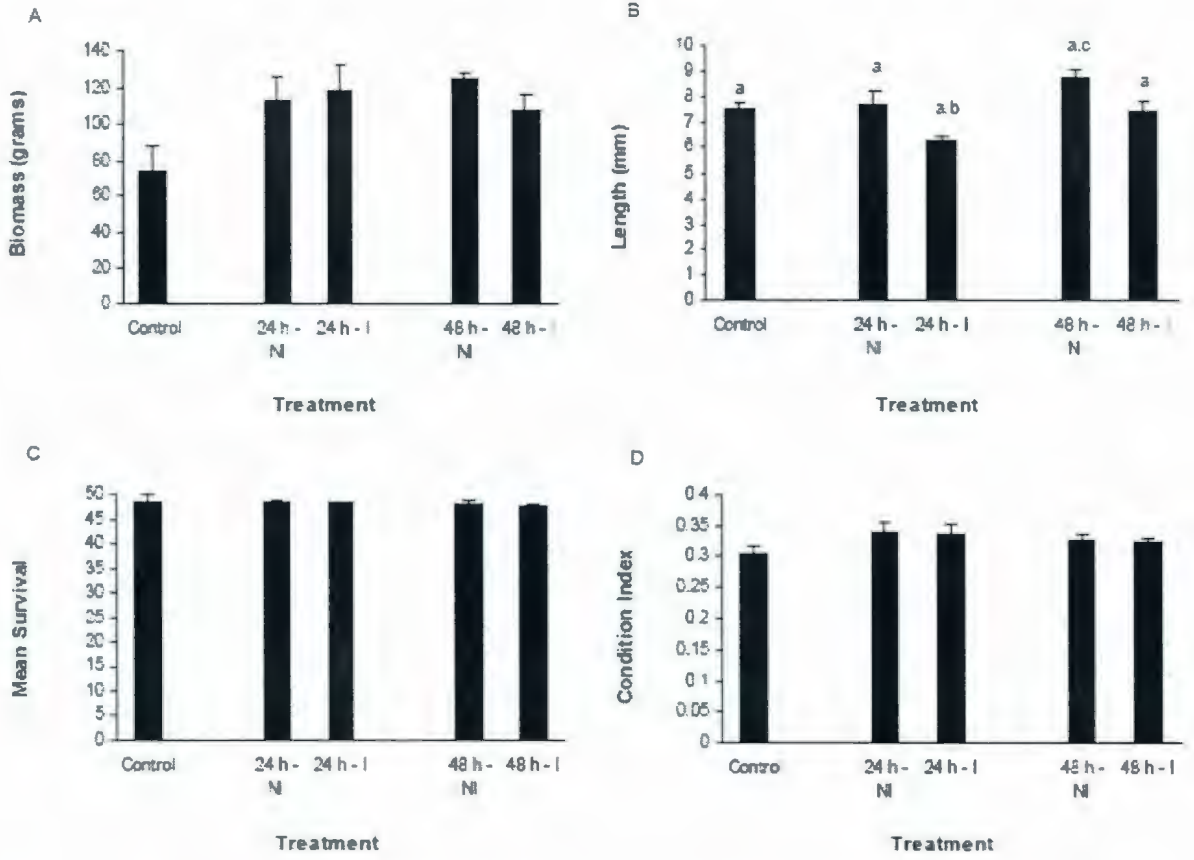

Figure 20. Effects of storage condition (on ice or ambient air temperature, 4 °C, 100% humidity) and storage time (0, 24, and 48 h) on the long-term performance of mussel seed A) biomass, B) length, (n = 25), C) survival, and D) condition index, (n = 30). Bars express the mean + S.E. Common letters indicate no significant difference among treatments (Tukey's b, $p>0.05$).

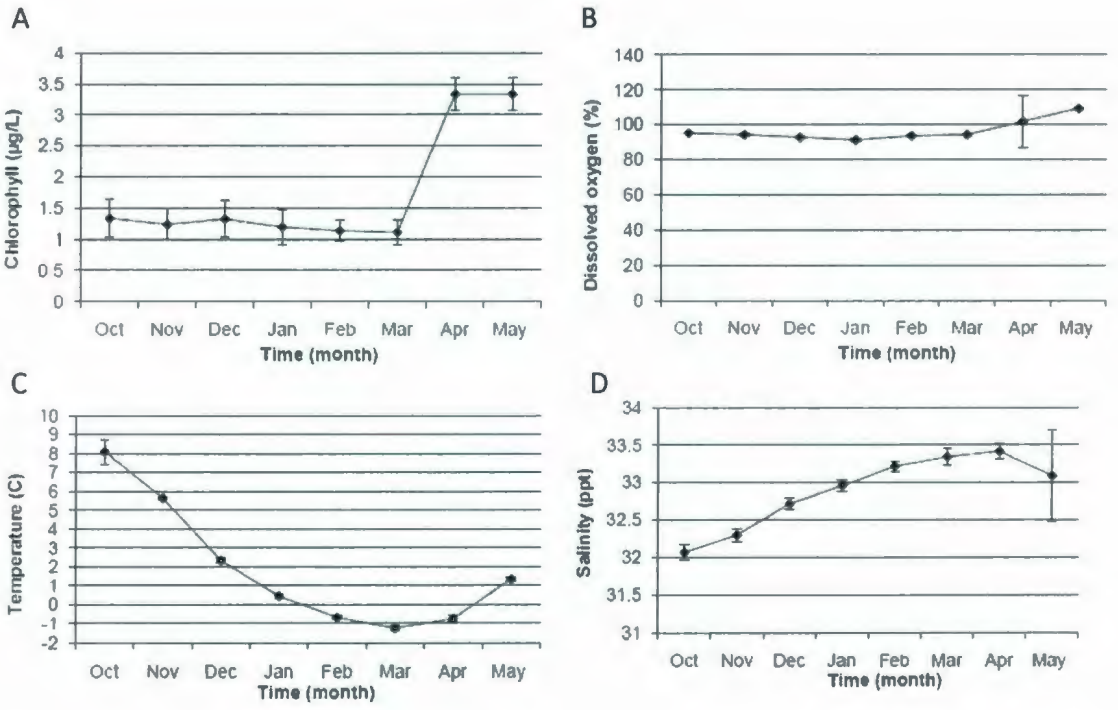

Figure 21. Mean monthly A) chlorophyll-*a* levels ($\mu g \cdot L^{-1}$), B) dissolved oxygen (%), C) water temperature (^{o}C), and D) salinity (ppt) in Cap Cove, Trinity Bay, Newfoundland for October, 2007 to May, 2008. Bars denote ± standard deviation.