Optimising the transport of *Crassostrea gigas* for aquaculture

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Abstract

Transporting oysters in air between growing sites is common practice in the New Zealand Pacific oyster (*Crassostrea gigas*) aquaculture industry. However, this process is detrimental to their post-transport survival, leading to significant stock losses. Additionally, the New Zealand oyster industry does not currently have techniques to quickly and accurately assess levels of physiological stress to allow for better decision-making (i.e. delaying transport or avoiding additional handling) in the production process. This work focused on two areas: (1) finding ways to improve post-transport survival, and (2) investigating straightforward techniques that could be used to assess stress levels of oysters in the field. Effects of handling and husbandry methods before, during and after transport on oyster post-transport survival and condition, as well as six different techniques for determining stress (biomarkers) were investigated. Two techniques (periodic air exposure and variations in stocking density) were investigated in an attempt to condition or "harden" oysters in the three weeks prior to transport. Oysters were subjected to either no air exposure (oysters continuously submerged), or air exposure periods of up to 69% of the week for the duration of either 1, 2 or 3 weeks. There were also two stocking densities used (6 litres and 13 litres of oysters per 37 litre mesh bag). Air exposure did not result in better post-transport survival. Lower stocking density resulted in a better condition index (ratio of meat to shell volume), suggesting that oysters stocked at a lower density were in a better condition to survive transport. Temperature and humidity in transit between growing sites were investigated to determine the optimal conditions for post-transport survival. Combinations of two temperatures (6°C and 12°C) and two relative humidity (RH) levels (low - 55% and high - 90% RH) were assessed. Conditions of 6°C and 90% RH imposed the least amount of stress on oysters and therefore were the most favourable for transport. On arrival at the destination farms, the return of oysters to the water is frequently delayed due to inaccessibility of the intertidal farm space at low tide. The effects of those delays on oysters were investigated. Delays of 2, 8, 22 and 46 hours were simulated and the highest survival rate was observed when oysters were returned to the water within 2 hours, followed by a two-fold decrease in survival after 8 and 22 hours. A delay of 46 hours decreased the survival eight-fold compared with the 2-hour delay. Growth and stress levels were also negatively affected by the delays. Throughout the study, the effects of various treatments on oyster stress levels were assessed with six different techniques: haemocyte counts, haemolymph pH, haemolymph refractive index and osmolality, weight loss in air and algae clearance rate. Haemolymph refractive index was the most reliable and accurate indicator of stress in this study, showing an increase in haemolymph density in response to stressors such as temperature, air exposure and grading. Its ease-of-use also makes it a promising candidate for measurements of shellfish stress in the field. A range of improvements to the current oyster transport methods are recommended, including avoiding high stocking densities prior to transport, keeping the transport temperature at 6°C and maintaining conditions of high humidity and expediting the return of oysters to the water upon arrival at the destination sites.

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1.0 General introduction

1.1 Background

Aquaculture in an important industry both as a valuable source of food, and economically as a means of employment for millions of people worldwide. Aquaculture is growing faster than any other major food production sector and has employed 19.3 million people in 2016 (FAO, 2018).

Molluscs account for 14% of worldwide marine production, with 89% of that coming from aquaculture. They are a sustainable and environmentally friendly species to produce, requiring no additional feeding and having beneficial effects on the environment through processes such as nutrient recycling. They also have nutritional benefits as a food source, being rich in vitamins and essential minerals (Wijsman *et al*., 2019).

However, there are still inefficiencies in aquaculture production of molluscs. Mortality after transport in air is one of the major problems for oyster producers (APEC, 1999; Wingerter *et al*., 2013). This project sought to address this issue and assessed how variations in handling procedures before, during and after transportation affected the physiology and survival of juvenile oysters.

1.2 Oyster aquaculture

Oysters have been used as a food source for thousands of years and have a very long history of being farmed. It is believed that the practice of cultivating oysters originated in ancient Rome and Greece, although there are reports of even earlier artificial oyster beds having been constructed in China (Laing, 2009).

Cupped oysters in the genus *Crassostrea* are important aquaculture species and accounted for the majority of worldwide mollusc aquaculture production in 2016 (FAO, 2018). Pacific oysters, *Crassostrea gigas* (Thunberg, 1793) are a focus of oyster aquaculture around the world and accounted for 3% of all mollusc production by weight in that year (FAO, 2018). Their nomenclature is also controversial, having been putatively changed to *Magellana gigas*. Both names are now in use throughout the scientific literature (Bayne *et al*., 2017). I refer to this species as Pacific oysters throughout this thesis.

In New Zealand, Pacific oyster culture is the third most important aquaculture industry after green-lipped mussels and Chinook salmon, making up 1.6% of aquaculture production by weight and 1.9% by value in 2017 (FAO, 2020). In 2017, 1834 tonnes were produced with a

value of US $15.7 million (FAO, 2020). Most New Zealand Pacific oysters produced are exported to Australia, Hong Kong, China, Japan and New Caledonia (Seafood New Zealand, 2017).

Bivalve aquaculture structures can also act as habitats for other marine species, and farmed shellfish help to remove excess nitrogen from the estuarine environments affected by excessive nutrient pollution (Shumway et al., 2003; Newell, 2004). Some oyster reefs may also sequester carbon, thus helping mitigate atmospheric build-up of CO_2 (Fodrie et al., 2017). However, oyster production can have negative environmental effects such as deposition of oyster waste that can change the nutrient characteristics of the seabed below oyster farms, accumulation of shell litter and alterations to the physical environment due to farm structures (Forrest et al., 2009).

1.3 General oyster biology

1.3.1 Biology

Oysters are bivalve molluscs, with two shells (valves) that enclose the soft inner body parts. The valves are joined together by a ligament hinge and can be tightly closed using a muscle (adductor muscle) attached to each valve. The internal organs are enclosed by a mantle which is involved in shell growth, sensation of the external environment and water current control (Galstoff, 1964; Bayne, 2017). Feeding is achieved by directing water flow containing suspended food particles over the ctendia (gills) where the particles are captured and transported in a flow of mucus to the labial palps and subsequently to the mouth (Bernard, 1974). Whereas food particles are transported to the mouth, unwanted particles such as inorganic material are rejected as pseudofaeces, along with faeces generated from the digestive process (Bougrier *et al*., 1997). Oysters respire using both the mantle and gills (Percy *et al*., 1971) and are also capable of limited respiration in air (Seaman, 1991). Food sources consist mainly of phytoplankton, bacteria, organic detritus (Brown, 1988; Barillé *et al.*, 1997) as well as other dissolved organic compounds (Fankboner & De Burgh, 1978; Manahan & Crisp, 1982; Jones & Iwama, 1991).

The primary response of bivalves to changes in the environment is the opening and closing of the valves, using their adductor muscles (Akberali & Trueman, 1985). Oysters can survive periodic exposure to unfavourable conditions (e.g. emersion) by closing their valves and altering their metabolic processes, without experiencing desiccation and unrecoverable hypoxia (Akberali & Trueman, 1985). During times of emersion a number of adaptive

strategies are employed. Metabolic depression lowers the rate of adenosine triphosphate (ATP) utilisation that can be sustained by production of ATP from the fermentation reactions alone, and a switch to anaerobic glycolysis processes occurs, with production of alternative end products such as alanine, succinate, propionate and malate (De Zwaan *et al*., 1991; Meng *et al*., 2018). The amount of enzymes present in tissues also changes (Le Moullac *et al*., 2007).

The oyster circulatory system is an open one in which haemolymph composed of plasma, haemocytes and a variety of proteins flows through a system of sinuses from the heart to the peripheral tissues, subsequently diffusing back into the circulatory system between the cells. Haemolymph plays a vital role in digestion, wound repair, nutrient transport, excretion and shell calcification (Schmitt, 2012).

1.3.2 Life cycle

Oysters are protandric hermaphrodites, usually maturing as males before changing into females later in life (Guo *et al*., 1998). They reach sexual maturity and spawn in their first year, with spawning in New Zealand occurring between the middle of spring to the end of summer. During the reproductive phase the gonad mass increases and comprises around 70% of the total dry meat weight in adult individuals (Miossec *et al*., 2009). Spawning in Pacific oysters is induced by the combination of elevated water temperature and the period of exposure to those higher temperatures (Dinamani, 1987). A temperature of at least 18°C is required for spawning, with the ideal range being 21 - 22°C (Héral *et al*., 1991). During spawning the eggs and sperm are released directly into the water where fertilisation occurs. The larvae thus formed swim and drift in the water, feeding on phytoplankton. After two - three weeks depending on environmental conditions, the larvae develop a foot, sink to the seabed and explore the sediment until they find a suitable place to permanently attach to. Next, they go through metamorphosis to become immature adults (spat). Subsequent growth depends on temperature and food availability, with market size of 70 to 100-gram live weight attained in 18 - 30 months (Laing, 2009).

1.4 Distribution of Pacific oysters

Pacific oysters are native to Pakistan, Japan, Korea, Sakhalin, North East China, Philippine islands, Borneo and Sumatra (Harry, 1985). They have been introduced to many regions around the world, both accidentally and with the aim of establishing an aquaculture industry (Shatkin *et al*., 1998; Beu *et al*., 2004).

Pacific oysters were unintentionally introduced to New Zealand in the 1960s, likely attached to the hulls or in the ballast water of ocean-going vessels (Beu *et al*., 2004). They were able to outcompete the native rock oyster *Crassostrea glomerata* for food and space and rapidly spread to most harbours and estuaries in Northland by the end of the 1970s. Additionally, due to higher fecundity and shorter time needed to achieve market size this species was widely adopted and almost totally replaced the native oyster on New Zealand farms by 1978 (Dinamani, 1987; Miossec *et al*., 2009).

1.4.1 Conditions for optimal growth

Optimum salinity range for Pacific Oysters is between 24 and 28 parts per thousand (ppt), although they can briefly survive salinity levels down to 5 and up to 50ppt (Shatkin *et al*., 1998; Miossec *et al*., 2009). They are also tolerant to a range of temperatures, achieving best growth at 15 - 19°C (Shatkin *et al*., 1998), but are able to survive temperatures as low as -2°C (Askew, 1972), and as high as 43°C for short periods of time (Shamseldin *et al*., 1997).

1.4.2 Production methods

There are many methods employed in commercially growing Pacific oysters. They range from wild spat collected and grown on sticks or ropes, to hatchery produced single-seed oysters. Wild spat collection involves deploying spat collectors such as ropes, wooden sticks, plastic sticks, cement fibreboard or bags of shell cultch during the spawning season. After several months the juvenile oysters are removed from the spat collectors and on-grown using methods such as on-bottom culture or mesh containers attached to, or suspended from, rigid structures in the intertidal zone. Alternatively, the spat can be left on the spat collectors for subsequent grow-out (Handley & Jeffs, 2003; Miossec *et al*., 2009).

Oysters are also produced in a hatchery environment. This allows for greater control over the spat supply and quality. When wild spat is unavailable, hatchery produced spat can be used to satisfy the industry demand (Robert & Gerard, 1999). Hatchery spat production involves the use of selected broodstock and cultured algae to produce oyster spat in several steps: broodstock spawning, larval culture and larval setting, with the process from fertilisation to setting taking approximately three weeks (Breese & Malouf, 1975). Hatchery production allows for selected broodstock to be used, which has several advantages over wild spat collection. Selective breeding can increase yields (Langdon *et al*., 2003), confer genetic resistance to pathogens such as Ostreid herpes virus type 1 (OsHV-1) (Castinel & Atalah, 2015), and produce triploid oysters for year-round harvest owing to their greatly reduced spawning behaviour (Guo *et al*., 1996; Nell, 2002).

1.5 Oyster production at Moana NZ

This project was carried out at Moana NZ facilities. In the mid-1980s, after a NZ High Court finding that the Crown failed to protect Māori commercial fishing interests, a settlement was agreed upon and Māori were returned fishing quota, shares in fishing companies and financial reimbursement. Out of this settlement, the iwi-owned fisheries company Aotearoa Fisheries Limited was formed. The company (trading as Moana New Zealand) is involved in production and processing of both wild-caught and aquaculture-produced fish, shellfish and crustaceans. This company will hereafter be referred to as Moana NZ.

Moana NZ is a significant contributor to New Zealand Pacific oyster industry with the oyster exports for 2018 being valued at NZ $15.1 million (Moana NZ, 2018). This project originated from the need to reduce transport related mortalities during production of oysters by Moana NZ. All oysters used in this project were triploids produced by Cawthron Institute in Nelson, and grown by Moana NZ from spat to marketable size. The company operates a nursery in Nelson and farms in the top of the South Island as well as Northland. Each year, production involves transporting juvenile Pacific oysters between farms on a weekly basis between the months of April and November using refrigerated trucks. The need for transfers originated from the emergence of Ostereid herpesvirus type 1 in New Zealand in 2010 (Castinel *et al*., 2015). The virus led to high oyster spat mortalities on the Northland farms (at the time the only farms operated by Moana NZ and its contractors). In order to combat the high mortalities, a farm operated by a contractor in Croisilles Harbour, Marlborough Sounds (area free from the virus at the time) was established. This farm receives spat from the nursery at approximately 8-10mm in length and on-grows them to 50mm over the space of one year. At this size, oysters are less susceptible to the virus (Castinel & Atalah, 2015) and are transported to oyster farms in Northland.

Two sites were involved in this study - Croisilles Harbour farm in Marlborough Sounds and Whangaroa Harbour farm in Northland (*Figure 1*). Croisilles Harbour farm is a sub-tidal one, where the oysters are held in floating baskets. The baskets are designed to keep the oysters either in or out of the water by having foam floats attached to one side of them. Oysters are regularly mechanically graded for size and given 24 hour periods of air exposure every two weeks for biofouling control (Fitridge *et al*., 2012). Animals are graded for uniform size approximately two weeks prior to transport after reaching sizes ranging from 30 to 70 mm in length and placed back in the baskets at double their usual density to facilitate harvest. On the day of the shipment the oysters are harvested, loaded into a refrigerated truck and taken to

final on-growing sites in Northland. The trip takes approximately two to three days, depending on the destination. On arrival, oysters are placed in mesh bags and attached to racks in the intertidal zone where are on-grown to marketable size.

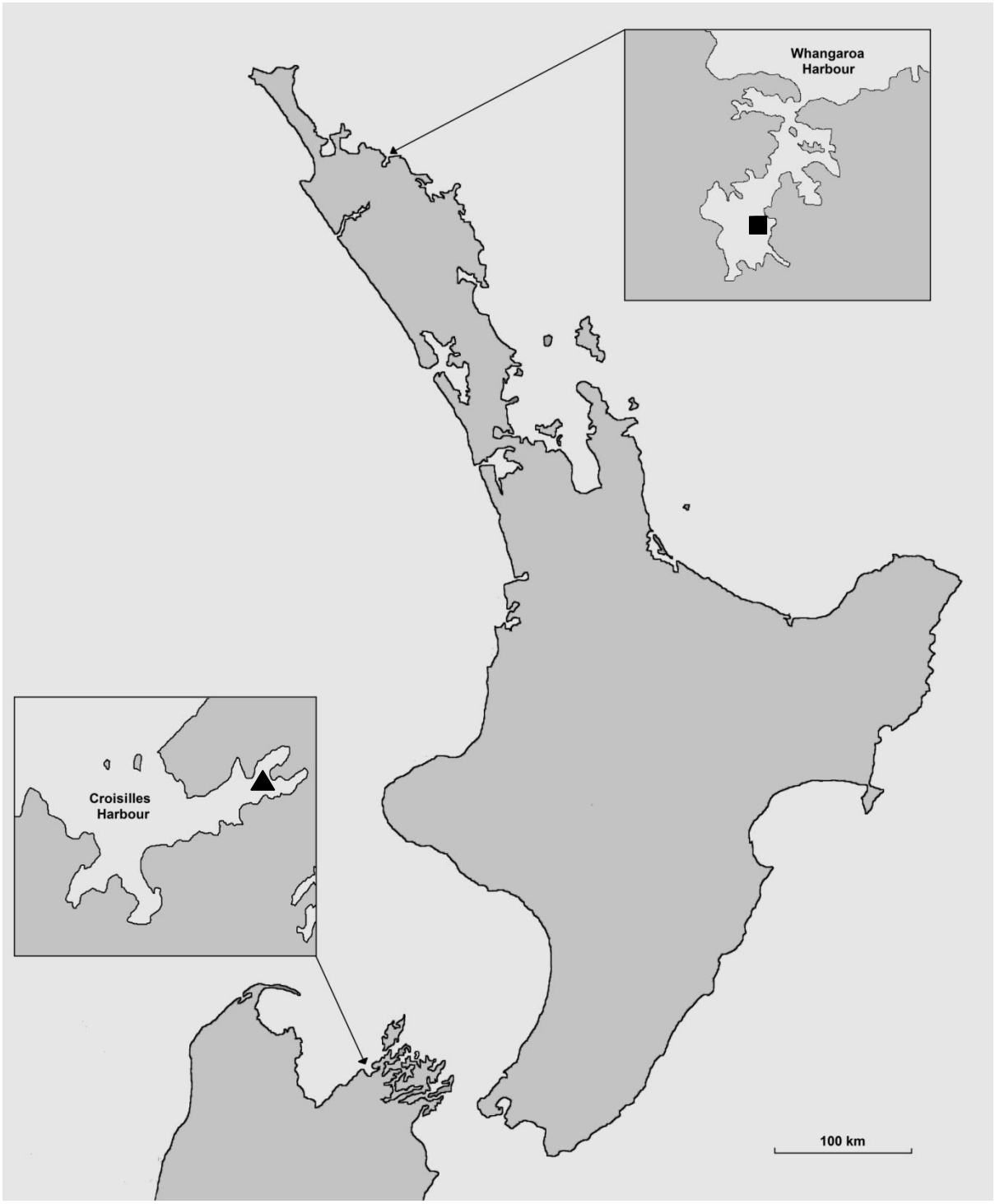

Figure 1: Locations of the two farms involved in this study - Croisilles Harbour (triangle) and Whangaroa Harbour (square).

1.5.1 The effects of transport on Pacific oysters

In 2016, a monitoring program was implemented by Moana NZ in order to assess the impact of transport on oysters. This program (which was separate from the work described in this thesis) assessed oyster mortality following each shipment of oysters. The data collected between March and September 2016 indicated that there were significant post-transport mortalities of 6.8% over all sites and formed the background for this research.

Survival was assessed two weeks after each shipment. Thirty-three shipments were tracked, with the destination site and time of year affecting survival. Oysters shipped on the same date to different sites experienced different levels of mortality. Additionally, factors including truck load size, number of days between grading and transport, oyster size at the time of transport, stocking density at the destination farms in Northland, variation in transport time and spawning batch were monitored to determine if they had an impact on post-transport mortality. None of those factors correlated with mortality, which narrowed down the potential causes of oyster mortality to be investigated.

It was hypothesized that varying processes employed by different farm crews such as delays in returning oysters to water after transport, excessive time the animals may be exposed to the sun during those delays (elevated temperature) or rough handling were responsible, as well as environmental variables such as water temperature, salinity, food abundance and OsHV-1 virus presence.

Based on the results of Moana NZ's monitoring program and a review of literature on oyster storage and transport, it was decided to investigate the effects of the following treatments on post-transfer mortality: increasing air exposure and reducing stocking density prior to transport in an effort to condition the oysters, modifying temperature and humidity conditions in the trucks as well as varying the time delay experienced by oysters between arrival and placement in water at the destination farm.

As well as reduction of transport-related mortality, a second area of interest for this project was the use of biomarkers in order to determine the sub-lethal stress levels of oysters after a particular handling event such as grading, harvest or transport, with an emphasis placed on finding biomarkers that are easily assessed in the field. A biomarker can be defined as an early warning signal that can be measured in the presence of one or more stressors that could include a physiological, biochemical or genotoxic response at the cellular, tissue or organism level (Chandurvelan *et al*., 2013). At present, there are no biomarkers used within the New

Zealand oyster industry that are able to quickly give an accurate assessment of the levels of physiological stress experienced by the animals and allow for better decision making around oyster handling on the farms, i.e. delaying transport or avoiding additional handling if the animals are already experiencing physiological stress.

Suitable biomarkers would allow for monitoring of the effectiveness of any given treatment undertaken as part of this project as well as predicting and possibly avoiding mortalities due to handling and husbandry techniques in commercial production. Development of easy-to-use field tests will allow farmers to quickly and accurately assess the health of their shellfish stock in the future.

1.6 Summary and aims of the project

Due to the large quantities of oysters being shipped every year, any changes leading to reduction in mortality would have a significant economic impact as well as contributing to better understanding of oyster husbandry. Additionally, biomarkers that allow for quick assessment of sub-lethal stress would enable production methods to be assessed and altered as needed to improve efficiency and productivity.

The aims of this research were:

- To determine which husbandry and handling techniques before, during and after the transport process lead to the best post-transport performance and survival
- To find suitable biomarkers that can be easily used in the field to determine the physiological stress and fitness level of oysters

This thesis is divided into six chapters:

1.0 General introduction - background information to the project.

2.0 Effects of air temperature and relative humidity during transport - variations in temperature and humidity during the transport process were investigated to test whether they affected post-transport mortality. Additionally, the suitability of five biomarkers of sub-lethal stress were assessed.

3.0 Effects of pre-transport conditioning - effects of pre-transport air exposure and stocking density on oyster growth, condition index, feeding performance and post-transport mortality were investigated.

4.0 Combined effects of pre-transport conditioning and post-transport recovery - a combination of pre-transport air exposure and delay between arrival at the destination farms and placement of oysters in water was used to assess the effect of those treatments on mortality, growth performance and stress levels.

5.0 Stress determination using haemolymph refractive index - the effect of mechanical grading on oyster stress levels was assessed, measured by a biomarker (haemolymph refractive index). Additionally, refractive index was used to determine recovery time from grading stress.

6.0 General discussion and conclusions - conclusions and recommendations to industry arising from the results of this project.

2.0 Effects of air temperature and relative humidity during transport

2.1 Introduction

2.1.1 Effects of temperature and relative humidity on oysters

Farming operations at Moana NZ involve multiple oyster transfers from the South to North Island. The transport process is thought to introduce stress and result in post-transfer mortality. Commercial transport process used by Moana NZ consists of shipping approximately 10 tonnes of oysters at a time in a refrigerated truck. The oysters are arranged in 45 litre perforated crates, with 30 to 35 crates stacked on a pallet. The temperature in the truck is set at 12°C and humidity is not controlled. The transport process takes approximately two to three days depending on location of the destination farm.

Temperature and humidity influence oyster survival and are mentioned in industry guides published for live transport of oysters as well as other shellfish and crustaceans (APEC, 1999; Barrento *et al*., 2013; Wingerter *et al*., 2013). While the Moana NZ transport temperature is currently kept low at 12°C, studies suggest that air storage temperatures between 0 and 7°C and conditions of high relative humidity (RH) are optimal for oyster survival (Wang & Amiro, 1977; Bird *et al*., 1995; Aaraas *et al*., 2004; Zhang *et al*., 2006; Song *et al*., 2007; Jiménez-Ruiz *et al*., 2015). Temperatures lower than 12°C may also reduce stress levels as determined by biomarkers (Zhang *et al*., 2006; Song *et al*., 2007; Jiménez-Ruiz *et al*., 2015).

A study conducted by Jiménez-Ruiz *et al*. (2015) investigated two simulated transport methods for live adult Pacific oysters, one involving refrigerated storage at 7°C, and the other at 24°C. Oysters were stored under the aforementioned conditions for 36 hours, with samples taken for adenylate energy charge (AEC), total carbohydrate content, glycogen and pH analyses (stress biomarkers). The oysters kept under refrigeration demonstrated a lower stress response. However, no follow-up observation was carried out to determine the longer-term response and survival.

Air storage at 7°C for 20 weeks resulted in survival of 80% of juvenile Pacific oysters (Seaman, 1991), indicating good potential for survival in air under appropriate storage/transport conditions. Pacific and Sydney rock oysters *Saccostrea glomerata* had greater survival at 5°C than at 23°C, with a two-fold increase in survival rate at the lower temperature (Bird *et al*., 1995). After 72-hour air exposure at 5°C, 15°C or 25°C, both juvenile and adult Pacific oysters held at 5°C recovered their lysosomal membrane stability

faster on re-immersion (lower stress response) than oysters at 15°C and 25°C (Song *et al*., 2007; Zhang *et al*., 2006). Storage or transport at low temperature also appears to be a useful method to improve survival in other studies on oysters (Boyd *et al.*, 1980; Hidu *et al*., 1988; Buzin *et al*., 2011). The majority of the studies and industry guides reviewed suggest that Pacific oysters stored at a temperature just above 0°C and in conditions of high humidity have the best chance of surviving for extended periods of time out of the water.

One potential risk with low transport temperatures could be temperature shock upon immersion. Zhang *et al* (2006) carried out a trial involving the exposure of Australian Pacific oysters to both rapid and gradual (2°C per day) water temperature changes of 10°C, between temperatures of 5°C, 15°C and 25°C (increase/decrease between 15°C and 5°C and increase/decrease between 15°C and 25°C). They reported that there was no difference between rapid and gradual temperature changes with regard to lysosomal membrane stability recovery times. This suggests that rapid temperature increase of 10°C (conditions experienced by Moana NZ oysters on immersion in seawater after transport) may not cause physiological stress, but more research is needed to establish whether this is consistent in New Zealand oysters.

Humidity during storage may also have an effect on oysters survival. High humidity conditions that reduce desiccation have been reported to be beneficial. Aaraas *et al*. (2004) reported that the soft tissue of air-stored flat oysters *Ostrea edulis* appeared dry after several days of storage at 1°C in polystyrene boxes with drainage and aeration holes, highlighting the desiccating effects of air exposure. High humidity at 2°C to 5°C was beneficial to long-term storage survival of eastern oysters *Crassostrea virginica* in a study carried out by Wang & Amiro (1977), where storage conditions resulting in less desiccation led to lower mortality. As well as the two studies by Aaraas *et al*. (2004) and Wang & Amiro (1977), most of the studies mentioned previously that discussed shellfish storage temperature also recommended conditions of high humidity or airtight enclosures (Hidu *et al*., 1988; Bird *et al*., 1995; Buzin *et al*., 2011).

2.1.2 Stress biomarkers

Accurate and expedient measurements of sub-lethal stress in shellfish are needed to detect stress and optimise oyster handing and transport conditions. Five biomarkers were assessed for accuracy of measuring stress: haemocyte count, haemolymph pH, haemolymph refractive index and osmolality as well as weight loss during air exposure.

Studies on shellfish and crustacean stress responses assessed the use of tissue or haemolymph pH as an indicator of increasing stress. In a study monitoring transport-related stress in Pacific oysters by Jiménez-Ruiz *et al*. (2015), tissue pH levels varied with transport temperature. It was suggested that this was due to differences in ATP degradation and lactic acid and amino compounds production in the different temperature treatments. Pacific oysters also displayed a difference in muscle pH values during air storage in response to different prior growing environments (Fratini *et al*., 2013). In a study observing the effects of air exposure on spiny lobsters *Panulirus interruptus*, Marquez-Rios *et al*. (2007) saw a decrease in muscle pH in animals exposed to air for one hour compared to those that remained in water. The difference was explained by a switch to anaerobic metabolism, where stored glycogen was catabolised into lactic acid. This mechanism was also observed to lower muscle pH in New Zealand abalone *Haliotis iris* after a period of air exposure (Baldwin *et al*., 1992).

As well as changes in muscle pH, haemolymph pH has also been reported to decrease in response to stress. Greenlip abalone *Haloitis laevigata* displayed a reduction in haemolymph pH in response to different transport conditions in air such as temperature and oxygen availability (Bubner *et al*., 2009), as have Pacific oysters in response to air exposure (Michaelidis *et al*., 2005).

Haemocyte count (the number of haemocytes per volume of haemolymph) has been used in studies involving stress monitoring in the Venus clam *Chamelea gallina* subjected to mechanical stress (Ballarin *et al*., 2003) and blue mussels *Mytilus edulis* subjected to copper pollution (Parry & Pipe, 2004; Pipe *et al*., 1999) where it was observed that the haemocyte count varied with stress exposure. Stress was also seen to have an effect on haemocyte counts in eastern oysters (Fisher *et al*., 2000) and in the soft-shell clam *Mya arenaria* (Gagne et al., 2008). However, the direction of change in haemocyte abundance in relation to increased stress has varied among studies, increasing in blue mussels while decreasing in the Venus clams for example (Pipe *et al*., 1999; Ballarin *et al*., 2003).

The constituents of shellfish haemolymph may also change due to stress. For example, Thompson *et al*. (1978) found an increase in haemocytes and plasma protein in California mussels *Mytilus californianus* after exposure to air at 21°C for 25 hours. Increased haemolymph protein levels have also been observed in blue mussels exposed to $CuCl_2$ stressor for 72 hours (Pickwell & Steinert, 1984), as well as in the Mediterranean mussels *Mytilus galloprovincialis* exposed to environmental heavy metal contamination (Auffret *et*

al., 2006). A study on the haemolymph protein and carbohydrate levels in response to longer-term (7 to 24 days) temperature and salinity stress in two separate populations of eastern oysters found that the response in terms of protein levels was inconsistent among the two groups, increasing in one population after exposure to stress while decreasing in the other (Fisher & Newell, 1986). However, no short-term response was investigated in that study.

Since protein levels have been reported to vary with stress, methods to measure those levels were investigated. Refractometry has been widely used in veterinary practice for measuring protein and total dissolved solids content in urine and blood plasma (George, 2001). It has been used in studies involving stress monitoring through tracking blood protein levels in crustaceans (Lorenzon *et al*., 2011), shrimp (Moore *et al*., 2000) and fish (Wells & Panhurst, 1999). Lorenzon *et al*. (2011) determined that a salinity refractometer was able to accurately detect changes in haemolymph protein content in seven different crustacean species by comparing protein levels obtained with a colorimetric method to those obtained using a refractometer, and Wells & Panhurst (1999) obtained the same result for plasma protein measurements in rainbow trout. As the protein levels in haemolymph increase, so does its density which can be determined by measuring the refractive index of a sample.

Osmolality levels can also be used as an indicator of dissolved solids such as proteins in a liquid biological sample (Craig *et al*., 1995), although determination of osmolality requires a more specialised device that is less suited for use in the field.

As well as haemolymph biomarkers, weight loss in oysters during air exposure in transport can be assessed to establish the levels of desiccation experienced during the different transport conditions, as air storage has been reported to lead to desiccation in soft tissues of oysters (Aaraas *et al*., 2004).

2.1.3 Stress challenge

To examine the effect of temperature and humidity during transport on survival, oysters were subjected to simulated transport followed by a stress challenge (air storage at a deliberately elevated temperature of 20°C, where mortality rate was monitored over a period of time). This was a variation of a stress on stress testing (Hellou & Law, 2003). Stress on stress assessment is done on the principle that stressed animals will be less tolerant to an additional stressor, in this case air exposure at an elevated temperature and will therefore die sooner than un-stressed animals.

The temperature and humidity treatments in this experiment that allowed the animals to survive for longer were deemed to have caused less stress. Mortality was gauged by observing gaping behaviour. When animals were observed to be gaping and failed to close their shell after being squeezed several times, they were considered to be dead. Closed oysters were considered to be alive. This method of monitoring mortality in air to assess the fitness of shellfish has been used in studies involving Pacific oysters (O'Meley, 1995; Meng *et al*., 2018), blue mussels (Altieri, 2006), green-lipped mussels *Perna canaliculus* (Zamora *et al*., 2019) and black mussels *Choromytilus meridionalis* (Webb, 1999).

A simulated transport trial was carried out at a temperature of 6°C and 12°C to determine if there are any potential benefits in changing the current, 12°C transport process. Additionally, there was a high (90% RH) and a low (55% RH) humidity treatment at each temperature.

2.2 Materials and methods

2.2.1 Experimental design

The oysters used were collected from Croisilles Harbour, Marlborough Sounds on 19th of July 2019. The average length was 54.3 ± 0.8mm and the weight was 14.0 ± 0.57g (mean ± SE, n = 30). Weights were measured with Mettler Toledo PB3002-5 electronic scales (Mettler Toledo, Columbus, USA) and shell lengths with Mitutoyo CD-6GM Vernier calipers (Mitutoyou, Kanagawa, Japan). These two instruments were used throughout the experiments outlined in this thesis whenever weight or length are reported. The transport conditions were simulated using refrigerated storage facilities at Cawthron Aquaculture Park in Nelson. During the simulated transport, oysters were held in either 6°C or 12°C environments, at either 90% relative humidity (RH) or 55% RH. The experimental design and stress biomarker assessments are shown in *Figure 2*.

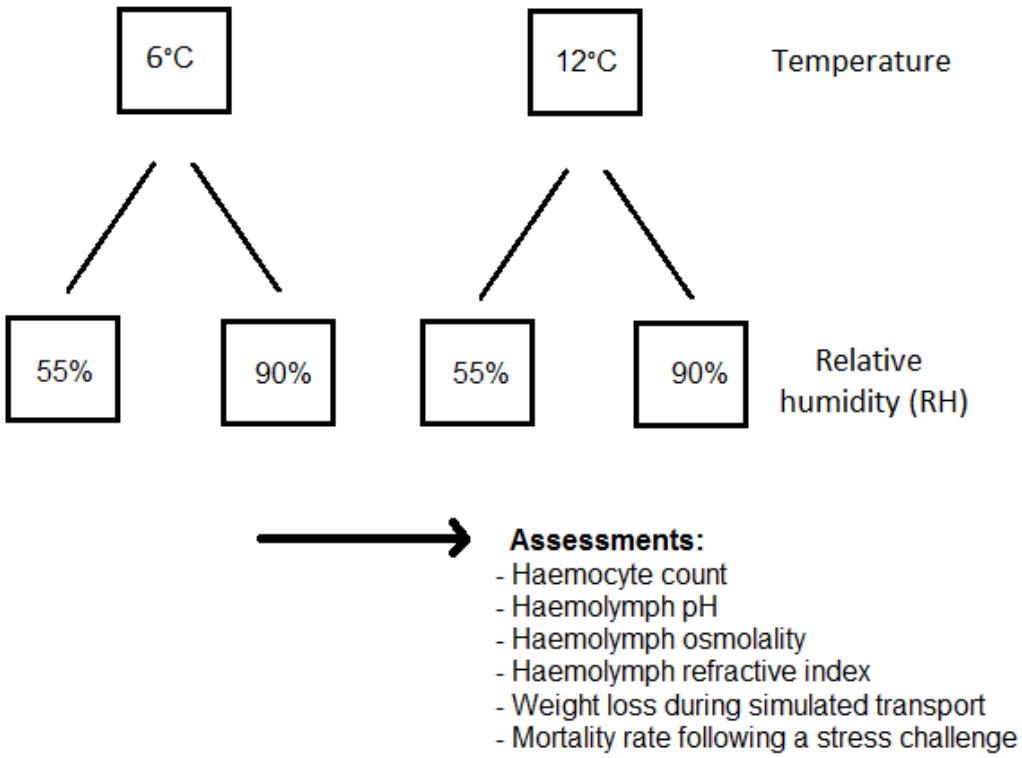

Figure 2: Combination of transport temperature and humidity treatments and the assessments to test the response of oysters to those treatments.

The temperatures were maintained by a domestic fridge and a refrigerated container/chiller. Humidity is not controlled during the current commercial transport process, with oysters being loaded onto a truck in open top crates and subjected to humidity conditions dictated by the weather and truck refrigeration systems. It was therefore decided to use two different humidity levels (55% and 90% RH) to observe their effect on the oysters during transport. The humidity levels used here were not determined prior to the experiment. The aim was to get the maximum possible difference in humidity levels with the set-up and equipment available.

The simulated transport period was 90 hours long and represented the maximum likely time period for oysters to be transferred from Marlborough Sounds to the most distant sites in the North Island.

There were 72 oysters used for each of the four temperature/humidity treatments, randomly assigned to 6 containers per treatment, with 12 oysters per container. Oysters were placed on polystyrene trays (*Figure 3*) to allow them to be kept in the same orientation - left valve down. Oysters were arranged in this way to stop any liquid trapped in the shell spilling and potentially leading to faster flesh desiccation in oysters lying on their side. Humid and dry conditions were created by placing either paper towels moistened with 100ml of water, or desiccant powder (280g of calcium chloride) in between the polystyrene trays in each plastic container (*Figure 3*). High humidity treatments were measured at 90% RH on average over the 90-hour period, and low humidity at 55% RH with Elitech RC-4HC (Elitech Technology, Milpitas, USA) temperature and humidity data loggers.

Figure 3: Plastic containers housing the oysters, with the low humidity treatment tray on the left (containing calcium chloride desiccant), and high humidity treatment on the right (containing wet paper towels).

During simulated transport, the oysters in each of the four treatments were placed inside separate plastic bags (*Figure 4*) to create a consistent set of humidity conditions for each treatment. There were two bags (humid at 90% RH and dry at 55% RH) placed in the fridge at 6°C, and two bags placed on a shelf in the refrigerated container at 12°C.

Figure 4: Arrangement of oyster containers placed in plastic bags for humidity control.

Prior to being placed inside the bags, 20 oysters were randomly selected from each treatment (each of the four bags) and weighed. This was done to observe any potential weight loss due to dessication during the experiment. The same oysters were weighed again immediately upon completion of the simulated transport period.

To monitor temperature and humidity during the treatments, two Elitech RC-4HC temperature/humidity data loggers were used with one being placed in the bag in the fridge (6°C) and the other in the refrigerated container (12°C), both set to record temperature and humidity at 10 minute intervals. Since only two loggers were available and there were four different conditions to monitor, loggers were placed in the low humidity treatments at both 6°C and 12°C. To get the humidity data for high humidity treatments, the 90-hour experiment was repeated with the two high humidity treatments identical to the ones done earlier but without oysters, just the containers with wet paper towels.

2.2.2 Stress challenge

To observe the effects of each transport method on survival, experimental oysters were placed in a challenging temperature environment of 20.0 ± 0.01°C at 74.4 ± 0.1% RH (mean ± SE) for the next two weeks immediately upon completion of the 90-hour transport experiment and monitored for mortality on a daily basis. Mortality was determined by observing oyster gaping behaviour - closed oysters were considered to be alive, gaping

oysters that did not close their shell in response to the shell halves being squeezed together several times were considered to be dead. To obtain a consistent temperature of 20°C for this part of the trial, the same refrigerated container/chiller was used, but the inside temperature was now maintained at 20°C instead of 12°C using an oil fin heater. Humidity was not controlled or maintained at a pre-determined level but was observed and recorded with an Elitech RC-4HC data logger. Plastic containers from each of the treatments were removed from bags they were stored in for simulated transport and randomly arranged on the shelves of the refrigerated container (*Figure 5*). The oysters were checked for mortalities daily and any dead ones were recorded and discarded.

Mortality was presented as cumulative mortality rate during the two weeks of exposure to 20°C, calculated by taking a percentage of the day's mortalities (number of mortalities in the treatment / total number of oysters in that treatment) and adding it to the previous day's total.

Figure 5: The arrangement of oysters in the refrigerated container during the 20°C stress challenge.

2.2.3 Biomarker assessments

Five biomarkers of sub-lethal stress that included haemolymph cell (haemocyte) count, haemolymph pH, osmolality, refractive index and weight loss were assessed.

On the day of arrival at the Cawthron Aquaculture Park, approximately four hours after being removed from water on the farm (due to the remote location of the Croisilles Harbour farm), 10 oysters were used to obtain baseline or un-stressed readings for haemocyte count, haemolymph pH, osmolality and refractive index. Hereafter, the term "un-stressed" is used throughout this thesis to refer to animals that have not yet been deliberately exposed to a stressor (i.e. they are representative of the population before the experimental stressor is applied). A knife was used to gently pry open the oyster shells, creating a gap several millimetres wide where a plastic wedge was inserted. This allowed for a haemolymph sample to be taken from the adductor muscle with a 25ga needle and a 1ml syringe. Approximately 0.2ml to 0.3ml of haemolymph was collected from each oyster. Each sample of haemolymph was divided among the four biomarkers due to very small sample volumes required for testing (10µl for osmolality, 50 µl for refractive index and 40 µL for haemocyte count). Testing for osmolality, refractive index and pH was done immediately, while the samples for haemocyte counts were preserved in equal parts of filtered seawater and 10% formalin solution for later analysis.

After the simulated transport treatments, 10 randomly selected oysters per treatment were removed from the trays and the same tests were carried out. In total, 50 oysters were tested, 10 before and 40 after the simulated transport. Testing for osmolality was done using a Wescor Vapro 5600 vapour pressure osmometer (Wescor Inc, Utah, USA), refractive index was measured with an Atago S-10 handheld salinity refractometer (Atago Co, Tokyo, Japan), pH was determined using a Mettler Toledo SevenCompact meter (Mettler Toledo, Ohio, USA) and haemocyte count was obtained using a haemocytometer.

The osmometer and the pH meter were calibrated with standard solutions prior to use, and the refractometer was calibrated with distilled water. The salinity refractometer used during this experiment had a salinity scale labelled in grams of salt per 100g of solution, unlike some instruments that also provide a density scale alongside the salinity one. Therefore the results obtained were recorded using that scale.

2.3 Statistical analyses

Mortality data were assessed using analysis of covariance with differences among treatments being assessed with Tukey's post hoc tests. Two-way analyses of variance (ANOVAs) were used to assess the effects of temperature (6°C and 12°C) and humidity (high = 90% RH and

low = 55% RH) on haemocyte counts, haemolymph pH, osmolality, refractive index and oyster weight loss.

Significant effects were examined using post hoc Tukey's tests to determine differences among groups within a factor. Oysters used to obtain baseline readings were not included in the statistical analysis because they were not held for the duration of the experiment, being sampled 90 hours earlier than the experimental animals. Furthermore, their inclusion un-balanced the analysis. Instead, the baseline oyster data was presented graphically to give an indication of how the treatments might have modified biomarker levels relative to a baseline (*Figure 7*). Data were assessed for normality and heterogeneity with Shapiro Wilk and Cochran's C tests, respectively. Data not meeting assumptions of normality and homogeneity was log(x+1) or arcsine transformed. Analyses were carried out in STASTISTICA 12 (Statsoft Ltd.).

2.4 Results

2.4.1 Temperature and humidity treatments

Temperatures and humidity in this experiment are reported as target values of 6°C, 12°C, 55% RH and 90% RH. However, the actual mean values recorded with data loggers varied among treatments (*Table 1*).

Table 1: Temperature and humidity readings (mean ± SE) over the space of the 90-hour simulated transport treatments.

Treatment	Temperature (°C)	Humidity (% RH)
12°C, high humidity	12.0 ± 0.009	93.9 ± 0.1
12°, low humidity	12.2 ± 0.008	57.7 ± 0.2
6°C, high humidity	5.7 ± 0.01	90.3 ± 0.1
6°C, low humidity	6.1 ± 0.02	58.9 ± 0.2

2.4.2 Mortality following the stress challenge

Mortality increased over time and varied among treatments ($F_{3, 51}$ = 8.37, p = 0.0001, *Figure 6*). The mortality rate increased more slowly in the two 6°C treatments compared to the 12°C,

55% RH treatment ($p < 0.01$). There were no significant differences between 12°C, 90% RH treatment and the other treatments.

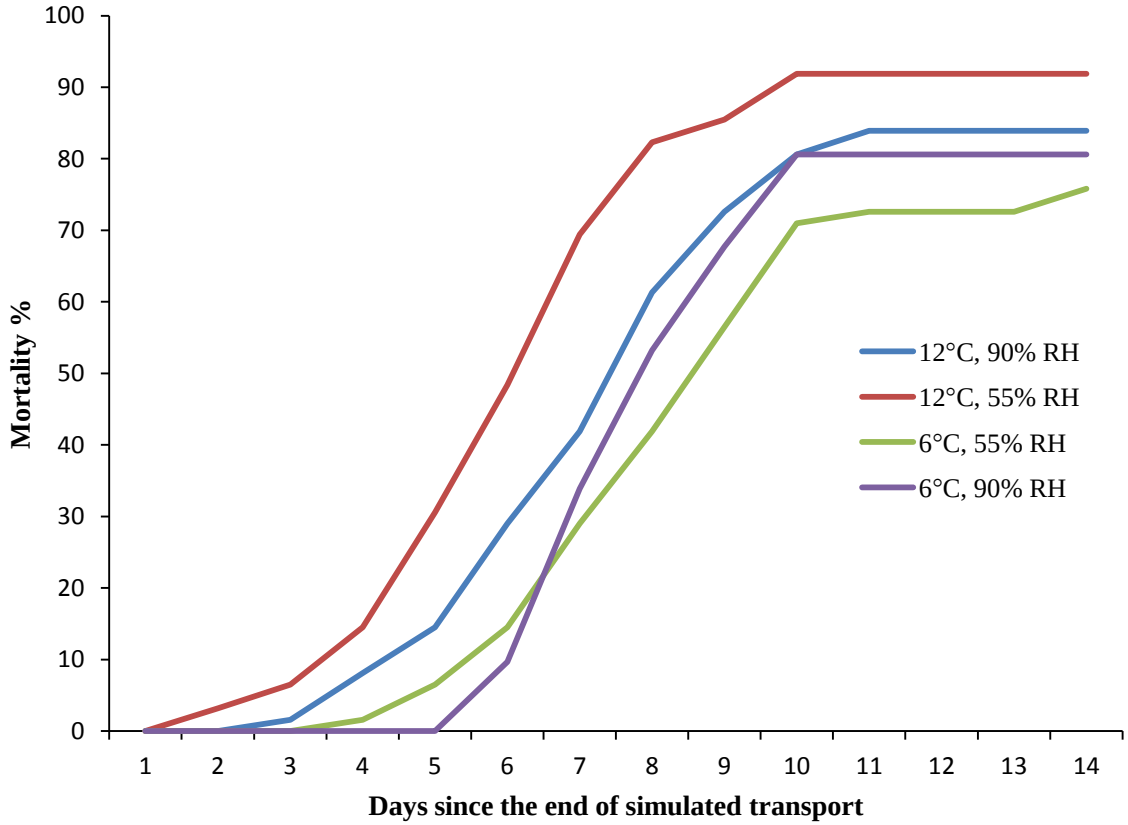

Figure 6: Cumulative daily mortality percentages in oysters from the four transport treatments.

2.4.3. Biomarkers

2.4.3.1 Cell count

At the end of the 90-hour experiment, the number of haemocytes per µl of haemolymph was greater in the 6°C (148.81 ± 17.98, mean ± SE, n = 20, *Figure 7A*) compared to the 12°C (85.74 ± 7.79, n = 20) treatment ($F_{1, 36} = 18.43$, $p = 0.0001$, *Table 2*). The number of haemocytes was also greater in the low (55% RH) compared to the high (90% RH) humidity treatments with haemocyte counts of 145.14 ± 17.93 (n = 20) and 89.42 ± 8.66 (n = 20) per µl respectively ($F_{1, 36} = 14.27$, $p = 0.0006$). There were no interactive effects of temperature and humidity (*Table 2*). Comparisons between the baseline oysters and the experimental treatments show that mean haemocyte counts were increased in all but the high humidity, 12°C treatment combination (green line in *Figure 7A* represents the baseline values).

2.4.3.2 pH

Haemolymph pH values at the end of the experiment did not significantly differ across treatments with temperature ($F_{1, 36}$ = 0.23, p = 0.633) or humidity ($F_{1,36}$ = 0.27, p = 0.606), with the value of 6.84 ± 0.02 (n = 40, *Figure 7B*) across the treatments. The pH values decreased after the 90-hour experiment compared to the baseline values (7.0 ± 0.05, n = 10, *Figure 7B*).

2.4.3.3 Osmolality

There were differences in haemolymph osmolality at the end of the experiment in response to both temperature ($F_{1, 36}$ = 15.63, p = 0.0003) and humidity ($F_{1, 36}$= 105.89, p < 0.0001). There was also an interactive effect between temperature and humidity ($F_{1, 36}$ = 8.84, p = 0.052). Osmolality was lowest in the two high humidity (90% RH) treatments (1006.05 ± 4.61mmol/kg, n = 20, *Figure 7C*) compared to 12°C, 55% RH (1154.70 ± 17.1mmol/kg, n = 10) and 6°C, 55% RH (1077.10 ± 10.4mmol/kg, n = 10) treatments. Results show that osmolality increased with an increase in temperature and decrease in humidity, and was higher in every treatment compared to baseline values.

2.4.3.4 Refractive index

At the end of the experiment, the haemolymph density (determined by the refractive index) was lower in the 6°C (3.93 ± 0.05g of salt per 100ml, n = 20, *Figure 7D*) compared to the 12°C (4.06 ± 0.06g of salt per 100ml, n = 20) treatment ($F_{1, 36}$ = 5.83, p = 0.021). Haemolymph density was also lower in the 90% RH (3.82 ± 0.03g of salt per 100ml, n = 20) compared to the 55% RH (4.17 ± 0.05g of salt per 100ml, n = 20) treatment ($F_{1, 36}$ = 41.48, p < 0.0001). There were no interactive effects of temperature and humidity (*Table 2*). Results show that the haemolymph density increased with higher temperature and lower humidity over the duration of the 90-hour simulated transport and was higher in every treatment as compared to the baseline values.

2.4.3.5 Weight loss

Weight loss during the experiment was higher in the 55% RH (10.79 ± 0.78%, n = 40, *Figure 7E*) than in the 90% RH (1.48 ± 0.44%, n = 40) treatments ($F_{1, 36}$ = 176.12, p < 0.0001).
There was no significant effect of temperature on weight loss ($F_{1, 36}$ = 2.53, p = 0.116).

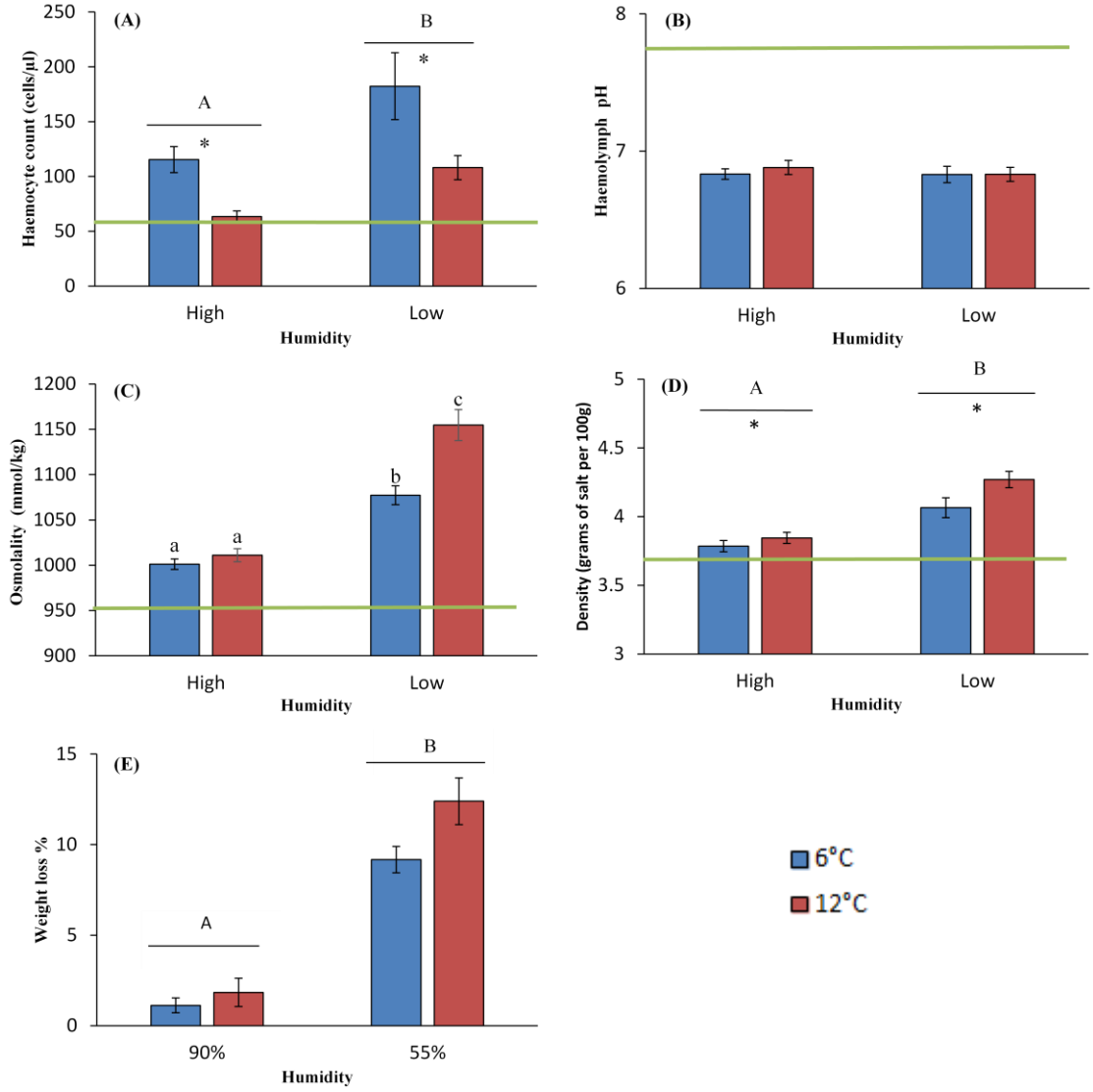

Figure 7: Biomarker results (n = 10) at the conclusion of a 90-hour simulated transport experiment. (A) = cell count; (B) = haemolymph pH; (C) = haemolymph osmolality; (D) = haemolymph refractive index (density); (E) = weight loss during simulated transport (mean ± SE). Capital letters indicate significant effects of humidity. Asterisks indicate significant differences between temperature treatments. Lowercase letters indicate differences between groups following a significant interactive effect of temperature and humidity. The green line indicates mean baseline values for oysters at the beginning of the trial (n = 10, see section 2.3 for more information).

Table 2: Results of the two-factor ANOVA. Text in bold represents significant differences (alpha = 0.05 or 0.01).

	d.f.	MS	F	p	d.f.	MS	F	p
		Cell count†				pH		
Temperature (T)	1	**0.495**	**18.43**	**0.0001**	1	0.006	0.23	0.6330
Humidity (H)	1	**0.383**	**14.27**	**0.0006**	1	0.007	0.27	0.6056
T × H	1	0.005	0.18	0.6733	1	0.006	0.21	0.6469
Residual	36	0.027			36	0.026		
		Refractive index†				Weight loss*††		
Temperature (T)	1	**0.001**	**5.83**	**0.0210**	1	0.017	2.53	0.1156
Humidity (H)	1	**0.009**	**41.48**	**0.0000**	1	**1.169**	**176.12**	**0.0000**
T × H	1	0.000	1.60	0.2139	1	0.008	1.15	0.2877
Residual	36	0.000			36	0.007		
		Osmolality*†						
Temperature (T)	1	**0.003**	**15.63**	**0.0003**				
Humidity (H)	1	**0.020**	**105.89**	**0.0000**				
T × H	1	**0.002**	**8.84**	**0.0052**				
Residual	36	0.000						

*Alpha 0.01 †Log(x + 1) transformed data ††arcsine(square root transformed)

2.5 Discussion

Overall, lower temperature (6°C) and higher relative humidity (90%) during simulated transport improved survival of Pacific oysters following the stress challenge and decreased sub-lethal stress as indicated by the biomarkers.

All 288 oysters used in the experiment survived the 90-hour simulated transport. Mortality rate was lower in the 6°C treatments compared to the 12°C, 55% RH treatment during the stress challenge. These results supports other studies on shellfish air transport and storage in that lower temperature and higher humidity is beneficial during air exposure (Bird *et al*., 1995; Zhang *et al*., 2006; Song *et al*., 2007; Jiménez-Ruiz *et al*., 2015). At the end of the challenge, 71.1% of oysters were determined to have died by observing shell gaping. At the time it was decided that the remaining 28.9% oysters were still alive. This may not have been the case however, and the pitfalls of trying to determine mortality based on gaping will be discussed in the next chapter (section 3.4.5).

The haemocyte counts were elevated in the 6°C treatments, and also in the treatments with higher humidity. This, in combination with the baseline readings suggested the transport conditions of 12°C and high humidity were least stressful to the oysters. While the haemocyte count results are in agreement with other biomarkers on the conclusion that higher humidity

is beneficial, they differ with regard to temperature. Haemocyte count data is difficult to interpret as the direction of change in haemocyte abundance in relation to increased stress varies among studies, increasing in blue mussels while decreasing in the Venus clams for example (Pipe *et al*., 1999; Ballarin *et al*., 2003). As a biomarker, haemocyte count proved to be time consuming to analyse with a lot of variation within treatments, which may limit its utility as an field stress test.

The results for haemolymph pH indicated no differences among experimental treatments, although post-transport readings were lower than baseline value. This is in agreement with other studies where haemolymph pH of shellfish decreased when exposed to stressful environments (Michaelidis *et al*., 2005; Bubner *et al*., 2009). This method may require further examination with larger sample size and larger animals as there were difficulties in consistently measuring the pH of a 0.2ml sample, since only a limited amount of haemolymph could be drawn from the juvenile oysters. As a biomarker, difficulties in measurement and the fragility of sensitive pH probes may make this method unsuitable as a field stress test.

Osmolality and refractive index (density) tests, both of which measured the quantity of dissolved solids in haemolymph, showed that higher temperature and lower humidity led to higher readings. This increase was expected due to dehydration under air storage conditions although there may have been several mechanisms causing these elevated readings: simple dehydration increasing the concentration of total dissolved solids, a change in blood chemistry with an increase in dissolved compounds such as proteins due to stress, or a combination of both. It was not possible to determine which mechanism led to the increased readings in this experiment. Despite that, haemolymph protein levels are known to increase in shellfish in response to stress (Thompson *et al*., 1978; Pickwell & Steinert, 1984; Auffret *et al*., 2006) and can be detected with refractometry (Lorenzon *et al*., 2011) and osmometry (Craig *et al*., 1995). It was therefore possible that the elevated osmolarity and refractive index indicated physiological stress rather than just dehydration. Due to the ease of use, refractometry may warrant further investigation as a biomarker and a field stress measurement tool. While osmolality readings also showed a similar response, the specialised and fragile equipment required to measure osmolality would make this method less suitable for use on the farms or in the field.

Oysters subjected to dry (55% RH) transport conditions were noticeably drier on the outside of the shell compared to oysters kept in humid (90% RH) conditions that were damp to the touch. Also, oysters in the dry conditions lost more weight (10.8% over 90 hours) compared to those held in more humid conditions (1.5% over 90 hours). Humid storage was, therefore, more beneficial to oysters, as desiccation and the resulting shell liquor loss can result in increased concentrations of toxic waste products derived from oyster metabolism (Wang & Amiro, 1977). As a biomarker, this method could be used to assess specific air storage conditions for their impact on oysters. It could also be used to assess the total gape time of oysters under air storage or transport (as a proxy for adductor muscle endurance time), since longer periods of gaping would lead to accelerated desiccation.

A consideration when interpreting the results was the fact that all oysters used in this experiment already spent a considerable amount time out of the water (4-6 hours) prior to any treatments or sampling taking place. This was due to the driving distance involved between the farm in Croisilles Harbour where they were collected and Cawthron Aquaculture Park where the assessments were carried out. This meant that some stress may have already been imposed on the animals, and may have influenced baseline readings taken before the 90-hour transport simulation began. If this experiment was to be repeated, taking baseline readings as soon as the animals are removed from the water on the farm would minimise any potential error.

In summary, based on the stress challenge and biomarker results the transport process at a temperature of 6°C and under conditions of consistently high humidity appeared to be more beneficial than the current 12°C commercial transport process where humidity is not controlled. Going forward, out of the stress assessment methods (biomarkers) considered only the haemolymph refractive index and weight loss tests will be used again due to the ease of use and sufficient sensitivity to pick up differences under realistic handling conditions encountered in commercial production.

3.0 Effects of pre-transport conditioning

3.1 Introduction

3.1.1 Effects of air exposure and stocking density on Pacific oysters

Emersion increases stress in oysters (Zhang *et al*., 2006) and it is likely that transporting oysters in air for farming operations induces stress and post-transport mortality. Wild intertidal shellfish are periodically exposed to air which contributes to their ability to depress their metabolism and close their shells in air to prevent desiccation (Akberali & Trueman, 1985). It helps with "hardening" of the oysters - allowing them to better deal with periods of emersion through thickening of the shell edges and thus resisting desiccation (Quayle, 1988). This technique of allowing some air exposure has been widely used in Japanese and Korean shellfish aquaculture (Takeo & Sakai, 1961; Quayle, 1988; Mondol *et al*., 2016). It was first introduced in Japan in the 1920's to deal with mass mortalities in the sub-tidal culture of Pacific oysters (Handley, 1997). However, no known studies have looked at the effects of short-term (weeks rather than months) periodic air exposure treatments on hardening of oysters for subsequent storage or transport operations. Studies that discuss or involve hardening tend to do so on a much longer time scale of 2 to 10 months (Fujiya, 1970; Graham, 1991; Handley, 1997; Samain & McCombie, 2008; Kang *et al*., 2010; Samain, 2011; Mondol *et al*., 2016). While there are positive effects, there can also be an economic cost involved in hardening of oysters as increased periods of emersion can lead to slower growth (Spencer, 1990; O'Meley, 1995). Increased air exposure over a period of weeks rather than months, however, is unlikely to result in significant financial loss through reduction in growth.

Aerial exposure can affect the growth and storage survival of Pacific oysters (O'Meley, 1995). Graham (1991) also reported that Pacific oysters cultured intertidally at 25-66% air exposure per day for 5 months survived longer when subsequently stored in air at 15°C than sub-tidal oysters (an average of 20.7 days versus 15.8 days), although again the focus was on long-term husbandry practices. Also supporting the idea of a hardening treatment, Asia-Pacific Economic Cooperation (APEC) Air Shipment of Live and Fresh Fish & Seafood Guideline recommends hardening oysters in the intertidal zone for up to 3 weeks prior to transport to reduce gaping and mortality (APEC, 1999), although no specific oyster species or source of the information were mentioned. Greater air exposure has been observed to decrease mortalities from OsHV-1, with Pacific oysters grown at higher intertidal sites

reported to experience lower mortalities than those grown at lower sites (Paul-Pont *et al*., 2013). An experiment by Meng *et al*. (2018) compared the performance of one year old intertidal and sub-tidal juvenile Pacific oysters (60-80mm in length). The parameters used to assess performance were survival in air as well as ATP production, anaerobic glycolysis gene expression and production of organic acids in response to oxygen exposure among others. Survival in air was assessed by leaving a sample of sub-tidal and a sample of intertidal oysters in air at 20°C for nine days while observing the mortality rate based on shell gaping. It was found that intertidal oysters survived longer and maintained ATP production better than sub-tidal ones. Similar results have been achieved with blue mussels, with animals conditioned in the intertidal zone for seven weeks surviving an extended period of emersion and submerged hypoxia better than sub-tidal ones (Altieri, 2006). In this experiment, the period of time that the oysters are exposed to air prior to transportation from the study site (Croisilles Harbour farm) is currently short (one 24-hour period every two weeks), done to reduce biofouling rather than to have an effect on oyster survival out of the water.

Another potential factor that may affect oyster survival during transport is their prior condition and energy reserves. The density of oysters in the mesh bags they are grown in may influence the amount of food available for individual oysters. Currently, two weeks before transport the oyster density in the mesh bags on the Croisilles Harbour farm is doubled to facilitate faster harvest and loading onto the trucks. Drinkwater and Howell (1985) observed slower growth and higher mortality as the stocking density of juvenile Pacific oysters increased and recommended a level at which the number of animals in an enclosure was low enough for them to only just be touching when spread out evenly. In contrast, the high stocking density on Croisilles Harbour farm prior to transport results in several layers of animals resting on top of each other within the mesh bags, while a lower density results in the oysters forming a single layer. Higher density may also have a detrimental effect on the growth and condition index of Pacific oysters (Marshall & Dunham, 2013; Chavez-Villalba *et al*., 2010). A similar result was obtained by Holliday *et al*. (1991) in regard to cultivation of Sydney rock oysters. It is worth noting that although a reduced stocking density may be beneficial for growth and survival, it can also lead to increased labour and equipment costs for the farmer (Holliday, 1995). Since stocking density is reported to be important, this experiment assessed the effects of pre-transport stocking density as well as air exposure on oyster performance.

Measurements of oyster condition (volume condition index and weight) were used to assess oyster health and energy reserves after application of air exposure and density treatments. Condition index is a widely used indicator, with a low value indicating poor growing conditions and a low meat weight in relation to shell volume (Lucas & Beninger, 1985). Volume condition index has been proposed as a measure of the effect of nutritive stress and argued to be a better indicator as compared to shell condition index (dry meat weight / dry shell weight), since it takes into account variations in shell cavity volume due to shell shape or variability in shell thickness (Crosby & Gale, 1990).

3.1.2 Biomarkers to determine stress

It is evident that pre-transport air exposure and stocking density may both have significant effects on oyster stress during the transport process. In this experiment stress was measured using algal clearance rate as a stress biomarker, a method previously used to measure stress in mussels exposed to cadmium (Chandurvelan *et al*., 2012), transport stress (Chandurvelan *et al*., 2013) or increases in acoustic levels from pile driving (Spiga *et al*., 2016). In these studies, clearance rate was measured as part of a "scope-for-growth" suite of tests or on its own, and in every case there was a significant effect on the clearance rate. Algal clearance rates are also easy to measure, and this technique may be promising as a biomarker that can be used commercially.

3.2 Materials and methods

3.2.1 Pre-transport treatments

Currently, the oysters designated for transport from Croisilles Harbour farm to the North Island are graded for size, then kept at twice their usual density for approximately two weeks before transport (to facilitate harvest and minimise handling on the day of shipping). As the Croisilles Harbour farm is a sub-tidal one, oysters are grown in mesh bags while continuously submerged. The bags are 37 litres in volume and have foam floats attached to one side. Air exposure is limited to what is required to control biofouling, with 24 hours of continuous air exposure every 14 days used on the farm. This is done by manually taking oyster bags out of the water.

This experiment was carried out to test the effects of air exposure and stocking density prior to transport on condition and survival of juvenile Pacific oysters who's mean length was 64.6 ± 1.6mm and the weight was 17.4 ± 1.1g (mean ± SE, n=30). The air exposure treatments in

this experiment consisted of seven air exposure regimes (no air exposure, 33% exposure per week for 1, 2 and 3 weeks, as well as 69% exposure per week for 1, 2 and 3 weeks). These particular treatments were chosen as they were higher than the approximately 7% air exposure the animals already get as part of the biofouling control, and were not so excessive as to risk high mortalities due to the treatment There were also two stocking densities (6 and 13 litres in 37 litre mesh bags) for each of the air exposure treatments for a total of 14 treatments. Two separate bags were assessed for each treatment to determine whether there was any spatial variation (*Figure 8*). This number of bags was used because of limitations on the time the farm crew was able to set aside to attend to the flipping/air exposure regime, as well as limitations on set-up and sampling time. Twenty-eight bags in total were used during the experiment (attached next to each other on a rope).

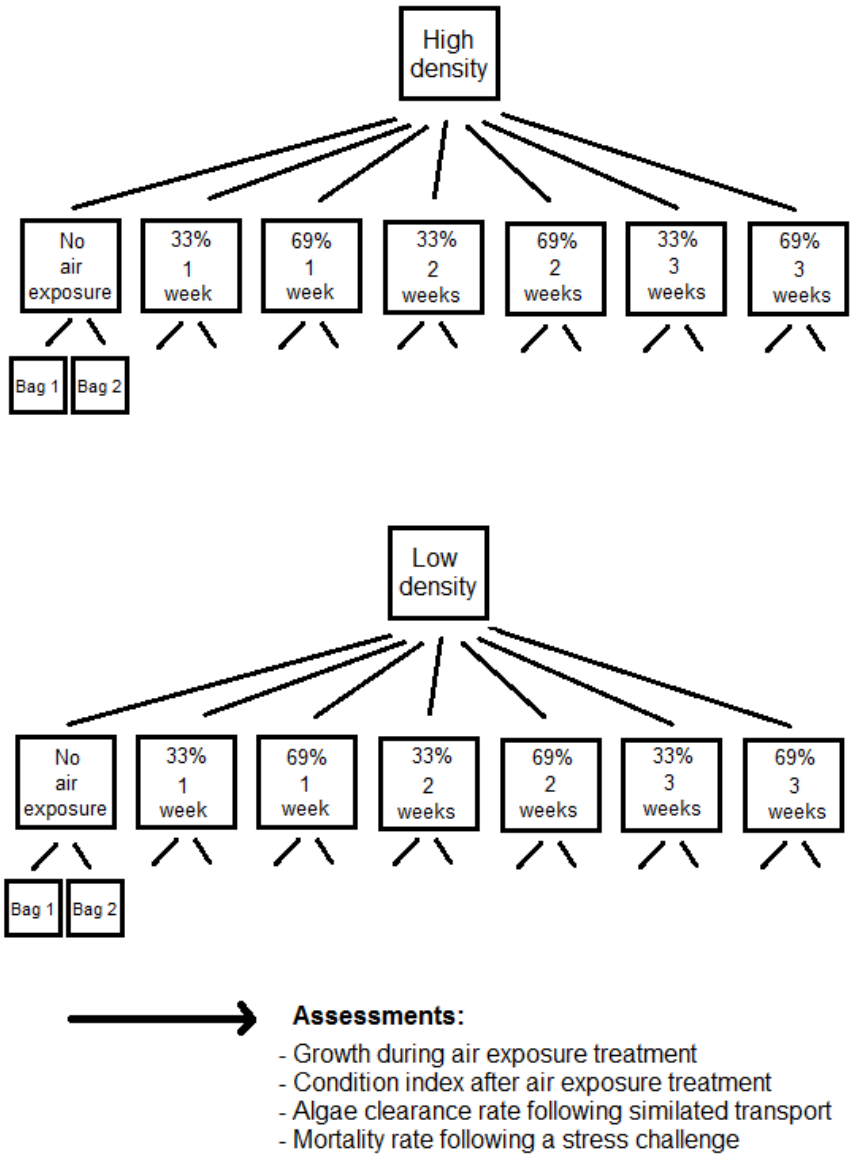

Figure 8: Experimental design for the trial that investigated the effects of pre-transport air exposure and stocking density on Pacific oysters. All treatment combinations are shown; air exposure (no exposure, 33% or 69%), density (high or low), duration of air exposure (1, 2 or 3 weeks) and replication of two bags per treatment. Assessments carried out are also shown.

The trial started on the 18th of July 2019 on the Croisilles Harbour farm, and was completed on 22nd of August 2019, when a sample of 50 oysters from each of the bags was transported to Cawthron Aquaculture Park for assessment of the air exposure and stocking density effects.

Three levels of air exposure were achieved by manually flipping the mesh bags containing the oysters out of the water. Two rows of oyster bags (*Figure 9A*) were used, attached to

either side of a rope (mussel line) approximately 30 metres long, supported above the water by buoys (*Figure 9B*). One row of bags was not involved in the experiment and only served as a platform to aid in floatation. The other row contained the experimental oysters. At various times of the week (*Table 3* and *Table 4*), bags containing experimental oysters were flipped over in order to allow for controlled times of air exposure. Flipping was achieved by manually turning the bags over, so they rested on top of the bags opposite to them, keeping the oysters above the water.

Figure 9: Photos of the flipping mechanism used to control pre-transport air exposure on the Croisilles Harbour farm. A = Oyster bag construction; B = an example of the air exposure method, with a single flipped bag in the foreground pointed out with an arrow resting on the constantly submerged row of oyster bags serving as a floatation platform.

Bags were either kept constantly submerged or flipped out of the water for periods of 56 or 116 hours per the week in total (maximum of 68 hours out of the water in any one continuous period) subjecting oysters to either 0%, 33% or 69% cumulative weekly air exposure (*Table 3* and *Table 4).* This schedule was designed to avoid excessive continuous periods of air exposure, while allowing for high cumulative air exposure in the space of a week. Farm crew

assisted with the set up of the experiment and carried out the air exposure treatments (bag flipping).

Table 3: 33% air exposure treatment schedule for the oyster pre-transport experiment.

Day and time	Action	Length of air exposure time
Mon-10am	submerged	
Tue-10am	taken out	Tuesday to Wednesday- 28 hours out of water
Wed-2pm	submerged	
Thu-10am	taken out	
Fri-2pm	submerged	Thursday to Friday- 28 hours out of the water
Sat	submerged	
Sun	submerged	

56h out / 168h total = 33% air exposure per week

Table 4: 69% air exposure treatment schedule for the oyster pre-transport experiment.

Day and time	Action	Length of air exposure time
Mon-10am	submerged	
Tue-10am	taken out	(Tuesday to Thursday- 48 hours out of water)
Wed	taken out	
Thu-10am	submerged	
Fri-2pm	taken out	
Sat	taken out	(Friday to Monday- 68 hours out of the water)
Sun	taken out	
Mon-10am	submerged	

116h out / 168h total = 69% air exposure per week

At the beginning of the trial and after being graded to achieve a uniform size, the oysters were placed into a total of 28 bags and allowed to remain submerged for 10 days prior to the beginning of air exposure treatments in order to recover from any grading and handling stress. Following that there were three weeks of air exposure treatments. On two occasions, once during the first week and once during the third week the flipping schedule was

interrupted due to the absence of staff on the farm. This meant that during the first week the oysters that required 33% and 69% air exposure only received 17% and 31% exposure respectively. During the third week they received 33% and 62% air exposure.

Water and air temperature were monitored for the duration of the trial with two HOBO UA-001-08 temperature data loggers (Onset Computer Corporation, Bourne, USA). One of the loggers was placed in a bag that was always submerged to monitor water temperature. The other logger was attached to a barge moored approximately 100m away and was used to monitor air temperature. Both loggers were programmed to record temperature at 5-minute intervals.

On conclusion of air exposure and stocking density treatments and prior to their transport to Cawthron Aquaculture Park, all oysters were allowed to recover from air exposure treatments by remaining submerged for 48 hours on the farm. A sample of 50 oysters was then taken from each of the 28 bags. The samples were transported back to Cawthron Aquaculture Park in insulated bins containing ice packs to keep the temperature low and reduce the stress on the animals.

3.2.2 Assessment of the effects of pre-transport treatments

The assessment regime for this experiment consisted of four assessments (*Figure 10*). Each assessment was further split into two bags per treatment (not shown in *Figure 10*) to evaluate spatial variation.

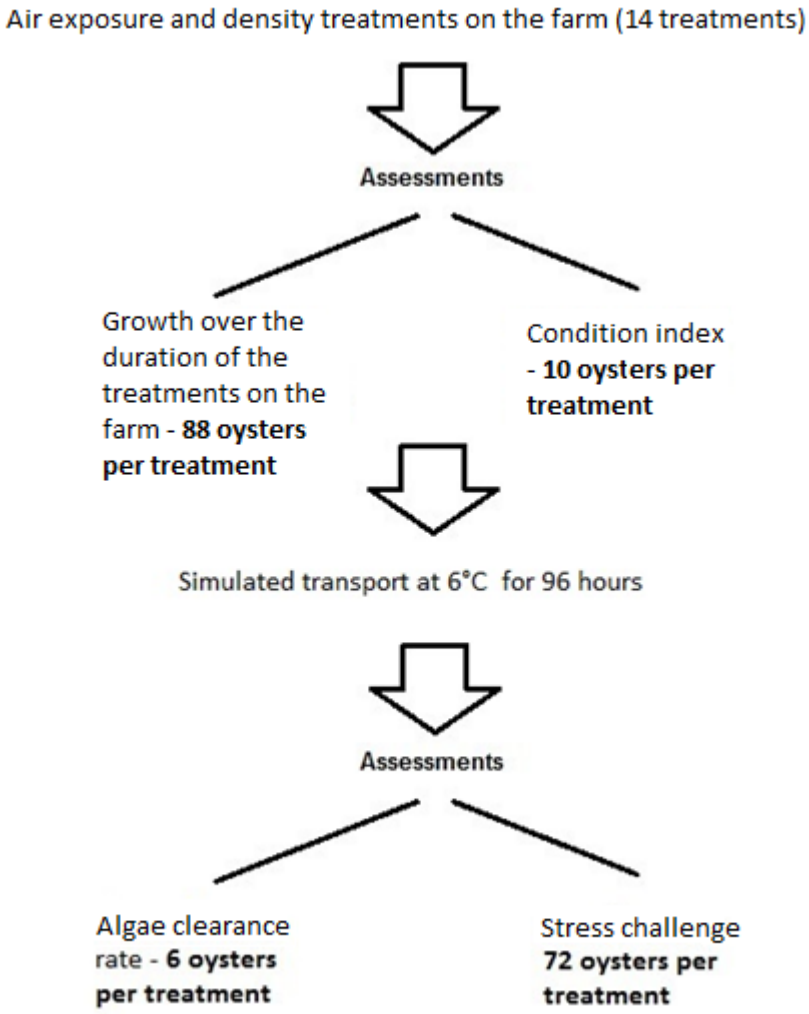

Figure 10: Sampling regime and assessments used to investigate the effects of pre-transport treatments on oyster condition and feeding.

3.2.2.1 Growth (weight gain)

Before the application of air exposure and density treatments, all experimental oysters were graded for uniform size by a mechanical grader. Following the air exposure and density treatments on the farm, 88 randomly selected oysters per treatment were weighed to determine mean wet weight and therefore compare weight gain among treatments. Measuring growth using weight instead of shell length was done as it was faster, since the sample sizes were large.

3.2.2.2 Condition index

Following air exposure and density treatments, 10 oysters per treatment were taken for condition index assessment. They were weighed whole to obtain the wet weight, with the

oyster shells superficially dried using paper towels beforehand to get rid of water present on the shell which could affect the calculation of the condition index. The flesh was then separated from the shell and both were dried and re-weighed. Flesh was dried in an oven at 80°C for 48 hours and shells were dried at 20°C for 4 days. Taking the wet weight as well as dry flesh and shell weights allowed for a volume condition index to be calculated. Volume was calculated as the difference between wet weight and dry shell weight, with water and oyster flesh assumed to have equal density (Lawrence & Scott, 1982). The following formula was used for volume condition index (Lawrence & Scott, 1982):

Volume condition index= (dry meat weight / (wet weight - dry shell weight)) x 1000

3.2.2.3 Simulated transport

Seventy-two oysters per treatment were subjected to simulated transport, carried out in the same manner as in previous experiment (see section 2.2.1). The temperature during simulated transport was 6.0 ± 0.02°C (mean ± SE). Humidity was not controlled, but was monitored with an Elitech RC-4HC data logger (83.1 ± 0.1% RH, mean ± SE).

This was done to determine the effects of air exposure and density treatments on the performance of oysters after transport. Performance was assessed by algae clearance rate and mortality observation after a stress challenge.

3.2.2.4 Algae clearance rate (feeding performance)

On completion of the 96-hour simulated transport period, 6 oysters per treatment were taken for individual algae clearance rate testing. *Isochrysis galbana* alga was used at a concentration of 30000 cells per ml, and a FluoroSense 2860-000-C handheld fluorometer (Turner Designs, San Jose, USA) measuring *in vivo* chlorophyll levels was used to determine clearance of algae over time. For clearance rate measurements, 3L tanks were filled with 13.5°C filtered seawater, and *Isochrysis galbana* algae was added followed by one oyster per container, and chlorophyll concentration readings were taken at 15 minute intervals for 1 hour to observe the depletion of algae over time (*Figure 11*). Due to poor feeding performance, a follow-up up experiment using 10 additional oysters randomly selected from the air exposure/density treatments was carried out over 2 hours. The aim was to see if the feeding performance would improve over a longer observation period.

Prior to actual clearance rate testing, several pilot studies were conducted. A sample of un-stressed oysters taken from Moana NZ's nursery on the Cawthron Aquaculture Park site was used to confirm the suitability of this concentration and species of algae for the experiment.

These pilot studies confirmed that *Isochrysis galbana* did not settle out over the time period of interest (1 hour) in a way that would affect fluorometer readings and did not require aeration or any additional agitation to stay in consistent suspension. The cell concentration of 30000 cells per ml was based on the clearance rate of un-stressed oysters of the same biomass per container over the same time period, so that the cell concentration would not fall below a detectable level during testing of stressed experimental oysters which were expected to have a lower clearance rate.

Figure 11: Experimental set-up for algae clearance rate testing.

A Multisizer 4 Coulter Counter (Beckman Coulter, Brea, USA) was used to correlate fluorometer readings with the actual cell count, to ensure that a fluorometer reading (in µg of chlorophyll per litre) was indicative of the number of cells present. To achieve this, 15 concentrations of *Isochrysis galbana* were tested. The Coulter counter was previously calibrated to detect the cells in the size range appropriate for the algae used in this experiment. The results showed that the fluorometer readings were closely related to the concentration of algae present in solution (*Figure 12*).

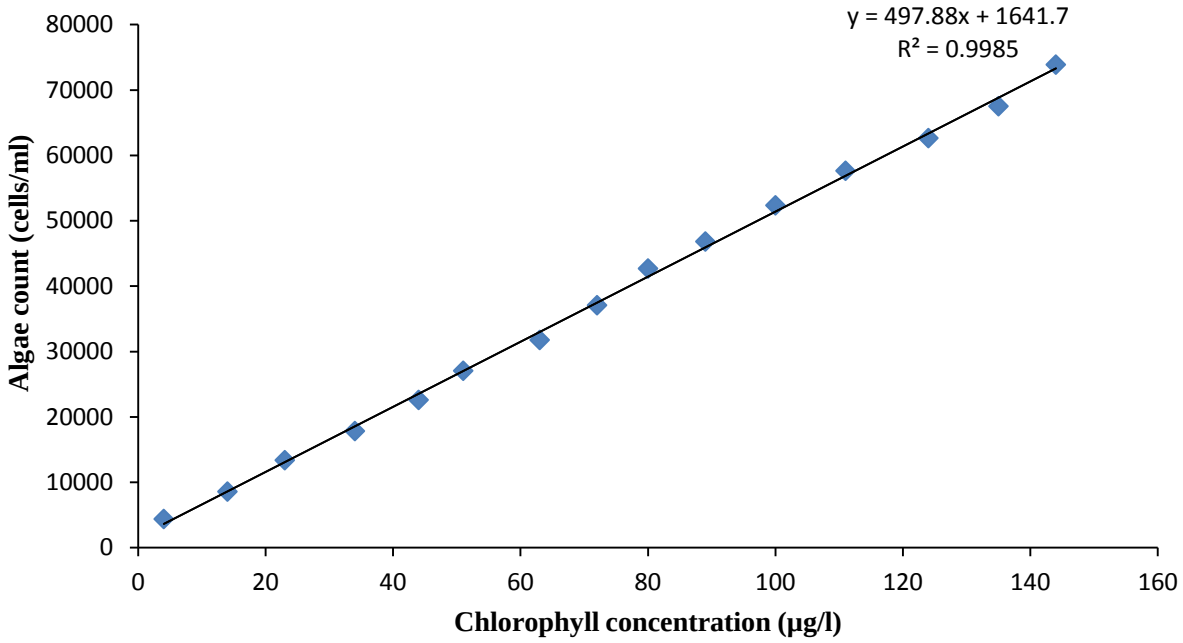

Figure 12: Regression showing a strong relationship between Coulter counter and handheld fluorometer readings of *Isochrysis galbana* concentrations.

In order to determine the clearance rate of animals which varied in size (between 18.7g and 36.4g), a clearance rate per gram of body weight formula was used

$$\textbf{Clearance rate (µg of chlorophyll L}^{-1}\textbf{ g}^{-1}\textbf{ h}^{-1}\textbf{)} = \frac{\mathbf{C1 - C2}}{\mathbf{W \times T}}$$

where **C1** and **C2** are initial and final algal concentrations in µg of chlorophyll per litre, **W** is the wet weight of the whole animal in grams and **T** is the time in hours.

3.2.2.5 Stress challenge

Upon completion of the simulated transport process, 72 oysters per treatment were exposed to a stress challenge at 20°C for 14 days (identical to the one used in Chapter 2, see section 2.2.2) to determine which pre-transport air exposure and density treatments allowed the oysters to better handle the transport process.

In addition to the stress challenge method as performed in section 2.2.2, oysters that remained shut at the end of the 14 days of stress challenge were immersed in seawater for one hour to assess whether they were dead or alive, as many (31.4%) stayed shut throughout the challenge and it was suspected that not all of those oysters were alive as assumed in the previous experiment. Oysters that gaped upon being placed in water but responded to touch by closing their shell were considered to be alive and those that gaped and did not respond to

touch were considered to be dead. Oysters that remained closed were considered to dead. Additionally, all oysters that remained closed at the end of the immersion period were manually opened to confirm this.

3.3 Statistical analyses

The effects of 7 air exposure treatments (0% emersion, 33% emersion for 1, 2 and 3 weeks and 69% emersion for 1, 2 and 3 weeks during a 3 week experiment) and 2 oyster density treatments (high = 13 L/bag and low = 6 l/bag) on three performance indicators (weight, condition index and clearance rate) were assessed with ANOVA. A 3-factor analysis was used to assess the crossed effects of air exposure and density. Bag was included as a random factor nested within the air exposure × density interaction to determine whether spatial variation within the treatments was important. There were two bags per air exposure/density combination with 44, 5 and 3 oysters per bag being assessed for weight, condition index and clearance rate respectively. Data were assessed for normality and heterogeneity with Shapiro Wilk and Cochran's C tests respectively. Data not meeting assumptions of normality and homogeneity were log(x+1) transformed. Significant effects were examined using post hoc Tukey's tests to determine differences among groups within a factor. Mortality data were assessed using analysis of covariance with differences among treatments assessed with Tukey's post hoc tests. Analyses were carried out in STASTISTICA 12 (Statsoft Ltd.).

3.4 Results

3.4.1 Environmental parameters

Water temperature during the trial was 11.54 ± 0.01°C, (mean ± SE, *Figure 13*). Air temperature during the trial fluctuated between 19.9°C and 1.2°C, with a mean temperature of 10.0 ± 0.04°C (*Figure 14*).

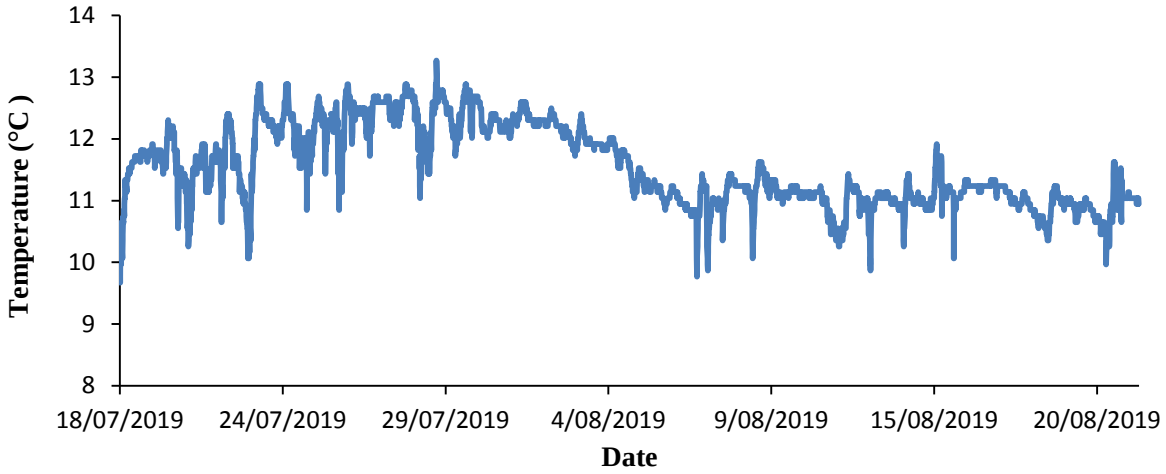

Figure 13: Water temperature during the trial on the Croisilles Harbour farm.

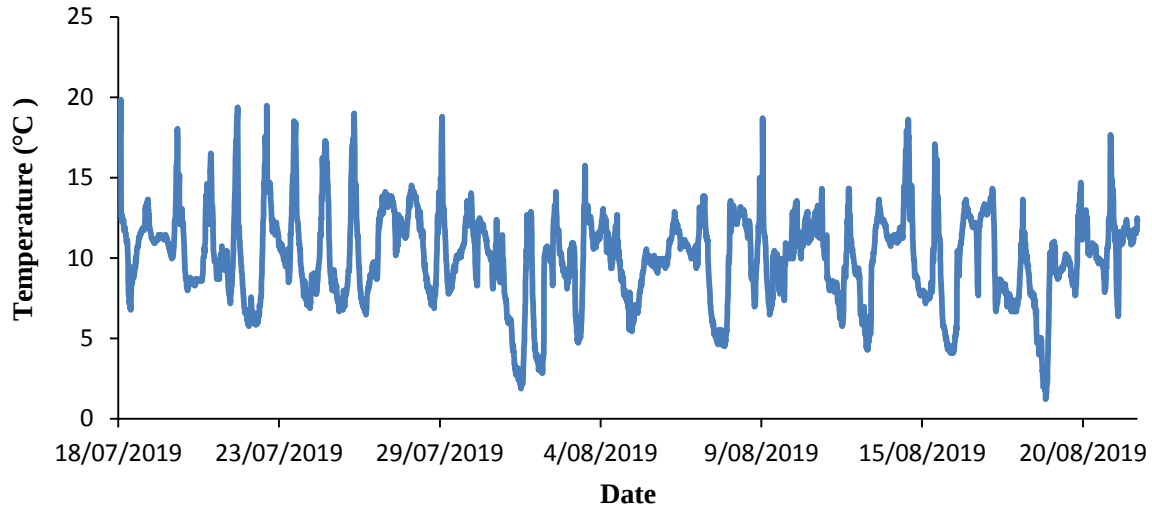

Figure 14: Air temperature during the trial on the Croisilles Harbour farm.

3.4.2 Effects of air exposure and density regimes on weight

At the end of the experiment, the weight increase of the oysters was greater at higher stocking density (23.35 ± 0.20g, mean ± SE, n = 616, *Figure 15*) compared to the lower stocking density (21.76 ± 0.20g, n = 616), treatment ($F_{1, 1204} = 12.15$, p = 0.0036, *Table 5*). There were also differences due to spatial variation between the two bags per treatment ($F_{14, 1204} = 2.34$, p = 0.0035). There were no significant differences between treatments due to air exposure ($F_{6, 1204} = 0.89$, p = 0.5257).

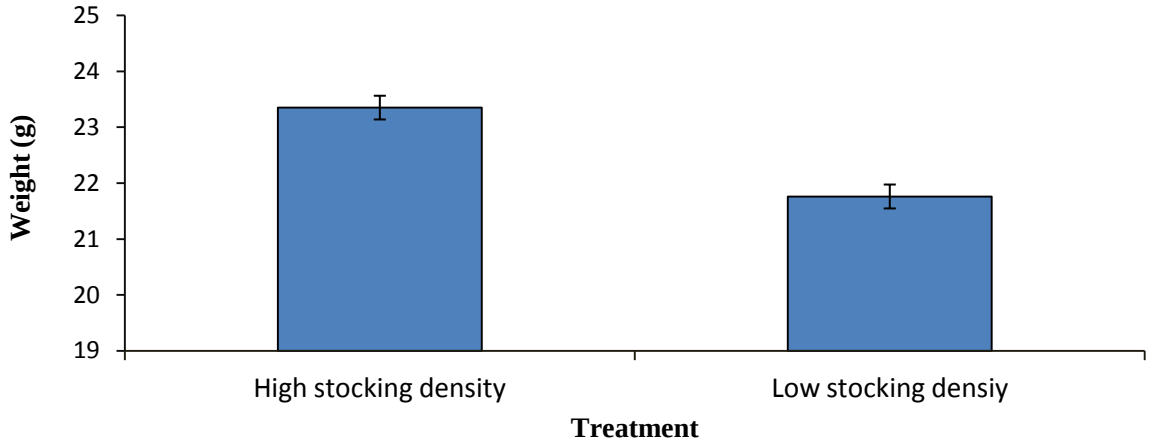

Figure 15: Mean weight of the oysters at the conclusion of the pre-transport air exposure and density treatments (air exposure treatments are combined). Mean (± SE) data are presented (n = 616).

3.4.3 Condition index

At the end of the experiment, the condition index of oysters was lower at the higher stocking density (27.40 ± 0.73, mean ± SE, n = 70, *Figure 16*) compared to the lower stocking density (29.37 ± 0.79, n = 70) treatment ($F_{1, 112}$ = 5.52, p = 0.034). There were no significant differences between treatments due to air exposure ($F_{6, 112}$ = 2.36, p = 0.0866) or between bags within the treatments ($F_{14, 112}$ = 0.67, p = 0.8034).

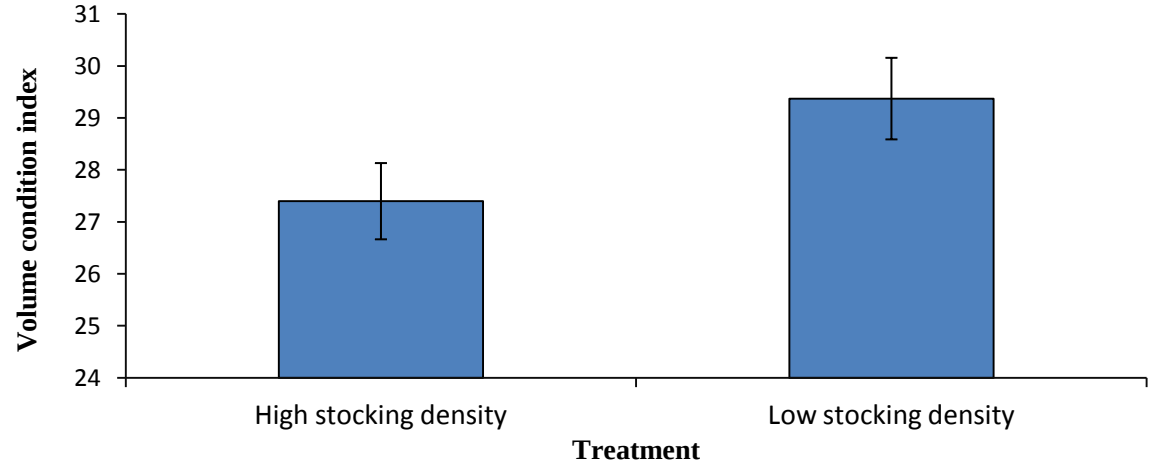

Figure 16: Volume condition index of the oysters at the conclusion of the pre-transport air exposure and density treatments (air exposure treatments are combined). Mean (± SE) data are presented (n = 70).

3.4.4 Algae clearance rate

There was a significant effect of air exposure regime on algae clearance rate ($F_{6, 56}$ = 4.38, p=0.0107). Tukey's post hoc test indicated that the clearance rate of oysters in the 69%, 3

week air exposure treatment (0.53 ± 0.10µg of chlorophyll L^{-1} g^{-1} h^{-1}, mean ± SE, n = 12, *Figure 17*) was greater than in both the 69%, 1 week treatment (0.18 ± 0.04µg of chlorophyll L^{-1} g^{-1} h^{-1}) and the 33%, 2 week treatment (0.175 ± 0.04µg of chlorophyll L^{-1} g^{-1} h^{-1}). There were no significant differences due to density ($F_{1, 56}$ = 2.48, p = 0.1376) or between bags within the treatments ($F_{14, 56}$ = 0.77, p = 0.6962).

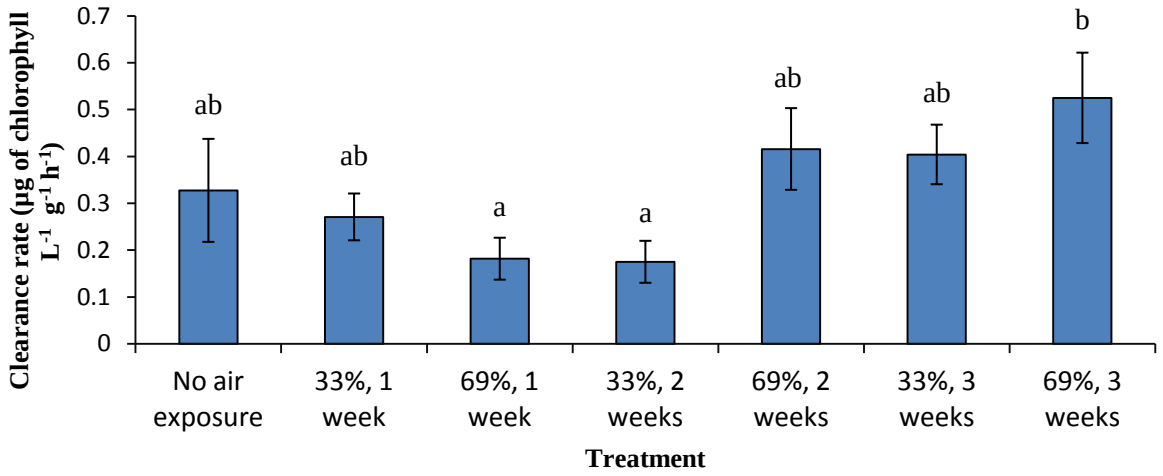

Figure 17: Algae clearance rate of the oysters at the conclusion of the simulated transport (stocking density treatments are combined). Mean (± SE) data are presented. Letters indicate significant difference between treatments, alpha = 0.05 (Tukey's post-hoc test), n = 12.

Table 5: Results of the three-factor ANOVA. Text in bold represents significant differences (alpha = 0.05).

	d.f.	MS	F	p	d.f.	MS	F	p
			Weight				Condition index*	
Air exposure (A)	6	57.21	0.89	0.5257	6	0.0129	2.36	0.0866
Density (D)	1	**778.11**	**12.15**	**0.0036**	**1**	**0.0301**	**5.52**	**0.0340**
A × D	6	21.79	0.34	0.9040	6	0.0132	2.43	0.0803
Bag (A × D)	14	**64.03**	**2.34**	**0.0035**	14	0.0054	0.67	0.8034
Residual	1204	27.42			112	0.0082		
			Clearance rate*					
Air exposure (A)	**6**	**0.0194**	**4.38**	**0.0107**				
Density (D)	1	0.0110	2.48	0.1376				
A × D	6	0.0095	2.13	0.1141				
Bag (A × D)	14	0.0044	0.77	0.6962				
Residual	56	0.0058						

*Log(x+1) transformed data

3.4.5 Mortality following the stress challenge

Mortalities started on day 4 and the cumulative mortality rate increased sharply from then on (*Figure 18*). There were no differences among treatments ($F_{13, 195} = 1.21$, $p = 0.272$). At the end of the experiment, 33.6% of the oysters (339 oysters) were still closed. When immersed in seawater for 1 hour, 46.7% of those opened and were clearly dead. The oysters that remained closed were manually assessed (opened) and were also dead. Thus 100% of the oysters in this experiment were dead after 14 days of air exposure during the stress challenge. Mortality observation based on gaping was highly inaccurate, and the true number of mortalities was not determined until immersion.

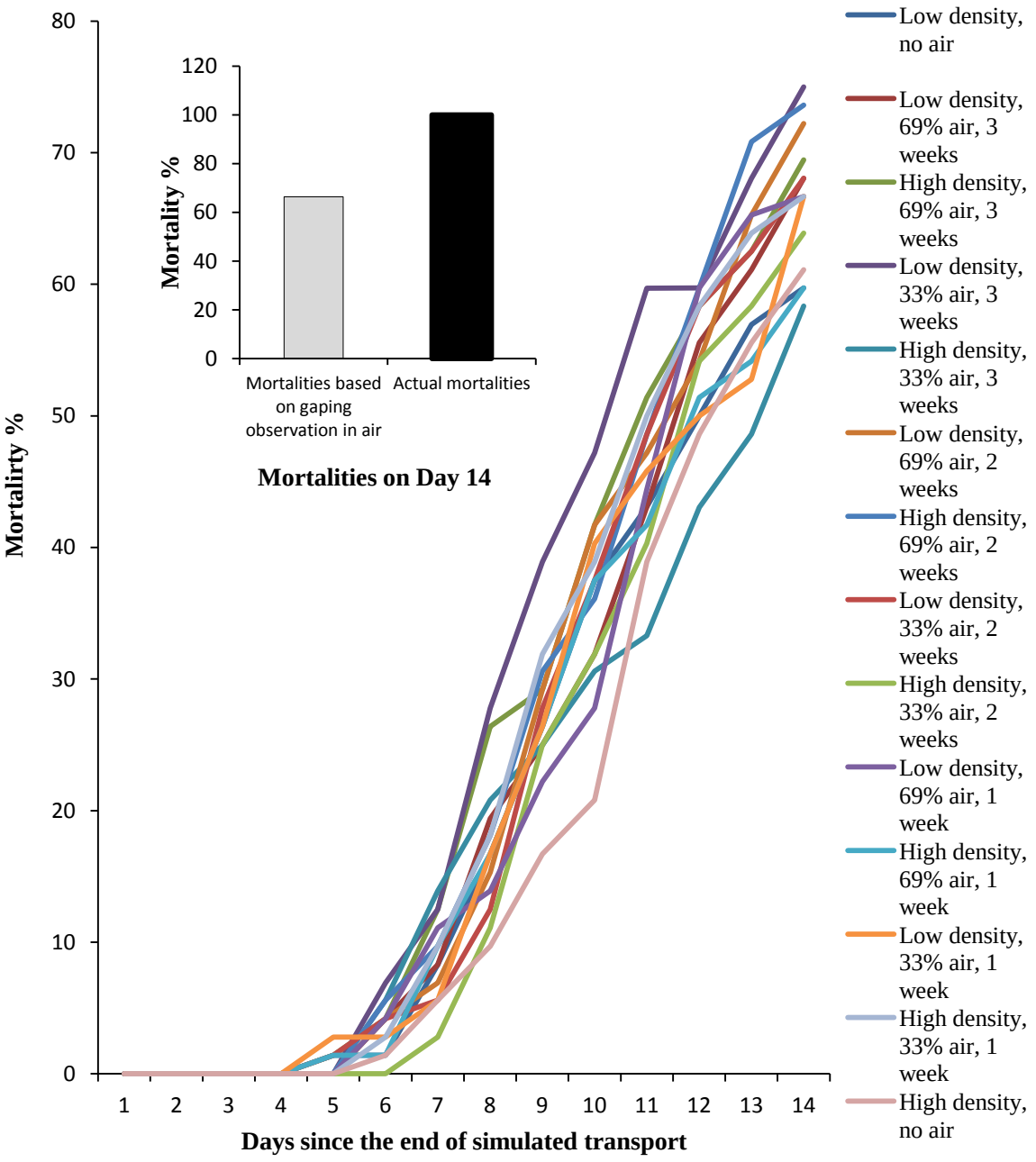

Figure 18: Cumulative oyster mortality percentage in the 14 treatments during the stress challenge. Insert shows the mortality percentage on Day 14 as obtained by the gaping observation method and after assessment in water (see section 3.2.2.5).

3.5 Discussion

The results of this experiment showed that higher density increased growth but decreased the condition index, while the highest air exposure treatment (69% per week for 3 weeks) resulted in the best feeding performance (algae clearance rate).

Studies into the effect of stocking density on growth (absolute weight gain over time) of oysters have suggested that high density is detrimental to growth due to factors such as reduced water flow and food availability (Drinkwater & Howell, 1985; Holliday *et al*., 1991; Marshall & Dunham, 2013; Capelle *et al*, 2019). However, depending on the extent of the density increase, oysters have also been reported to maintain equivalent growth at higher densities due to compensatory mechanisms such as an increase in clearance rate, decrease in pseudofeces production and increase in pre-ingestive selection of organic material (Honkoop & Bayne, 2002).

In this experiment higher stocking density was not observed to be detrimental and in fact led to greater growth during the pre-transport treatments (there was no effect of air exposure). No explanations for higher growth rates under conditions of higher density have been found in literature. Additional trials with a greater range of densities may need to be carried out to further investigate this relationship between density and growth rate.

There was also a difference in growth between the bags within the treatments. This may be explained by environmental differences such as variation in water currents and therefore food quantities despite only a short distance separating the two bags per treatment (less than 15 metres). Another source of variation could have been biofouling (some biofouling was present, attached to the mesh oyster bags), the amount of which can vary from bag to bag. Biofouling has the effect of slowing down oyster growth by competing for nutrients (Marshall & Dunham, 2013). This may mean that any treatments performed on the farm have to be assessed in conjunction with environmental variables (presence of biofouling organisms, phytoplankton abundance, water flow) which can vary depending on location within a single farm (Strohmeier *et al*., 2008; Mallet *et al*., 2009). The experimental design may have also affected the results since oysters were not individually weighed before air exposure and stocking density treatments. Instead, all oysters were assumed to be of approximately the same weight after mechanical grading at the start of the experiment. It is therefore possible that there was a difference in weight between treatments at the start of the experiment. If this experiment was to be repeated, tracking the weight gain of individual oysters over time would give more accurate results.

Volume condition index of oysters in this experiment was affected by density, but not by air exposure. The condition index decreased with an increase in density. This is in agreement with other studies on Pacific oysters grown at varying densities (Honkoop & Bayne, 2002;

Chavez-Villalba *et al*., 2010; Marshall & Dunham, 2013; Capelle *et al*, 2019) that report lower condition indices at higher densities. This may be due to less food available per individual and the added stress of contact with other oysters that can increase the potential for shell damage at higher densities. The energy expenditure to repair that damage lowers the potential for somatic growth (Chavez-Villalba *et al*., 2010). A low condition index indicating nutritive stress and lower energy reserves (Crosby & Gale, 1990) may be detrimental to oysters prior to an additional stress imposed by transport. Therefore, avoiding high stocking densities prior to transport may be beneficial to oyster survival.

It is important to note that the density effects on growth and condition index presented here were a result of 5 weeks of stocking density treatment, as there was time allocated before and after the 3-week air exposure treatment to allow oysters to recover from handling. The commercial transport process involves only two weeks at a higher stocking density, therefore the results may not be directly comparable.

The results showed that the air exposure treatments had a statistically significant effect on clearance rate, with the highest air exposure treatment (69% per week for 3 weeks) resulting in the best feeding performance. However, due to extremely poor performance of the oysters during the testing and only 6 samples per treatment, this result should be interpreted with caution. During a pilot study carried out using un-stressed oysters from Moana's nursery before the algae clearance rate testing in order to establish a baseline feeding rate, oysters opened their shells and started feeding within several minutes of being placed in water containing algae. A significant drop in algae concentration was observed after 15 minutes and was maintained throughout the 1-hour time period. However, the stressed (experimental) oysters in almost all of the 84 observations (6 samples x 14 treatments) were much slower to open their shells, and no reduction in algae concentration was observed after 30 minutes. Some feeding took place between 30 and 60 minutes, but it was very slow (2/3 of the animals cleared less than 16% of available algae). It was suspected that oysters needed time to "acclimatise" to being back in the water after having just gone through a 96-hour simulated transport, and this may have contributed to the slow feeding performance. Therefore, at the conclusion of the clearance rate experiment 10 spare randomly selected oysters that went through the simulated transport were used to conduct a 2-hour follow-up clearance rate experiment to confirm whether this was the case, but presented an equally slow feeding rate. Time periods of over 2 hours would be needed for more accurate assessments which would make this method excessively time consuming as a biomarker. Given the difficulties

encountered with this method, it may not be a good biomarker for stress assessments in a commercial setting. What this assessment showed, however, was that transport severely impacted oyster feeding behaviour, indicating that it was a significant stressor.

An important finding in this study was that a commonly used method (gaping) to assess oyster mortality was found to be highly inaccurate. At the end of the stress challenge, the average observed mortality (based on gaping) over all treatments was 66.4%, whereas the additional immersion assessment showed that every single oyster was dead. On immersion in seawater, some oysters opened their shells, while others stayed shut until they were opened manually. Upon manual opening, all animals were visibly dead and partially decomposed. Clearly, mortality data based on gaping during the 14 days was unreliable since 33.6% of mortalities were not captured. It appeared the oyster shells stuck together as the shell liquor dried out and congealed and remained closed in many cases regardless of whether the oyster was dead or alive. Even when oysters do gape, this may not accurately indicate the time of death, and may simply indicate the time when the spring action of the hinge ligament overcomes the adhesive action of congealed shell liquor. Additionally, the adhesive action is strong enough to prevent some oysters from opening even when placed in water. It is suspected that in the first several days while the oysters have not had a chance to dry out, the mortality monitoring based on gaping may be more reliable and therefore may still be taken as an indication of true mortality. However, the only way to ensure accurate mortality results are obtained after air storage is to either open the oysters and visually assess them or pry the shells open and note if there is any resistance from the adductor muscle.

In the previous experiment (Chapter 2, section 2.4.2), 28.5% of the animals remained closed at the end of the 14-day stress challenge and were considered to be alive with no further investigation. The result of this experiment meant that mortality data from Chapter 2 may not be reliable either, since in that case oysters were not inspected at the end of the trial to confirm whether they were indeed alive.

There are other studies that used this method of observing mortality rate in air based on gaping in Pacific oysters (Bird *et al*., 1995; O'Meley, 1995; Meng *et al*., 2018) as well as in mussels (Webb, 1999; Altieri, 2006; Zamora *et al*., 2019) and didn't report any difficulties or inconclusive results. Meng *et al*. (2018) used this method to compare survival of Pacific oysters from sub-tidal and intertidal populations in air and reported total mortality after 9 days at 20°C using gaping observation to assess mortality. No oysters were reported as failing

to open. This is of particular interest because the animals used (juvenile Pacific oysters) and the 20°C temperature regime were identical to this trial. Experiments carried out by Bird *et al*. (1995) and O'Meley (1995) also used the same mortality monitoring technique on Pacific oysters to determine their shelf life in air. The results of those studies may have been affected by the failure of gaping observation to accurately detect mortality.

Water temperature for the duration of the trial remained stable at 11.54 ± 0.01°C (mean ± SE), while air temperature fluctuated significantly between 1.2°C and 19.9°C with a mean of 10.0 ± 0.04°C. If this experiment is to be compared to other studies or future experiments involving air exposure, these temperatures may need to be considered due to their influence on oysters such as reducing growth in colder conditions (Spencer, 1990).

In summary, due to difficulties with both the algae clearance rate and the stress challenge, and their failure to determine which of the pre-transport conditioning treatments allowed the oysters to better handle simulated transport, the conclusions that can be drawn from this experiment are that increased stocking density results in faster growth but reduced condition index of the animals. A low condition index may be detrimental to oysters prior to any additional stress imposed by transport and therefore lowering stocking densities before transport may be beneficial.

4.0 Combined effects of pre-transport conditioning and post-transport recovery

4.1 Introduction

In the previous chapter the effects of pre-transport air exposure and stocking density treatments on algae clearance rate and resilience to a stress challenge were assessed, but these were only weakly affected by the treatments. The following is an additional experiment to further assesses air exposure in the context of pre-conditioning, with the effects of added post-transport handling methods also investigated.

4.1.1 Post-transport delay effects

Commercial transport process used by Moana NZ involves the transfer of oysters from Croisilles Harbour to Whangaroa Harbour by truck, where they are unloaded and placed back in the water on the farm. However, there is often a time delay between arrival on the Whangaroa Harbour farm and the return of oysters to the water due to the farm being accessible by barge only at high tide. If the arrival of the truck doesn't coincide with the high tide, there is a time delay where oysters are stored out of the water (at ambient temperature in a shed) until the following day. Reducing this time delay may be beneficial to oyster survival, especially since refrigerated storage facilities are not available in Whangaroa Harbour.

Zhang *et al*. (2006) carried out an experiment exposing adult Pacific oysters to air temperatures of 5°C, 15°C and 25°C for 72 hours. Following that, the animals were put into 15°C water to recover. To quantify the stress levels, lysosomal membrane stability was assessed. Haemolymph samples were taken at 0, 0.5, 1.5, 3, 6, 12, 24, 48 and 72 hours after the start of air exposure. The results indicated that both longer air exposure times and higher temperatures induced higher stress levels in the animals, which in turn demonstrated the importance of avoiding periods of non-refrigerated storage whenever possible. Also, when refrigeration is not possible, better survival of eastern oysters was observed when duration of air exposure to temperatures of 12°C to 19°C was reduced (Clements *et al*., 2018).

The following experiment was carried out in two parts: pre-transport air exposure treatments carried out on the Croisilles Harbour farm followed by transport to Whangaroa Harbour, and post-transport recovery treatments after arrival at the Whangaroa Harbour farm. Additionally, physiological assessments were carried out to monitor oyster survival and performance throughout the experiment.

Weight loss in air was one of those assessments. Air exposure has been reported to help with strengthening of the adductor muscle and thickening of the shell edges of oysters thus improving their survival in air by reducing the rate of desiccation (Quayle, 1988; O'Meley, 1995). For this reason weight loss was used to assess if air exposure helped the oysters to stay closed more efficiently and for longer in air and avoid harmful desiccation and increased concentrations of toxic waste products derived from oyster metabolism (Wang & Amiro, 1977).

4.2 Materials and methods

The experimental design and assessments carried out at each step are shown in *Figure 19*.

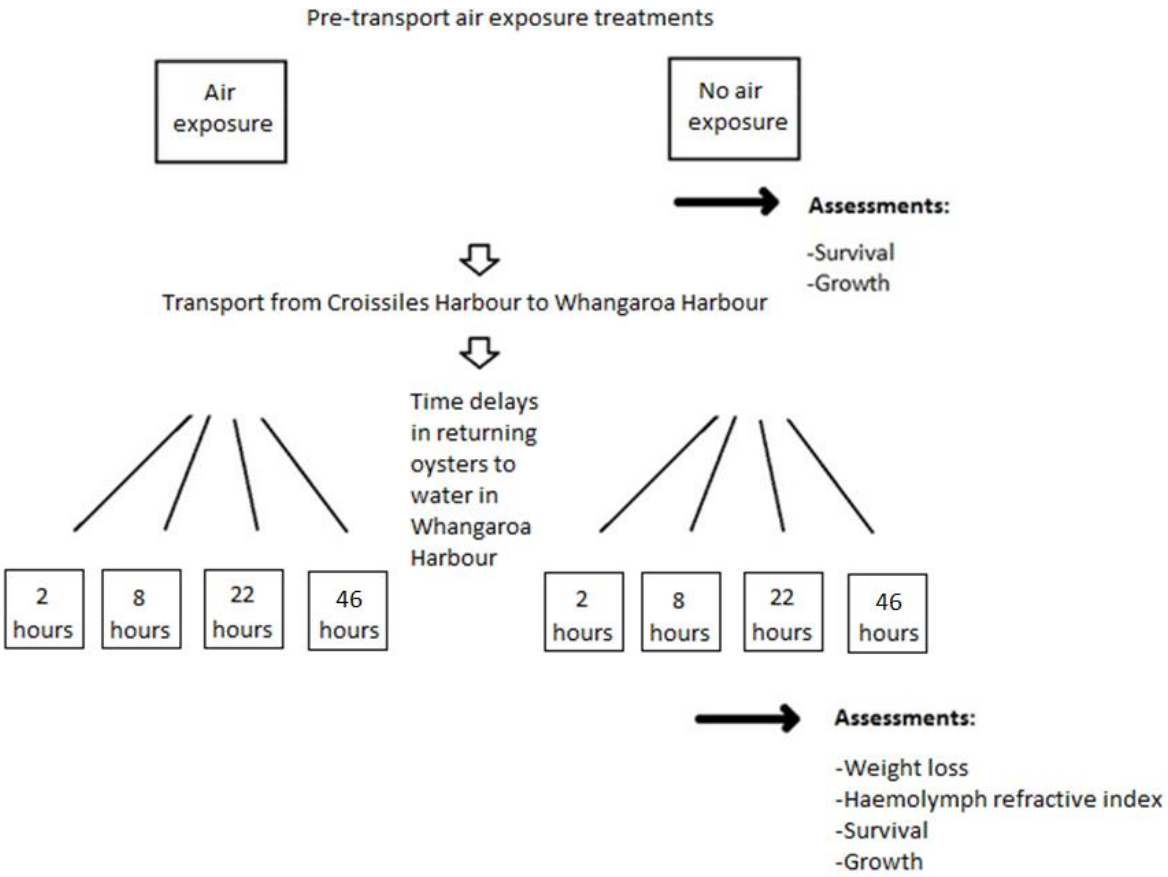

Figure 19: Combinations of air exposure pre-treatments and post-transport time delay treatments investigated as well as the assessments carried out.

4.2.1 Pre-transport air exposure

Juvenile Pacific oysters 46.86 ± 0.65mm long and weighing 9.89 ± 0.39g (mean ± SE, n = 50) were placed in the water on the 1st of October, after being mechanically graded for uniform size in the previous week. They were kept submerged for a week before the air exposure treatments started, to allow them to recover from handling stress.

The air exposure trial was carried out between the 7th of October 2019 and the 31st of October 2019. Oysters were allocated to eighty 37 litre mesh bags, as in previous experiment (*Figure 9A*, section 3.2.1). The bags were stocked by volume with 13 litres of oysters (between 650 and 950 animals per bag), the density which in the previous experiment represented the "high stocking density" treatment. The 80 experimental bags were attached to a mussel line in the same way as was done in the previous experiment, with additional 80 bags used on the opposite side of the mussel line as floatation platforms (*Figure 9B*, section 3.2.1). Air exposure procedure was also similar to that in the previous experiment (methods in section 3.2.1). Oysters were allocated to either an air exposure treatment group, or a continuously submerged group for 3 weeks prior to shipping.

Air exposure was carried out over 3 weeks and consisted of the bags being flipped out of the water on a Monday afternoon, and flipped back into the water on a Friday morning (92 hours out of the water). This resulted in oysters receiving 55% of continuous air exposure per week. This schedule was chosen as it was logistically viable to carry out on the farm compared to the one used in the previous experiment and could realistically be incorporated into commercial production. The schedule used previously relied on daily attendance by the farm crew which wasn't always possible.

On the 25th of October all bags were flipped back into the water and remained submerged until being removed for transport on the 1st of November in order to recover from any potential air exposure-induced stress before being handled.

As the air exposure phase of the trial got underway from the 1st of October to the 1st of November, rainfall levels in the Croisilles Harbour area were monitored through access to data collected by a weather station approximately 5km away from the farm site. Data was accessed through http://www.data.hyquestsolutions.co.nz. This was done to monitor for any severe rain events in the area (especially immediately prior to shipping) that may have imposed stress on the oysters through a change in water salinity (Gagnaire *et al.,* 2006). It

was not possible to monitor water salinity directly during this experiment, so rainfall data was used as a proxy (as heavy rainfall would reduce water salinity in the area).

Temperature data was also collected. A HOBO water temperature V2 data logger (Onset Computer Corporation, Bourne, USA) was placed into one of the air exposure bags for the duration of October 2019, recording both water and air temperature the oysters were exposed to in 10-minute intervals. Additionally, water temperature data for the whole oyster transport period for 2019 (between March and November) was obtained from a submerged (1 meter deep) temperature data logger on the farm in one-hour intervals. This data was collected to determine how much the water temperature in Croisilles Harbour changes throughout the year, as experimental results obtained at a certain time of the year may be specific to that time period due to seasonal temperature variations.

4.2.1.1 Growth and mortality assessments after the air exposure treatments

Five bags from each of the two treatments were taken aside near the start of the pre-transport air exposure experiment (9th of October) and stocked with exactly 800 live oysters, while removing any empty shells. This was to allow for mortalities resulting from the air exposure treatments to be assessed on the conclusion of the treatments on the 1st of November (percentage of dead to live oysters). The bags used to monitor mortality were evenly distributed throughout the mussel line to avoid any localised effects.

Gaping oysters that did not shut in response to touch were considered to be dead. Unlike the oysters in sections 2.2.2 and 3.2.2.5 that were stored in air, leading to their shells sticking together and making mortality determination unreliable, these animals were assessed immediately after being removed from water. Therefore, their shells did not have a chance to dry out and stick together.

Difference in growth (weight gain) over the duration of pre-transport air exposure treatments was assessed by measuring the wet weight of 60 oysters from each of the two treatments at the end of the pre-transport treatments.

4.2.1.2 Transport process

On the 1st of November, experimental oysters were removed from the water and placed in 45 litre plastic crates for shipping. Separate crates were used for the air exposure and non-air exposure oysters. The transport process from Croisilles Harbour to Whangaroa Harbour took 44 hours, with a refrigerated truck used for this purpose.

Temperature and humidity during transport were monitored with an Elitech RC-4HC temperature/humidity data logger.

4.2.2 Post-transport recovery

Upon arrival at the destination farm in Whangaroa Harbour 44 hours later, the oysters were subjected to four different time delays (hereafter referred to as delay treatments) between arrival and placement in the water. These were:

1. Placement in the water in long-line baskets 2 hours after arrival
2. Placement in bags on an intertidal rack 8 hours after arrival
3. Placement in bags on an intertidal rack 22 hours after arrival
4. Placement in bags on an intertidal rack 46 hours after arrival

These four treatments were separated according to their air exposure and non-air exposure groups for a total of eight treatments (*Figure 19*). The long-line basket method in deeper water was chosen as it allowed immediate (within 2 hours) placement in the water after arrival, independent of the tide (which limits barge access to the intertidal racks at certain times on the Whangaroa Harbour site). The long-line baskets (*Figure 20A*) stayed continuously submerged (approximately 1 metre deep) on the long-line (*Figure 20B*) without regular exposure to air compared to oyster bags on intertidal racks.

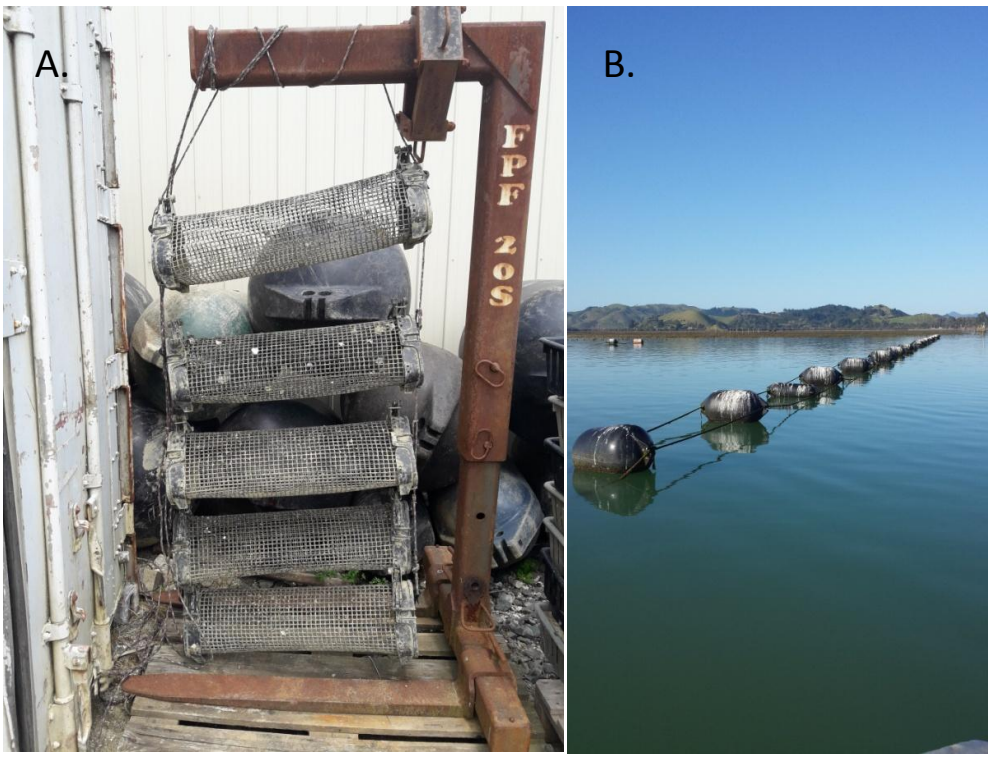

Figure 20: Images of the long-line system used during the post-transport recovery trial. A = Basket units housing the oysters; B = long-line the baskets were attached to in Whangaroa Harbour.

The intertidal method (*Figure 21*), involved oyster placement on the intertidal rack in bags 8 and 22 hours after arrival and approximated the standard farm practice of returning the oysters to water after transport (time delay between arrival and return to water being dependent on the tide). The last treatment that delayed the return to water by 46 hours is not normally encountered in the course of commercial operations but was chosen to deliberately stress the oysters to assess the effects of pre-transport air exposure.

Figure 21: Images of the intertidal system used during the post-transport recovery trial, depicting the rack housing and the oyster bags in the three intertidal delay treatments.

After arrival, the oysters were stored in the shade in a steel shed for the duration of being kept out of the water. Temperature and humidity during this period were monitored with an Elitech RC-4HC temperature/humidity data logger.

The bags for each of the three intertidal treatments were labelled with colour tags for identification and were arranged in an alternating pattern on the rack, to reduce any localised effects on the oysters. Treatments were evenly spread out across the occupied length of the rack. Intertidal and long-line oysters were approximately 300 metres apart due to the layout of the farm.

Both the long-line baskets and the bags were stocked at a density normally used for commercial growing. The baskets were stocked with exactly 100 oysters each, and the bags with 150. The number of oysters in each of the eight treatments (air exposure and non-air exposure treatments × 4 delay treatments) was set at 1500. Therefore, there were more long-line baskets used than bags per treatment (15 baskets versus 10 bags). The total number of bags across all treatments was 60, and the number of baskets was 30. The total number of individual oysters used in the experiment was 12000.

4.2.2.1 Stocking and collection methods

Upon arrival from Croisilles Harbour, oysters were immediately allocated into the long-line baskets and intertidal bags. Only live oysters were stocked. Before being placed into an individual basket/bag, the oysters were weighed (total weight of either 100 or 150 oysters) in order to observe subsequent growth rate (weight gain). Individual baskets/bags within treatments were not labelled due to time limitation on the day of experiment set-up, therefore the weight increases were only tracked for the entire treatments (1500 oysters). The baskets and bags were labelled to differentiate between air exposure treatments and between different delay treatments. The long-line baskets were placed in the water at 12pm on the 3rd of November, followed by the first lot of intertidal bags at 6pm the same day, then 8am the following day and 8am a further 24 hours later. Oysters were then collected for mortality and growth assessments in the same order eight days later, starting with the long-line baskets at 8am on the 11th of November, then the intertidal bags at 12pm the same day, and the rest of the bags in the early afternoon of the 12th and 13th of November. Oysters in each treatment group stayed in the water for 8 days (192 hours).

Salinity and water temperature data from a sensor buoy in Whangaroa Harbour (accessed through www.wai.co.nz) were collected. This was done to see if there were any periods of low salinity in the harbour while the trial was underway that may have imposed additional stress on the oysters (Gagnaire *et al*., 2006).

At the conclusion of the experiment, all surviving oysters were placed back on the farm as part of commercial stock.

4.2.2.2 Assessments during the post-transport period

The assessments carried out during the post-transport period consisted of monitoring weight loss and haemolymph refractive index after transport as well as growth and survival rates (*Figure 22*).

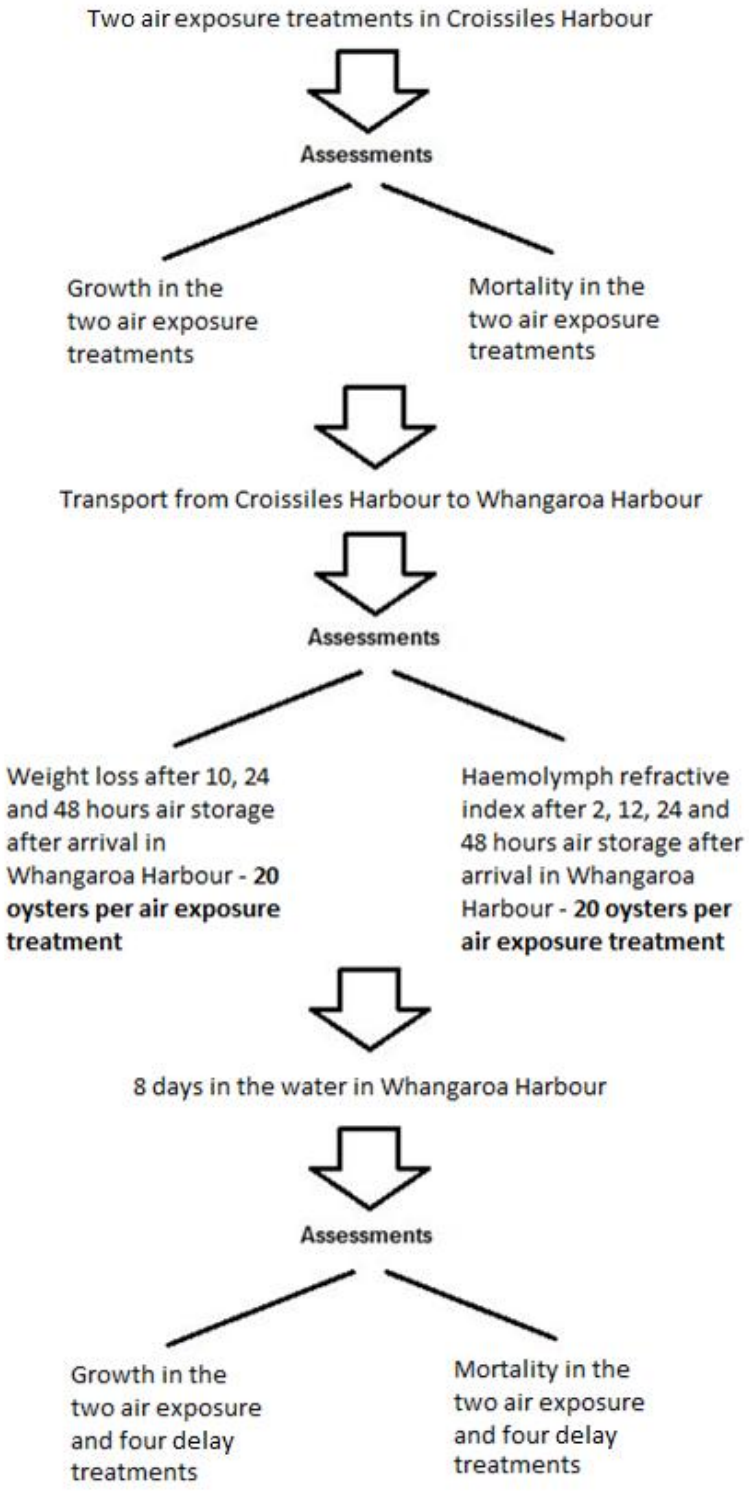

Figure 22: Sampling regime and assessments of the effects of air exposure and time delay treatments.

4.2.2.3 Weight loss

Sixty oysters were taken from each air exposure treatment (i.e. an air exposure and a no air exposure treatment) on 1st of November and placed into individual mesh bags (*Figure 23*)

prior to being loaded onto the truck in Croisilles Harbour for transport to Whangaroa Harbour. The bag size was chosen so that it did not prevent individual oysters from gaping. The 120 oysters were weighed immediately after arrival in Whangaroa Harbour.

Figure 23: One of the 120 individually bagged oysters used for weight loss assessment.

Following this, 40 oysters (20 from each of the air exposure treatments) were weighed 10, 24 and 48 hours later, and a percentage of weight lost over each period was calculated. This was a variation of the weight loss assessment used in section 2.2.1.

4.2.2.4 Haemolymph refractive index

An assessment was carried out to determine if pre-transport air exposure reduced stress levels experienced by oysters during the transport process. Haemolymph refractive index was determined for 160 oysters to track the change in refractive index (and the density derived from that) over time. A sample of 20 oysters from each of the air exposure treatments was used, and haemolymph taken and analysed 2, 12, 24 and 48 hours after arrival in Whangaroa Harbour. Different oysters were sampled each time.

Approximately 50µl of haemolymph was taken from the adductor muscle of an oyster and placed on a salinity refractometer (of a generic brand, *Figure 24*). This test was similar to the one carried out in section 2.2.3. Out of the total of 120 oysters allocated for haemolymph testing, 7 were unable to be used due to inability to get a sufficient haemolymph sample.

Figure 24: Temperature-compensated salinity refractometer used for measuring haemolymph refractive index.

4.2.2.5 Mortality

After eight days in the water on the Whangaroa Harbour site, the bags and baskets containing the oysters were collected in the same order they were initially placed in the water, with each treatment receiving 192 hours in the water. Mortality was assessed by counting dead oysters in each container.

4.2.2.6 Growth (weight gain)

Growth was assessed by weighing all oysters in each of the treatments together on arrival in Whangaroa Harbour before the oysters were placed in the water. The same oysters were weighed again after 192 hours in the water. As individual bags and baskets were not labelled, there was no replication. The weight gain was only able to be determined per treatment as a whole (total weight gained by 1500 oysters), rather than per container.

4.3 Statistical analyses

Two-sample T-tests were used to analyse mortality and growth after air exposure treatments on the Croisilles Harbour site.

The effects of air exposure (2 treatments: air exposure and no air exposure) and recovery (4 treatments: 2, 8, 22 and 46 hour delays in returning oysters to water) on three performance indicators (weight loss, haemolymph refractive index and mortality in Whangaroa Harbour)

were assessed with ANOVA. A 2-factor analysis was used to assess the crossed effects of air exposure and recovery. Data were assessed for normality and heterogeneity with Shapiro Wilk and Cochran's C tests, respectively. Data not meeting assumptions of normality and homogeneity were log(x+1) transformed. Significant effects were examined using Tukey's post hoc tests to determine the differences among groups within a factor. Analyses were carried out in STASTISTICA 12 (Statsoft Ltd.).

4.4 Results

4.4.1 Pre-transport air exposure: environmental parameters

There were no significant rainfall events in Croisilles Harbour in the week prior to shipping and therefore no expected salinity changes that could be associated with increased stress (*Figure 25*).

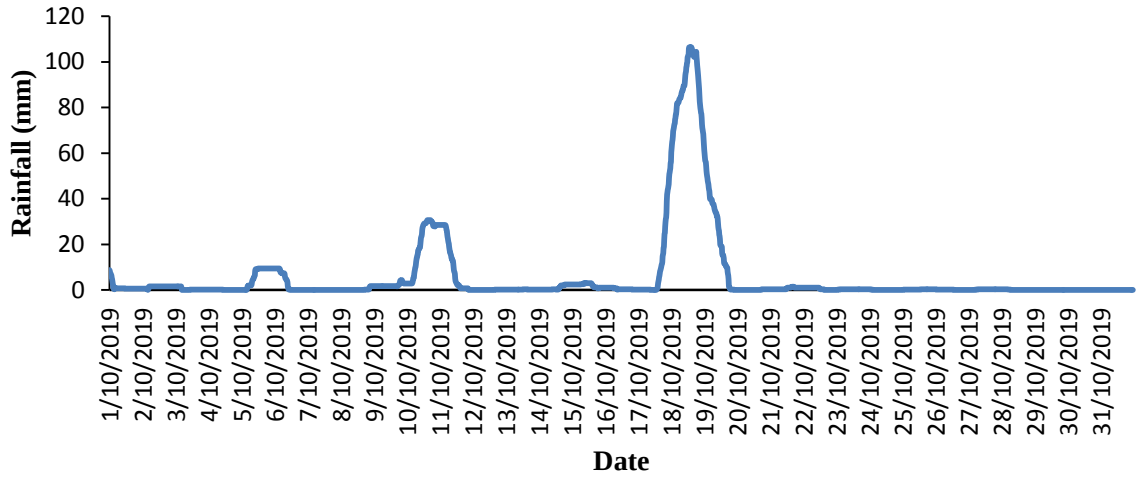

Figure 25: Rainfall data for the duration of the pre-transport phase of the experiment in Croisilles Harbour.

Water temperature in Croisilles Harbour during the experiment varied from 11.3°C to 17.0°C with a mean of 14.0 ± 0.02°C (mean ± SE, *Figure 26* un-shaded sections), and the air temperature during air exposure periods (*Figure 26* shaded sections) varied from 8.4°C to 24.0°C with a mean of 14.0 ± 0.05°C.

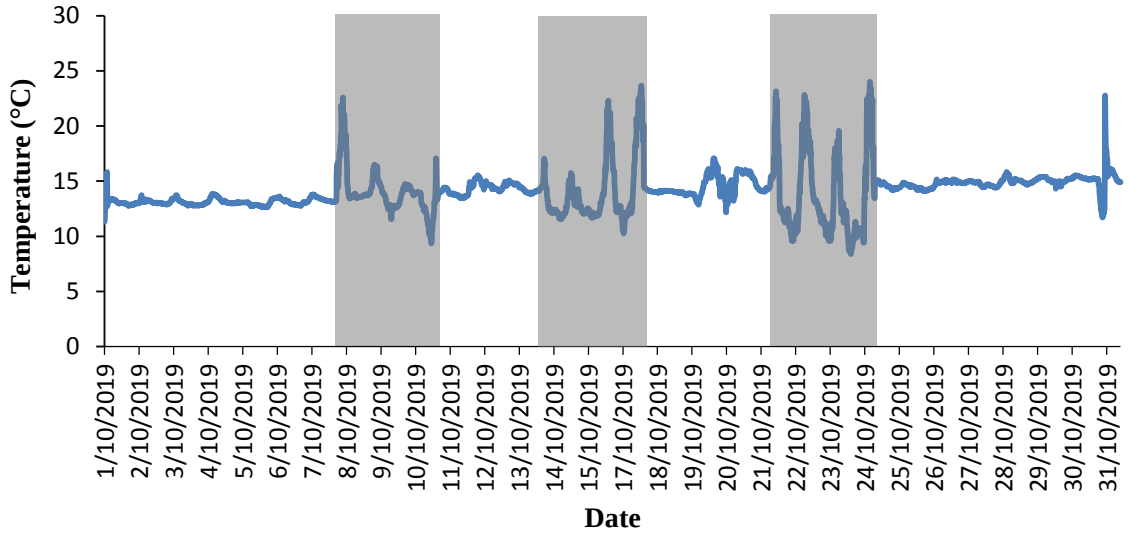

Figure 26: Water and air temperature data (monitored in one of the air exposure treatment bags) for the pre-transport phase of the experiment in Croisilles Harbour (shaded areas indicate periods of air exposure).

Water temperature during the whole of the annual oyster transport period between March and November 2019 varied between 10.1°C and 21.0°C (*Figure 27*).

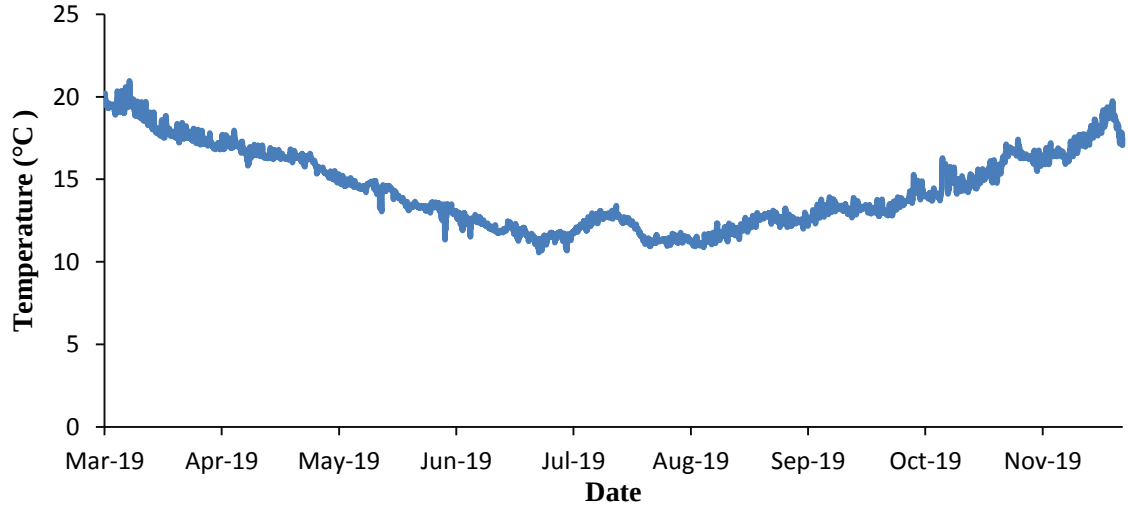

Figure 27: Water temperature on the Croisilles Harbour farm for the time period from March to November 2019.

4.4.2 Pre-transport air exposure: mortality and growth

There was a higher rate of mortalities (*Figure 28*) in the air exposure (2.33 ± 0.32%, mean ±SE, n = 5) compared to no air exposure (1.15 ± 0.3%, n = 5) treatment (T_8 = 2.67, p=0.028).

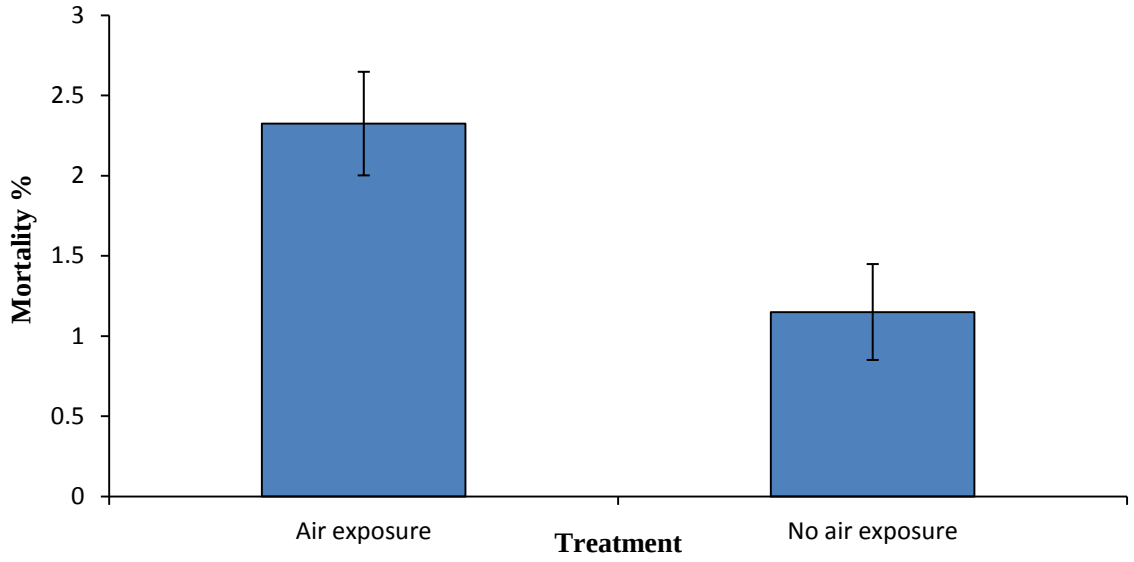

Figure 28: Percentage of oyster mortalities (mean ± SE) in the two pre-transport air exposure treatments in Croisilles Harbour, (n = 5).

Oysters in the air exposure treatment weighed less (17.11 ± 0.34g, mean ± SE, n = 60) than oysters in the no air exposure treatment (18.65 ± 0.40g, n = 60, *Figure 29*), (T_{118} = 2.93, p = 0.004).

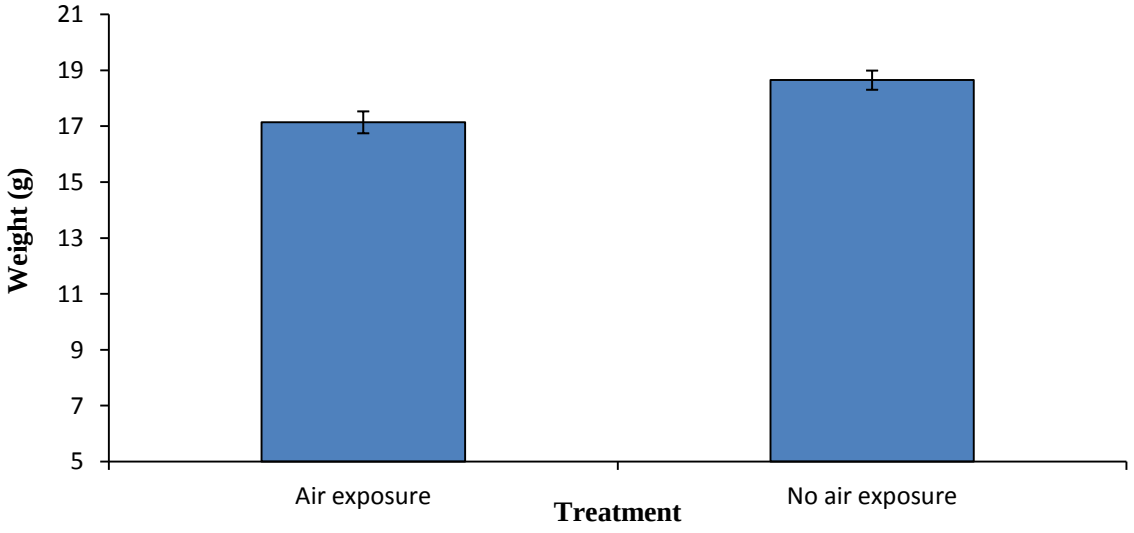

Figure 29: Whole individual oyster wet weights (mean ± SE) at the end of the air exposure phase of the experiment (n = 60).

4.4.3 Post-transport recovery: environmental parameters

The temperature during the 44-hour transport period in the refrigerated truck was 16.2 ± 0.07°C, mean ± SE (*Figure 30* shaded section) and mean humidity was 89.4 ± 0.3% RH (*Figure 31* shaded section). Following the transport, during 46 hours of storage the

temperature fluctuated between 11.2°C and 32.2°C, with a mean value of 20.8 ± 0.2°C (*Figure 30* un-shaded section). Humidity also fluctuated between 87.4% RH and 41.6% RH with a mean value of 72.2 ± 0.4% RH (*Figure 31* un-shaded section).

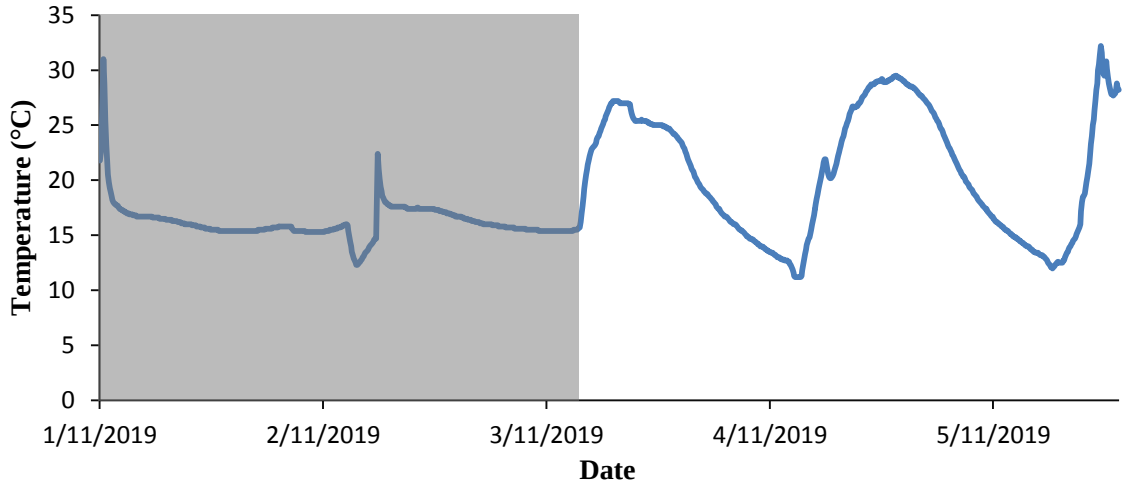

Figure 30: Temperature for the duration of the transport and 46-hour storage in Whangaroa Harbour (shaded areas indicate the transport period, un-shaded areas indicate storage time up to the placement of the last delay treatment oysters in the water).

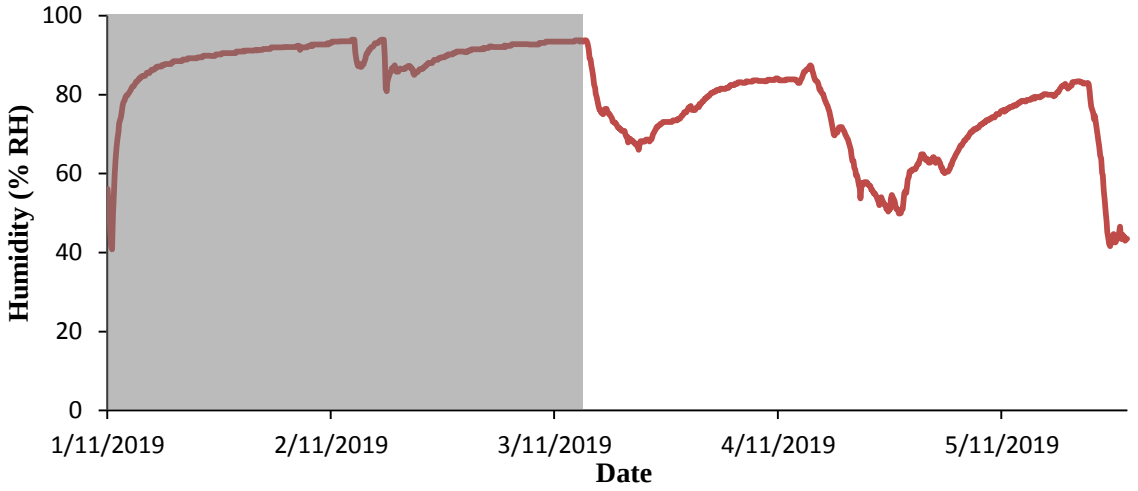

Figure 31: Relative humidity for the duration of the transport and 46-hour storage in Whangaroa Harbour (shaded areas indicate the transport period, un-shaded areas indicate storage time up to the placement of the last delay treatment oysters in the water).

The mean salinity in Whangaroa Harbour for the duration of the experiment was 33.8 ± 0.04 parts per thousand (ppt) (*Figure 32*). There was no reduction in salinity below 30.7ppt over that time period.

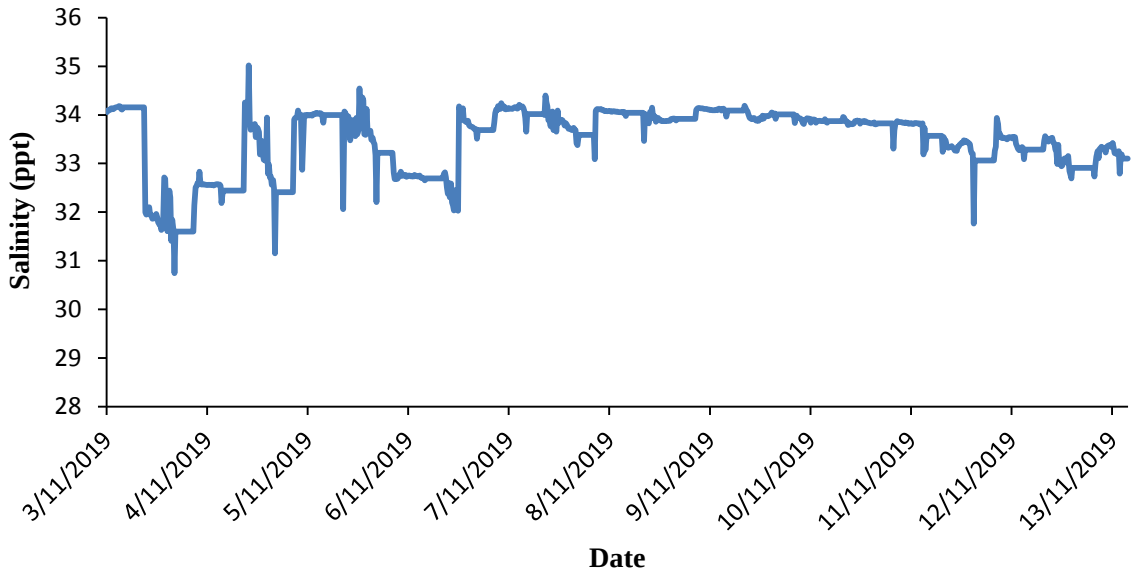

Figure 32: Salinity levels in Whangaroa Harbour for the duration of the experiment.

The mean water temperature in Whangaroa Harbour for the duration of the experiment was 19.2 ± 0.07°C (*Figure 33*), with a minimum of 8.98°C and a maximum of 26.94°C.

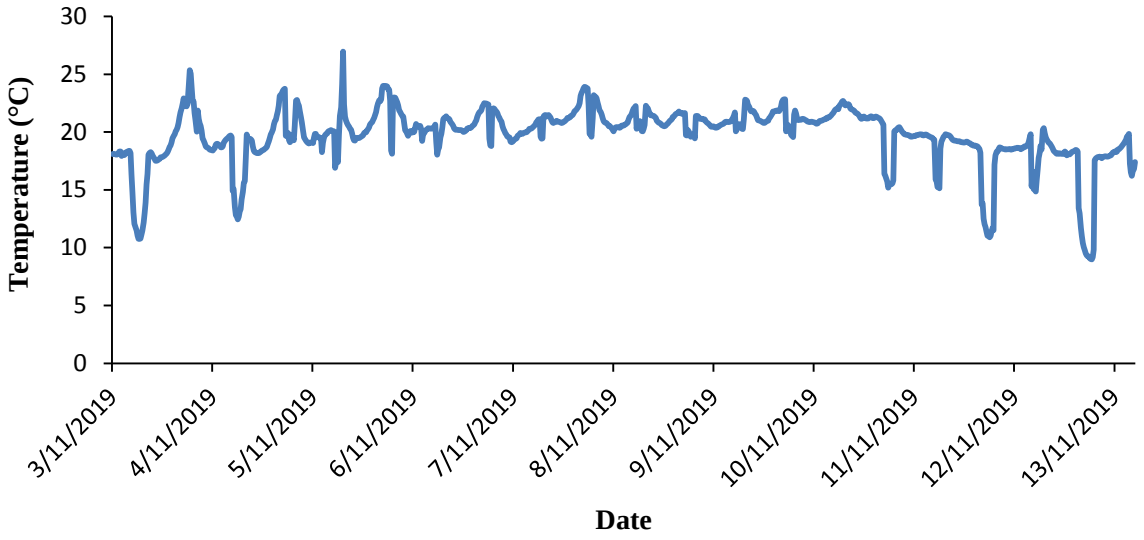

Figure 33: Water temperature in Whangaroa Harbour for the duration of the experiment as measured by a continuously submerged sensor on a buoy.

4.4.4 Post-transport recovery: weight loss, refractive index, mortality and growth

At the end of the experiment the weight loss was lowest (4.44 ± 0.47%, mean ± SE, n = 40) in the 10 hour delay post-transport treatment, followed by the 24 hour (7.63 ± 0.88%, n = 40) and 48 hour (13.41 ± 1.15%, n = 40) treatments ($F_{2, 114}$ = 32.17, p < 0.0001, *Figure 34, Table 6*). There was no significant effect of air exposure ($F_{1, 114)}$ = 1.17, p = 0.281).

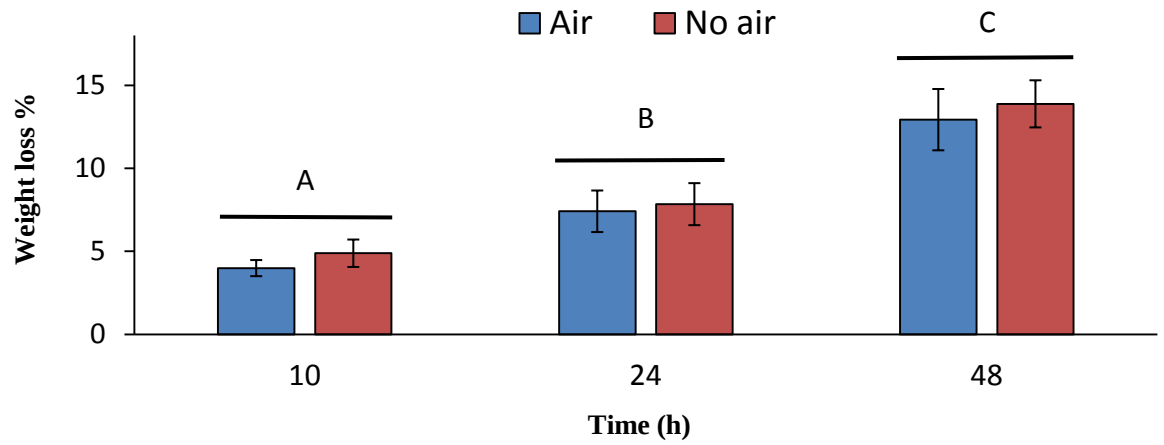

Figure 34: Weight loss percentage in oysters 10, 24 and 48 hours after arrival in Whangaroa Harbour (n=20). Letters indicate significant effects of the time delay. 'Air' = animals that experienced air exposure on the farm prior to transport. 'No Air' = animals that did not experience air exposure on the farm prior to transport.

Pre-transport air exposure ($F_{1, 183}$ = 13.18, p = 0.0004) and the post-transport delay treatments ($F_{1, 183}$ = 68.67, p < 0.0001) both had significant effects on the haemolymph density (as measured by the refractive index). There was also a significant interaction between air exposure and recovery treatments ($F_{4, 183}$ = 5.05, p = 0.0007). Haemolymph density increased faster over time in the pre-transport air exposure treatment, and was significantly higher (1.035 ± 0.0001g/ml, mean ± SE, n = 18, *Figure 35*) in the 48-hour delay treatment than in the no air pre-transport treatment (1.032 ± 0.0001g/ml, n = 18).

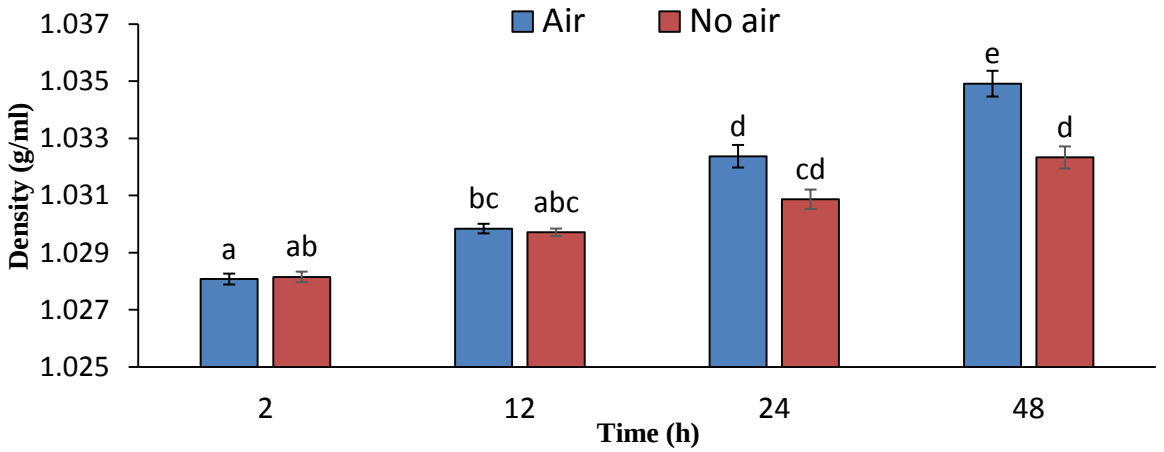

Figure 35: Density of oyster haemolymph (as determined by refractive index) in the two pre-transport (air exposure) and four post-transport time delay treatments (indicated by the time axis), mean ± SE, n=18-20. Letters indicate differences between groups following a significant interactive effect of pre-transport air exposure and post-transport recovery.

Oyster mortality increased as the time out of water increased. At the end of the experiment the mortality percentage was lowest (0.82 ± 0.16%, mean ± SE, n = 30, *Figure 36*) in the 2-hour delay post-transport treatment, followed by the 8-hour (2.05 ± 0.31%, n = 20) and 22-hour (1.95 ± 0.30%, n = 20) treatments which did not differ from each other, followed by the 48-hour treatment (7.62 ± 0.70%, n = 20) which had the highest mortality percentage ($F_{3, 82}$ = 50.18, p < 0.0001). There was no significant effect of air exposure ($F_{1, 82}$ = 0.04, p = 0.8484).

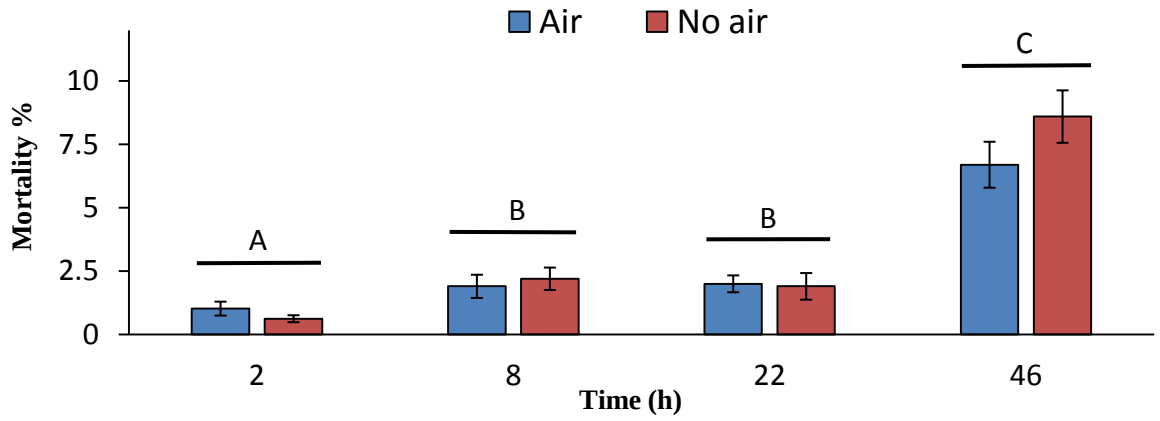

Figure 36: Mortality percentage (mean ± SE) in the two pre-transport (air exposure) and four post-transport time delay treatments (indicated by the time axis) , n=10-15. Letters indicate significant effects of the time delay.

Table 6: Results of the two-factor ANOVA. Text in bold represents significant differences (alpha = 0.01).

	d.f.	MS	F	p	d.f.	MS	F	p
			Weight loss*				Refractive index*	
Air exposure (A)	1	0.0009	1.17	0.2810	**1**	**0.0000**	**13.18**	**0.0004**
Time (T)	**2**	**0.0239**	**32.17**	**0.0000**	**4**	**0.0000**	**68.67**	**0.0000**
A × T	2	0.0000	0.06	0.9414	**4**	**0.0000**	**5.05**	**0.0007**
Residual	114	0.0007			183	0.0000		
			Mortality*					
Air exposure (A)	1	0.0001	0.04	0.8484				
Time (T)	**3**	**0.1673**	**50.18**	**0.0000**				
A × T	3	0.0039	1.17	0.3276				
Residual	82	0.0033						

*Log(x+1) transformed data, alpha = 0.01

Total weight gain was higher in the 2-hour delay post-transport treatment at 2.616kg for the air exposure and 2.89kg for no air exposure treatments, and lower in the 8, 22 and 46-hour

post-transport delay treatments with the weights of 1.468 and 0.774, 1.5 and 1.559, 1.166 and 1.171kg respectively (*Figure 37*).

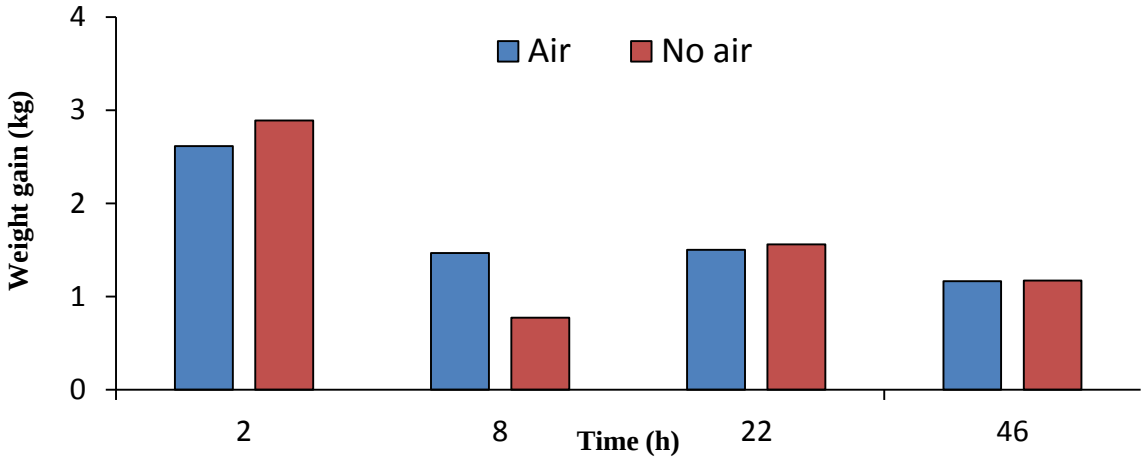

Figure 37: Total combined weight gain in the two pre-transport (air exposure) and four post-transport time delay treatments (indicated by the time axis) for oysters in each treatment, n=1450-1500.

4.5 Discussion

Air exposure treatment in this experiment doubled the rate of mortality (from 1.15% to 2.33%) and reduced the growth of oysters prior to transport. The reduced growth rate in response to air exposure is in agreement with other studies on culture of Pacific oysters at different tidal heights, although increased mortality is not (Spencer *et al*., 1978; Drinkwater & Howell, 1985; Spencer, 1990). However, the experimental design may have affected the growth results in the same way as in Chapter 3 (section 3.2.2.1). Oysters were not individually weighed before air exposure treatment and instead all oysters were assumed to be of approximately the same weight after mechanical grading at the start of the experiment. The increased mortality rate may indicate that the air exposure schedule chosen for this trial imposed a significant stress on the animals, likely due to long periods of continuous exposure of up to 92 hours at a time.

Increased stress due to air exposure was also evident from the results of the haemolymph refractive index assessment. Air exposure resulted in increased haemolymph density levels in oysters (indicating higher levels of stress based on the results in section 2.4.3.4) during the post-transport delays. Oysters from the air exposure treatment showed a trend of increasingly higher stress levels after 12 and 24 hours compared to those that remained submerged, although difference in stress levels only became significant after the longest delay period of 48 hours. This indicates that even if animals are already stressed prior to the transport

process, there is a window of opportunity to avoid more severe effects of it by returning oysters to the water as soon as possible and within the first 24 hours after arrival at the destination sites. Faster return to water was also beneficial for oysters not subjected to air exposure, as haemolymph density progressively increased over time indicating detrimental effect of extended air storage on all animals.

Air exposure treatment had no effect on the weight loss in oysters during the post-transport period. With reports of it helping to strengthen the adductor muscles and thicken the shell edges in oysters thus improving their survival in air (Quayle, 1988; O'Meley, 1995), it was assumed that this treatment may help oysters stay closed more efficiently and for a longer time period while in transport and avoid the harmful effects of desiccation (Wang & Amiro, 1977). However, this was not evident from the weight loss assessment. This was likely either due to the specific air exposure schedule used in this experiment not having the expected conditioning effect on adductor muscles and shell edges due to factors such as excessively long continuous exposure periods, or because gaping behaviour is influenced by more than just adductor muscle endurance. Weight loss in all experimental oysters increased with storage time. This is yet another indication that oyster condition is negatively affected by extended air storage, with a loss of 13.41 ± 1.15% body weight in the space of 48 hours.

The post-transport mortality rate on the Whangaroa Harbour farm was not affected by the air exposure pre-treatment. It was however affected by the by the length of the time delay treatments. The mortality rate increased as the length of the delays increased, with the 46-hour delay resulting in an eight-fold mortality rate increase compared to the 2-hour delay. This is consistent with the results of the weight loss and haemolymph refractive index assessments, both of which point to the negative effects of the longer time delays even on a short time scale of 48 hours used in this experiment.

The shortest (2-hour) time delay before placing oysters in the water resulted in the fastest growth over 8 days on the Whangaroa Harbour farm. However, this may have been due to the fact that 2-hour delay treatment oysters were placed into a sub-tidal farming system rather than an intertidal one. Sub-tidal systems which keep the oysters continuously submerged may allow for longer feeding time and faster growth rate (Spencer *et al*., 1978; Sumner, 1981). Therefore, weight gain in the two farming systems is not directly comparable. There was no trend seen in post-transport growth among the 8, 22 and 46 hour delay treatment animals that were all placed in the same, intertidal farming system. This meant it was difficult to

determine if the time delay between arrival and placement in water affected the post-transport growth rate. Also, since there was no replication within treatments and only the total weight of all oysters per treatment was measured, factors such as variability within the treatments could not be determined.

The temperature and humidity data collected over the duration of the transport process as well as the post-transport delay period in Whangaroa Harbour indicated that the temperature conditions in the truck (16.2 ± 0.07°C) were not ideal when considering the results of the transport temperature and humidity experiment in Chapter 2 (section 2.4.3). Those results showed that lower air storage temperatures (6°C as compared to 12°C) were more beneficial. It is unclear whether this unusually high temperature was the result of the refrigeration unit in the truck not functioning as intended during this particular shipment, or being set to a higher temperature than the usual 12°C. The humidity in the truck was high (89.4 ± 0.3% RH) and therefore favourable. The air storage temperature during the time delay treatments was high at 20.8 ± 0.2°C, which may have put oysters under additional stress and contributed to the increase in mortality seen as the time delays got longer. The high temperature also likely contributed to a much faster weigh loss during storage in this experiment (13.41 ± 1.15% body weight in the space of 48 hours) as compared to weight loss seen in section 2.4.3.5 of between 1.48 ± 0.44% to 10.79 ± 0.78% in the space of 90 hours when oysters were stored at 6°C to 12°C respectively. These results indicate that the temperatures involved in the oyster transport process were too high and likely had a negative impact on oyster health.

The rainfall and salinity level monitoring indicated that there were no low salinity events either in the week before transport from Croisilles Harbour to Whangaroa Harbour, or on the Whangaroa Harbour site itself. Therefore, it was unlikely that the results obtained in this experiment were affected by low salinity stress.

The higher mean water temperature in Whangaroa Harbour (19.2 ± 0.07°C) compared with Croisilles Harbour (14.0 ± 0.02°C) may influence oyster performance after transport, as the animals acclimatise to a new environment. However, water temperature data for Whangaroa Harbour must be interpreted with caution as the data logger may have been exposed to air due to the action of the tides, as evidenced by large variations in temperature. Water temperatures in Croisilles Harbour between March and November 2019 varied between 10.1°C and 21.0°C, indicating that results of the experiments into oyster husbandry may vary according

to the time of year during which they were conducted as temperature has a significant effect on oyster performance (Gagnaire *et al*., 2006).

In summary, this experiment indicated that the pre-transport air exposure schedule used was detrimental to oysters and is not recommended as a pre-transport conditioning treatment. Different schedules (particularly ones involving shorter continuous periods of exposure that occur more often), may be more favourable as they would better approximate the natural action of the tides that oysters encounter in the wild.

Minimising delays as much as possible when returning oysters to the water after arrival is recommended, as this was shown to reduce weight loss, stress and mortality levels. This is particularly important if no refrigerated facilities for oyster storage are available on the destination farms (as was the case on the Whangaroa Harbour site). Both shorter delay times and refrigerated storage would be optimal for oyster survival. If it is not possible to achieve both, either factor on its own would also be beneficial.

5.0 Stress determination using haemolymph refractive index

5.1 Introduction

Storage conditions and handling associated with transport caused variations in the haemolymph refractive index of oysters in the previous experiments (Chapters 2 and 4). It was decided to test whether this biomarker can also be used to detect stress imposed on oysters by the type of mechanical grading used by Moana NZ (vibration-based sorting on perforated screens), as well as the length of time the animals would need to recover from that stress. Mechanical grading has been determined to be a stressor for Pacific oysters in studies by O'Meley (1995) and Spencer *et al.* (1992). It was therefore hypothesized that grading will alter (increase) the haemolymph refractive index of graded oysters compared to un-graded ones.

5.2 Materials and methods

Grading stress and subsequent recovery over a 7-day period was evaluated. Grading was simulated using a vibrating platform (Action Vibration Trainer, model S001942V) to which containers housing the oysters were attached (*Figure 38*).

The experimental set-up approximated a commercial grading process of passing oysters along a vibrating mesh platform submerged in a shallow layer of water, where the grade of the oysters separated out is determined by the mesh size. The simulated grading period was chosen to be 10 minutes long, done to approximate the length of time of the commercial grading process.

Figure 38: Equipment used to simulate grading (the vibrating platform and containers housing the oysters).

Two 500ml containers were attached to the platform and each container was filled with 100ml of filtered seawater at 21°C. As well as approximating a commercial grading method, this eliminated the possibility of water loss from the oysters due to shaking and the possible change in haemolymph refractive index through desiccation rather than through haemolymph chemistry change.

Oysters 57.5 ± 0.7mm long and 24.54 ± 0.54g in weight (mean ± SE, n = 42) were collected from Moana NZ's nursery on the 13th of December 2019. The oysters used were considered to be in an un-stressed state due to absence of prior handling. A total of 42 oysters were used in this experiment, 21 were graded and 21 remained un-graded (un-stressed). Grading consisted of two oysters being placed in each of the containers attached to the vibrating platform, for a total of four animals undergoing the simulated grading procedure at a time. The platform induced a reciprocating vertical lift of 2-3cm at a point where the containers were attached. However, the frequency of the vibration was not able to be determined as the device displayed the vibration "speed" in arbitrary units on a scale of 1 to 100. The device was set to its highest setting of 100 for this experiment.

Immediately after the simulated grading, a 0.1ml sample of haemolymph was taken from the adductor muscle of each oyster and approximately 50µl of it was used for refractive index determination using the temperature-compensated salinity refractometer used in the previous trial (section 4.2.2.4, *Figure 24*). A sample was taken from each of the 42 oysters (both graded and un-graded). Haemolymph sampling was done in a way that minimised damage to oyster shell and tissues, using a small plastic wedge to hold the shell halves apart (gap of 4-5mm) while a sample was taken. After sampling the animals were individually labelled and placed in a bucket of filtered seawater before being transferred back into the Moana NZ's nursery facility for recovery and observation. The nursery was kept at the conditions used for commercial production - water temperature of approximately 19°C with algae feed provided. Water temperature in the nursery tank containing the experimental oysters was recorded daily.

Recovery of the oysters was monitored for the duration of 7 days after simulated grading. It consisted of taking a second haemolymph sample from each oyster, with 6 oysters tested each day (3 graded and 3 un-graded individuals). As the oysters were individually labelled, this allowed for a daily change in haemolymph refractive index in 3 oysters at a time to be observed over the period of 7 days (*Figure 39*).

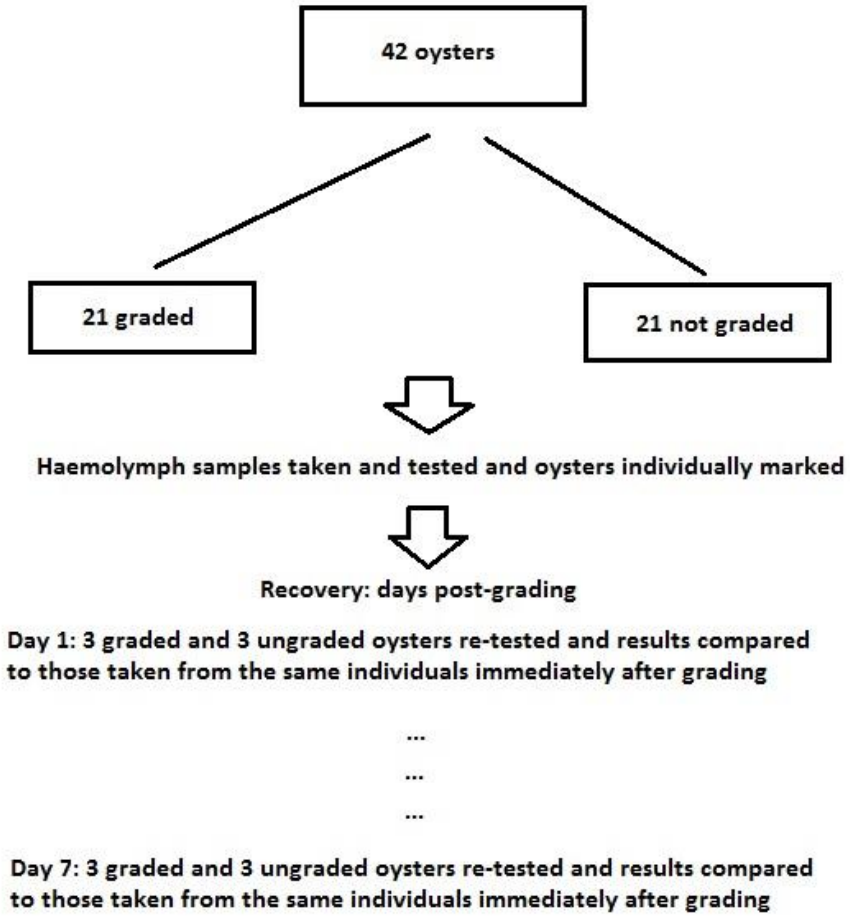

Figure 39: Summary of experimental procedures and samples taken during the oyster grading recovery trial.

Sampling individual oysters twice allowed for the effect of haemolymph sampling itself (if any) on the stress levels of un-graded oysters to be observed. Experimental oysters were also monitored for mortality for 1 month following the conclusion of this experiment.

5.3 Statistical analyses

A two-sample T-test was used to analyse refractive index data after the grading treatment. The effects of grading (2 treatments: graded and un-graded) and recovery (7 treatments: days 1 through to 7) on haemolymph refractive index was assessed with ANOVA. A 2-factor analysis was used to assess the crossed effects of grading and recovery. Data were assessed for normality and heterogeneity with Shapiro Wilk and Cochran's C tests, respectively. Significant effects were examined using Tukey's post-hoc tests to determine differences among groups within a factor. Analyses were carried out in STASTISTICA 12 (Statsoft Ltd.).

5.4 Results

There was a difference in haemolymph density (determined by the refractive index) between oysters subjected to simulated grading and those that were not (T_{40} = 2.20, p = 0.034). The oysters subjected to simulated grading had a higher haemolymph density (1.0326 ± 0.0003g/ml, n = 21, mean ± SE, *Figure 40*) than un-graded ones (1.0317 ± 0.0003g/ml).

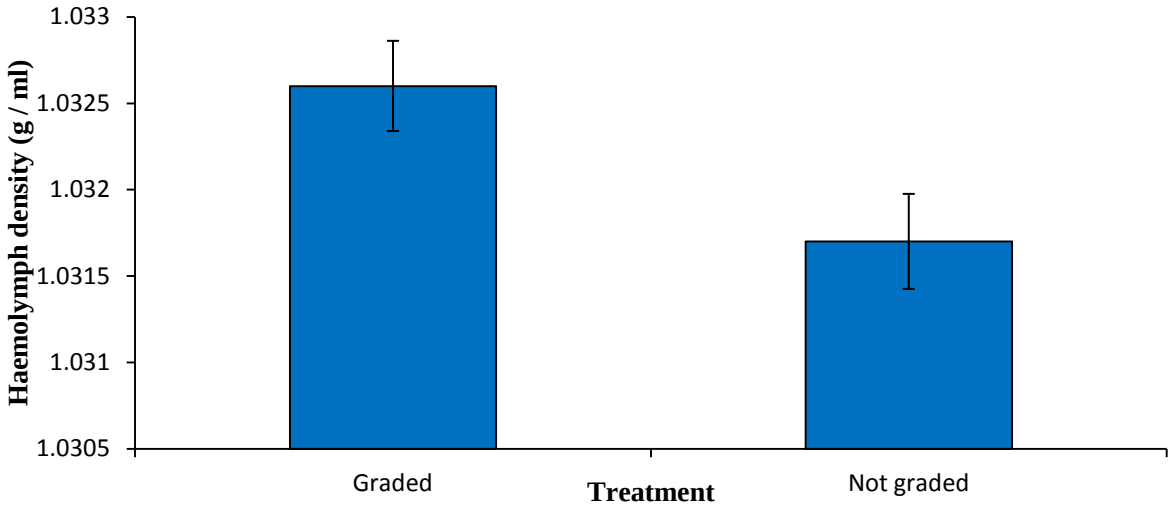

Figure 40: Density of oyster haemolymph (mean ± SE) in graded and un-graded animals immediately after grading (n = 21).

Water temperature during recovery in the nursery tank gradually decreased from 20.6°C on day 1 to 17.8°C on day 6, increasing again to 18.6°C on day 7 (*Figure 41).*

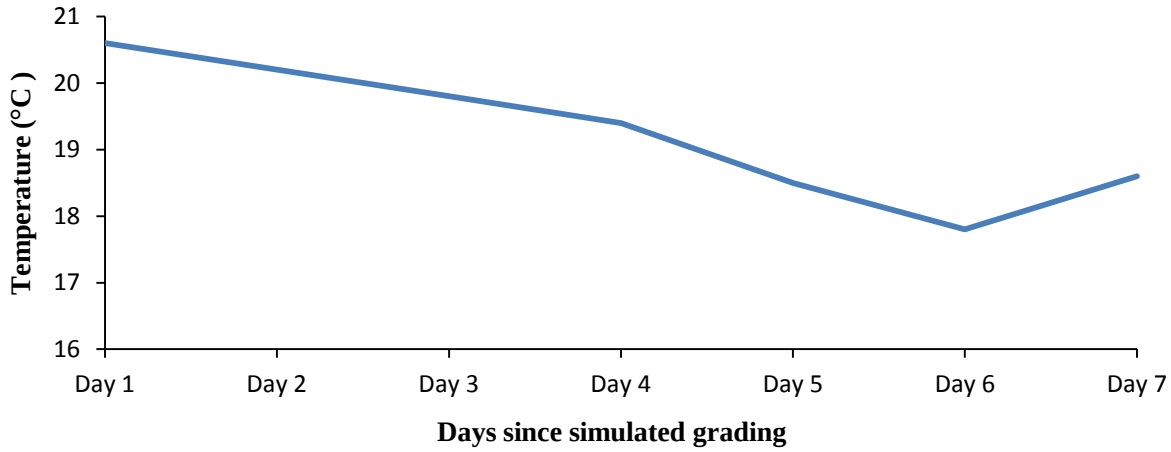

Figure 41: Water temperature in the nursery tank for the duration of the experiment.

As the recovery time progressed, final haemolymph density readings decreased as compared to the initial ones after the third day in both graded and un-graded treatments (except on day 6), from a change of 0.00175 ± 0.0004g/ml (mean ± SE) on the third day, to -0.0011 ± 0.0003g/ml on the fourth day (*Figure 42*). Only the recovery time had an effect on haemolymph density change ($F_{6, 28}$ = 5.57, p = 0.0007, *Table 7*). There was no significant effect due to grading ($F_{1, 28}$ = 3.24, 0.0826).

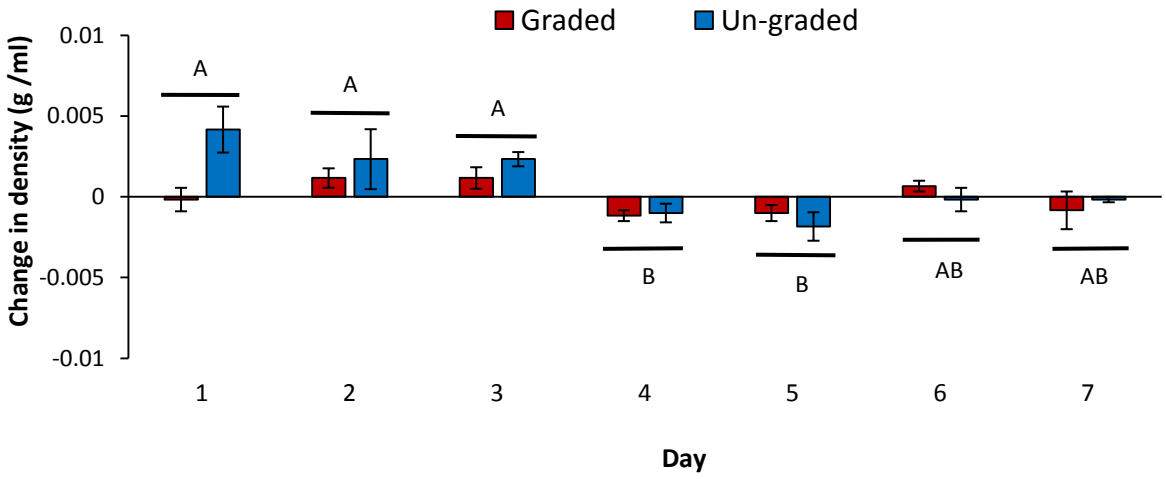

Figure 42: Change in haemolymph density (mean ± SE) between initial sampling on the day of grading and a subsequent delayed sampling (indicated by the day axis), n=3. Letters indicate significant effects of the time delay.

Table 7: Results of the two-factor ANOVA. Text in bold represents significant differences (alpha = 0.05).

	d.f.	MS	F	p
Grading (G)	1	0.00001	3.24	0.0826
Recovery (R)	**6**	**0.00001**	**5.57**	**0.0007**
G × R	6	0.00000	2.06	0.0912
Residual	28	0.00000		

Oysters were observed for 1 month following the trial and no mortalities occurred during that time. However, there were some shell abnormalities seen in both graded and un-graded oysters, with around half of all animals developing a "double shell" after the experiment.

5.5 Discussion

The results of this experiment indicate that the stress imposed on the animals after a 10-minute simulated grading in water can be detected by using a salinity refractometer. It was

also demonstrated that this test can be used to determine the recovery time of oysters to a pre-stressed state after they have been subjected to a stressful event, in this case - 3 days. However, there were no significant differences between haemolymph densities of graded and un-graded oysters during the 7-day recovery period. This may be due to the small sample size (n = 3) taken per grading treatment per day as well as a large variability in readings within treatments.

An unexpected increase in haemolymph density readings was seen in both treatments on day 6. This coincided with a drop in temperature in the nursery tank, although it is difficult to say if the change in temperature was the cause of the increase in readings.

The observed recovery time of 3 days is consistent with results obtained by Zhang *et al*. (2006) in an experiment where Pacific oysters were exposed elevated to air temperatures. In that study, lysosomal membrane stability of the oysters was analysed for a period of time after the initial exposure to a stressful environment and it was determined that the recovery period ranged from 24 to 72 hours depending on the temperature used (with progressively higher temperatures resulting in higher stress levels and longer recovery).

Haemolymph analyses in Chapters 2 and 4 could not rule out haemolymph density changes due to desiccation. The results of this experiment confirmed that haemolymph density varies through a change in blood chemistry rather than just desiccation, as grading was carried out in water to prevent this from happening.

There was an indication that the haemolymph sampling process itself induced stress in the animals as seen from the increase in haemolymph density during the follow-up sampling of oysters that have not been subjected to simulated grading. The density readings during the recovery process of un-graded oysters remained higher than the initial sampling ones for the first 3 days, suggesting that although the animals did not go through grading, the stress of haemolymph sampling itself was significant. Therefore, if this method of stress testing is to be used, taking a haemolymph sample from each animal only once would eliminate the possibility of the results being affected by the sampling procedure.

If refractometry is to be used to assess stress commercially, baseline levels of haemolymph density would need to be determined above which the animals would be considered to be experiencing stress. These baseline readings could vary for different populations of oysters and among environments (Fisher and Newell, 1986). If the variation between populations and

environmental conditions is found to be too great, a procedure involving maintenance of "stress-free" stock at each site/farm location to be exclusively used for comparison during testing could be implemented. Water salinity in the environment the oysters came from would also need to be taken into account when testing haemolymph refractive index since oysters are osmoconformers (Bayne, 2017) and different environmental salinities will have an impact on the readings.

There were no mortalities observed during the 1-month monitoring period following this experiment, demonstrating that this method of stress testing allows the animals to stay alive, reducing waste and costs if a large number of animals are to be tested in a commercial setting. However, care would need to be taken during testing as the small amount of shell damage sustained by the oysters during the procedure was seen to induce shell growth abnormalities (double shells).

In order to better understand the process by which the haemolymph density increases due to stress, further testing may need to be done combining the refractive index and colorimetric analyses of haemolymph for the levels of protein and possibly other solutes.

To summarise, further work is needed to determine the mechanism behind the increase in haemolymph density, establish a baseline "un-stressed" level of density (if one exists) and compare the responses of different populations. This method could develop into a viable way of measuring stress in oysters during commercial production.

6.0 General discussion and conclusions

The aims of this study were to determine which husbandry and handling techniques before, during and after transport led to the best post-transport performance and survival of Pacific oysters, and to find suitable biomarkers for assessing physiological stress levels in the field. The results of this research can be used by the oyster industry to improve the husbandry practices around live transport.

The results of the experiment into different transport environments indicated that a temperature of 6°C (compared to the currently used temperature of 12°C) and high humidity conditions of 90% RH formed the best combination in terms of causing the least amount of stress to the oysters. As temperature increased and humidity decreased, oysters were seen to experience greater levels of stress. While both cooler and more humid conditions are preferable, if it is not possible to control both of those parameters, keeping either the temperature low or the humidity high will still be beneficial to oysters. In Chapter 4 (section 4.4.3) it was observed that the humidity in the truck used to transport the oysters from Croisilles to Whangaroa Harbour was high (89.4% RH). However, the humidity was only measured during one transport event, and either further measurements to confirm consistently high humidity in transport or steps to control it may need to be taken.

As a pre-transport conditioning treatment, air exposure in this study was determined to either have no effect or a negative effect on the oysters. The results from Chapter 3 indicated that air exposure had no effect on the growth or condition index of oysters. The results from Chapter 4 showed that air exposure was detrimental in terms of increased mortality and slower growth on the Croisilles Harbour site and also had a detrimental effect on the oysters once they arrived in Whangaroa Harbour with higher stress levels observed. The combined results from Chapters 3 and 4 indicate that the air exposure schedules chosen in this study did not result in better oyster performance leading up to and after transport. Given that multiple studies which suggest air exposure treatments for the purpose of "hardening" or conditioning oysters (Fujiya, 1970; Graham, 1991; Handley, 1997; Samain & McCombie, 2008; Kang *et al*, 2010; Samain, 2011; Mondol *et al*., 2016) refer to placing oysters in the intertidal zone to obtain this exposure, it may be that the long periods (up to 92 hours at a time) of continuous air exposure in this experiment were the cause behind the lack of beneficial effects. The 3-week period that air exposure treatments were applied over may also have been too short, as the treatment time frames used in the studies that recommend air exposure are between 2 and

10 months (Fujiya, 1970; Graham, 1991; Handley, 1997; Samain & McCombie, 2008; Kang *et al*., 2010; Samain, 2011; Mondol *et al*., 2016).

There was a difference between results obtained in Chapter 3, where there was no effect on the growth (weight gain) of oysters due to air exposure in Croisilles Harbour, and Chapter 4, where oysters grew slower as a result of air exposure. This may be explained by differences between the air exposure regimes used in the experiments. Oysters were subjected to longer continuous periods of air exposure (92 hours out of the water) in Chapter 4, compared to a maximum of 68 hours out of the water in Chapter 3. This further supports the idea that long continuous periods of air exposure may be responsible for the lack of beneficial effects from air exposure in these experiments. Higher air and water temperatures in Croisilles Harbour during the experiment in Chapter 4 may also have contributed to the differences in growth. Overall, it was found that air exposure was not a beneficial pre-transport treatment and it is not recommended that industry adopts this process based on these results. Further experiments at different times of the year, since water temperature in Croisilles Harbour between March and November 2019 varied by 10.9°C and with different air exposure schedules are required to determine if there still is merit in short-term air exposure treatments prior to transport.

Oysters held at a high density (13 as compared to 6 litres of oysters in 37 litre mesh bags) had a greater total weight gain but lower condition index. Greater total weight gain could not be explained, but may be due to experimental design since oysters were graded but not weighed at the beginning of the experiment. Due to stress imposed by the transport process, oysters with higher condition indices and therefore higher energy reserves are likely to perform better during and after transport than those with lower condition indices, and commercial operators may benefit from keeping stocking densities low prior to transport.

The longer time delays in returning oysters to water after arrival on the destination farms were found to be detrimental, increasing mortality rate from around 1% after 2 hours to 8% after 48 hours. This likely stemmed from the combination of the time spent emersed and the high temperature during emersed storage in Whangaroa Harbour. Longer delays resulted in greater stress and weight loss. Therefore, it would be advisable for commercial operators to minimise the delays and store oysters under refrigeration at all times while out of the water. If it is not possible to achieve both, either reduced delays or reduced temperature in storage would also be beneficial on their own.

The assessment of oyster mortality based on gaping during air storage was found to be highly unreliable due to the issue of the shells sticking together from congealed shell liquor. The mechanism leading to the shells sticking together could also affect gaping observations in other shellfish such as mussels. Therefore, this method should be avoided and alternative methods of mortality determination need to be investigated.

Of the six biomarkers assessed (haemocyte count, haemolymph pH, osmolality and refractive index, weight loss during emersion and algae clearance rate), haemolymph refractive index was the most reliable indicator of sub-lethal stress. It showed an increase in haemolymph density (indicated by the increase in refractive index) in response to stressors such as elevated temperature, air exposure and grading. Elevated haemolymph density readings were consistently observed in different groups of oysters as a result of exposure to stressful conditions or handling events in Chapters 2, 4 and 5. In addition, this method used a minimal amount of equipment, was easy to use and did not require the animals to be killed for testing. With further work to establish baseline "un-stressed" levels of haemolymph density and compare the response of different populations of oysters, this method could develop into a highly useful measure of stress in oysters during commercial production.

References

Aaraas, R., Hernar, I. J., Vorre, A., Bergslien, H., Lunestad, B. T., Skeie, S., Slinde. E., & Mortensen, S. (2004). Sensory, histological, and bacteriological changes in flat oysters, *Ostrea edulis L.*, during different storage conditions. Journal of Food Science, 69(6), 205-210.

Akberali, H. B., & Trueman, E. R. (1985). Effects of environmental stress on marine bivalve molluscs. Advances in Marine Biology, 22, 101-198.

Altieri, A. H. (2006). Inducible variation in hypoxia tolerance across the intertidal–subtidal distribution of the blue mussel *Mytilus edulis*. Marine Ecology Progress Series, 325, 295-300.

APEC Fisheries Working Group. (1999). APEC Air Shipment of Live and Fresh Seafood Guidelines: A Manual on Preparing, Packaging and Packing Live and Fresh Fish and Seafood Air Shipments Along with Customs and Inspection Guidelines for Six APEC Member Economies. Westhampton Beach, New York: First Coastal Corporation.

Askew, C. G. (1972). The growth of oysters *Ostrea edulis* and *Crassostrea gigas* in Emsworth Harbour. Aquaculture, 1, 237–259.

Auffret, M., Rousseau, S., Boutet, I., Tanguy, A., Baron, J., Moraga, D., & Duchemin, M. (2006). A multiparametric approach for monitoring immunotoxic responses in mussels from contaminated sites in the Western Mediterranean. Ecotoxicology and Environmental Safety, 63(3), 393-405.

Baldwin, J., Wells, R. M. G., Low, M., & Ryder, J. M. (1992). Tauropine and D-Lactate as Metabolic Stress Indicators during Transport and Storage of Live Paua (New Zealand Abalone) (*Haliotis iris*). Journal of food science, 57(2), 280-282.

Ballarin, L., Pampanin, D. M., & Marin, M. G. (2003). Mechanical disturbance affects haemocyte functionality in the Venus clam *Chamelea gallina*. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 136(3), 631-640.

Barillé, L., Prou, J., Héral, M., & Razet, D. (1997). Effects of high natural seston concentrations on the feeding, selection, and absorption of the oyster *Crassostrea gigas* (Thunberg). Journal of Experimental Marine Biology and Ecology, 212(2), 149–172.

Barrento, S., Lupatsch, I., Keay, I., & Shields, S. (2013). Protocol on Best Practice and handling Transportation of Live Mussels. Technical Report Supported by Project No. 243452, FP7-SME MusselAlive.

Bayne, B. L. (2017). Biology of oysters (Vol. 41). United Kingdom: Academic Press.

Bayne, B. L. Ahrens, M. Allen, S. K. D'Auriac, M. Anglès Backeljau, T. Beninger, P. Bohn, R. Boudry, P. Davis, J. Green, T. (2017). The proposed dropping of the genus *Crassostrea* for all Pacific cupped oysters and its replacement by a new genus *Magallana*: a dissenting view. Journal of Shellfish Research, 36, 545 - 547.

Bernard, F. R. (1974). Particle sorting and labial palp function in the Pacific oyster *Crassostrea gigas* (Thunberg, 1795). The Biological Bulletin, 146(1), 1-10.

Beu, A. G., Alloway, B. V., Pillans, B. J., Naish, T. R., & Westgate, J. A. (2004). Marine Mollusca of oxygen isotope stages of the last 2 million years in New Zealand. Part 1: Revised generic positions and recognition of warm-water and cool-water migrants. Journal of the Royal Society of New Zealand, 34(2), 111-265.

Bird, P. D., Glenda, A. J., Holliday, J. & Boronowsky, A. (1995). Effect of storage on the quality of purified live Pacific and Sydney rock oysters. Conference Internationale sur la Purification des Coquillages. Rennes (France), archimer.ifremer no. 1624, 315-322.

Bougrier, S., Hawkins, A. J. S., & Héral, M. (1997). Preingestive selection of different microalgal mixtures in *Crassostrea gigas* and *Mytilus edulis*, analysed by flow cytometry. Aquaculture, 150(1-2), 123–134.

Boyd, N. S., Wilson, N. D. C. & Hall, B. I. (1980). Storage of live Pacific oysters out of water. New Zealand Journal of Science, 23, 171-176.

Breese, W. P., & Malouf, R. E. (1975). Hatchery manual for the Pacific oyster. Oregon State University Sea Grant College Program. Publication no. ORESU-H-75-002, 22.

Brown, J. R. (1988). Multivariate analyses of the role of environmental factors in seasonal and site-related growth variation in the Pacific oyster Crassostrea *gigas*. Marine Ecology Progress Series, 45, 225-236.

Bubner, E. J., Harris, J. O., & Bolton, T. F. (2009). Supplementary oxygen and temperature management during live transportation of Greenlip abalone, *Haliotis laevigata* (Donovan, 1808). Aquaculture Research, 40(7), 810-817.

Buzin, F., Baudon, V., Cardinal, M., Barillé, L., & Haure, J. (2011). Cold storage of Pacific oysters out of water: biometry, intervalval water and sensory assessment. International Journal of Food Science & Technology, 46(9), 1775-1782.

Capelle, J. J., Hartog, E., Creemers, J., Heringa, J., & Kamermans, P. (2019). Effects of stocking density and immersion time on the performance of oysters in intertidal off-bottom culture. Aquaculture International, 28, 249-264.

Castinel A., Atalah J. (2015). Ostreid herpes virus in New Zealand: making sense of the environment. A report prepared for the Ministry of Business, Innovation and Employment. Cawthron Report no. 2570.

Castinel, A., Dhand, N., Fletcher, L., Rubio, A., Taylor, M., & Whittington, R. (2015). OSHV-1 mortalities in Pacific oysters in Australia and New Zealand: the farmer's story. A report prepared for the Ministry of Business, Innovation and Employment. Cawthron Institute, Report no. 2567.

Chandurvelan, R., Marsden, I. D., Gaw, S., & Glover, C. N. (2012). Impairment of green-lipped mussel (*Perna canaliculus*) physiology by waterborne cadmium: relationship to tissue bioaccumulation and effect of exposure duration. Aquatic Toxicology, 124, 114-124.

Chandurvelan, R., Marsden, I. D., Gaw, S., & Glover, C. N. (2013). Field-to-laboratory transport protocol impacts subsequent physiological biomarker response in the marine mussel, *Perna canaliculus*. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 164(1), 84-90.

Chang, E. S., Stentiford, G. D., Neil, D. M., & Chang, S. A. (2005). Crustacean hyperglycemic hormone and hemolymph metabolites: Stress responses in two lobster species. Aquaculture and Pathobiology of Crustacean and Other Species, 32, 77-85.

Chavez-Villalba, J., Arreola-Lizárraga, A., Burrola-Sánchez, S., & Hoyos-Chairez, F. (2010). Growth, condition, and survival of the Pacific oyster *Crassostrea gigas* cultivated within and outside a subtropical lagoon. Aquaculture, 300(1-4), 128-136.

Clements, J. C., Davidson, J. D., McQuillan, J. G., & Comeau, L. A. (2018). Increased mortality of harvested eastern oysters (*Crassostrea virginica*) is associated with air exposure and temperature during a spring fishery in Atlantic Canada. Fisheries research, 206, 27-34.

Craig, J. P., Simmons, P. A., Patel, S., & Tomlinson, A. (1995). Refractive index and osmolality of human tears. Optometry and vision science, 72(10), 718-724.

Crosby, M. P., Gale, L. D. (1990). A review and evaluation of bivalve condition index methodologies with a suggested standard method. Journal of Shellfish Research, 9, 233-237.

De Zwaan, A., Cortesi, P., Van den Thillart, G., Roos, J., & Storey, K. B. (1991). Differential sensitivities to hypoxia by two anoxia-tolerant marine molluscs: a biochemical analysis. Marine biology, 111(3), 343-351.

Dinamani, P. (1987). Gametogenic patterns in populations of Pacific oyster, *Crassostrea gigas*, in Northland, New Zealand. Aquaculture, 64(1), 65-76.

Drinkwater, J., & Howell, T. R. W. (1985). Experiments on the cultivation of oysters in Scotland. Scottish Fisheries Research report no. 35.

Fankboner, P. V., & De Burgh, M. E. (1978). Comparative rates of dissolved organic carbon accumulation by juveniles and pediveligers of the Japanese oyster *Crassostrea gigas* Thunberg. Aquaculture, 13(3), 205–212.

FAO (2020) Food and Agriculture Organization, Fisheries and Aquaculture Information and Statistical Service. http://www.fao.org/fishery/statistics/global-aquaculture-production/query/en. Retrieved 7th of January 2020.

FAO. (2018). The State of World Fisheries and Aquaculture 2018 - Meeting the sustainable development goals. Rome: FAO

Fisher, W. S., & Newell, R. I. (1986). Seasonal and environmental variation in protein and carbohydrate levels in the hemolymph from American oysters *(Crassostrea virginica* Gmelin). Comparative Biochemistry and Physiology Part A: Physiology, 85(2), 365-372.

Fisher, W. S., Oliver, L. M., Winstead, J. T., & Long, E. R. (2000). A survey of oysters *Crassostrea virginica* from Tampa Bay, Florida: associations of internal defense measurements with contaminant burdens. Aquatic Toxicology, 51(1), 115-138.

Fitridge, I., T. Dempster, J. Guenther, De Nys R. (2012). The impact and control of biofouling in marine aquaculture: a review. The Journal of Bioadhession and Biofilm Research, 28, 649-669.

Fodrie, F. J., Rodriguez, A. B., Gittman, R. K., Grabowski, J. H., Lindquist, N. L., Peterson, C. H., & Ridge, J. T. (2017). Oyster reefs as carbon sources and sinks. Proceedings of the Royal Society B: Biological Sciences, 284(1859), 20170891.

Forrest, B. M., Keeley, N. B., Hopkins, G. A., Webb, S. C., & Clement, D. M. (2009). Bivalve aquaculture in estuaries: review and synthesis of oyster cultivation effects. Aquaculture, 298(1), 1-15.

Fratini, G., Medina, I., Lupi, P., Messini, A., Pazos, M., & Parisi, G. (2013). Effect of a finishing period in sea on the shelf life of Pacific oysters (*C. gigas*) farmed in lagoon. Food research international, 51(1), 217-227.

Fujiya, M. (1970). Oyster farming in Japan. Helgoländer Wissenschaftliche Meeresuntersuchungen, 20(1), 464-479.

Gagnaire, B., Frouin, H., Moreau, K., Thomas-Guyon, H., & Renault, T. (2006). Effects of temperature and salinity on haemocyte activities of the Pacific oyster, *Crassostrea gigas* (Thunberg). Fish & Shellfish Immunology, 20(4), 536-547.

Gagne, F., Blaise, C., Pellerin, J., Fournier, M., Durand, M. J., & Talbot, A. (2008). Relationships between intertidal clam population and health status of the soft-shell clam *Mya arenaria* in the St. Lawrence Estuary and Saguenay Fjord (Québec, Canada). Environment international, 34(1), 30-43.

Galstoff, G. F. (1964). The American Oyster. US Department of the Interior, Fish and Wildlife Service. Fishery Bulletin, 64.

George, J. W. (2001). The usefulness and limitations of hand-held refractometers in veterinary laboratory medicine: an historical and technical review. Veterinary Clinical Pathology, 30(4), 201-210.

Graham, M., 1991. A Situation Analysis of the Australian Oyster Industry with Particular Reference to the Tasmanian Pacific Oyster. A report prepared for the Tasmanian Aquaculture, Co-operative Society and The Business School, University of Tasmania, Hobart, Australia.

Guo, X., DeBrosse, G. A., & Allen, S. K. (1996). All-triploid Pacific oysters (*Crassostrea gigas* Thunberg) produced by mating tetraploids and diploids. Aquaculture, 142(3-4), 149–161.

Guo, X., Hedgecock, D., Hershberger, W. K., Cooper, K., & Jr, S. K. A. (1998). Genetic determinants of protandric sex in the Pacific oyster, *Crassostrea gigas* Thunberg. Evolution, 52(2), 394-402.

Handley, S. J. (1997). Optimizing subtidal oyster production, Marlborough Sounds, New Zealand: *Spionid polychaete* infestations, water depth, and spat stunting. Journal of Shellfish Research, 16(1), 143-150.

Handley, S., & Jeffs, A. (2003). Assessment of future expansion of Pacific oyster (*Crassostrea gigas*) farming in Northland. NIWA Client Report: AL2003-027, Project: ENT03101. National Institute of Water and Atmospheric Research, New Zealand

Harry, H. W. (1985). Synopsis of the supraspecific classification of living oysters (Bivalvia: *Gryphaeidae* and *Ostreidae*). Veliger, 28, 121-158.

Hellou, J., & Law, R. J. (2003). Stress on stress response of wild mussels, *Mytilus edulis* and *Mytilus trossulus*, as an indicator of ecosystem health. Environmental Pollution, 126(3), 407–416.

Héral, M., & Deslous-Paoli, J. M. (1991). Oyster culture in European countries. Estuarine and Marine Bivalve Mollusk Culture, 154-190.

Hidu, H., Chapman, S. R., & Mook, W. (1988). Overwintering American oyster seed by cold humid air storage. Shellfish Research, 7(1), 47-50.

Holliday, J. E. (1995). Nursery culture of Sydney rock oysters, *Saccostrea commercialis* (Iredale & Roughley, 1933) and Pacific oysters, *Crassostrea gigas* (Thunberg 1793). Unpublished PHD Thesis, Department of Aquaculture, University of Tasmania, Australia.

Holliday, J. E., Maguire, G. B., & Nell, J. A. (1991). Optimum stocking density for nursery culture of Sydney rock oysters (*Saccostrea commercialis*). Aquaculture, 96(1), 7-16.

Honkoop, P. J.C ., & Bayne, B.L . (2002). Stocking density and growth of the Pacific oyster (*Crassostrea gigas*) and the Sydney rock oyster (*Saccostrea glomerata*) in Port Stephens, Australia. Aquaculture, 213(1-4), 171–186.

Jimenez-Ruiz, E., Marquez Rios, E., Cardenas-Lopez, J., Montoya-Camacho, N., Castillo-Yanez, F., Duarte-Figueroa, M., Ruiz-Cruz, S., Balois-Morales, R. & Ocano-Higuera, V. (2015). Impact of two commercial in vivo transport methods on physiological condition of the Japanese oyster (*Crassostrea gigas*). Journal of Chemistry, 6, 145-161.

Jones, T. O., & Iwama, G. K. (1991). Polyculture of the Pacific oyster, *Crassostrea gigas* (Thunberg), with chinook salmon, *Oncorhynchus tshawytscha*. Aquaculture, 92, 313–322.

Kang, D. H., Chu, F. L. E., Yang, H. S., Lee, C. H., Koh, H. B., & Choi, K. S. (2010). Growth, reproductive condition, and digestive tubule atrophy of Pacific oyster *Crassostrea gigas* in Gamakman Bay off the southern coast of Korea. Journal of Shellfish Research, 29(4), 839-846.

Laing, I. (2009). Oysters–Shellfish Farming. Encyclopedia of Ocean Sciences 2nd ed., Oxford: Academic Press, 274-286.

Langdon, C., Evans, F., Jacobson, D., & Blouin, M. (2003). Yields of cultured Pacific oysters *Crassostrea gigas* Thunberg improved after one generation of selection. Aquaculture, 220(1-4), 227–244.

Lawrence, D. R., & Scott, G. I. (1982). The Determination and Use of Condition Index of Oysters. Estuaries, 5(1), 23-27.

Le Moullac, G., Quéau, I., Le Souchu, P., Pouvreau, S., Moal, J., René Le Coz, J., & François Samain, J. (2007). Metabolic adjustments in the oyster *Crassostrea gigas* according to oxygen level and temperature. Marine Biology Research, 3(5), 357-366.

Lorenzon, S., Martinis, M., & Ferrero, E. A. (2011). Ecological relevance of hemolymph total protein concentration in seven unrelated crustacean species from different habitats measured predictively by a density-salinity refractometer. Journal of marine Biology, 2011, 1-7

Lucas, A., & Beninger, P. G. (1985). The use of physiological condition indices in marine bivalve aquaculture. Aquaculture, 44(3), 187-200.

Mallet, A. L., Carver, C. E., & Hardy, M. (2009). The effect of floating bag management strategies on biofouling, oyster growth and biodeposition levels. Aquaculture, 287(3-4), 315-323.

Manahan, D. T., & Crisp, D. J. (1982). The role of dissolved organic material in the nutrition of pelagic larvae: amino acid uptake by bivalve veligers. American Zoologist, 22(3), 635-646.

Marquez-Rios, E., Gomez-Jimenez, S., Ocano-Higuera, V. M., Castillo-Yanez, F. J., & Pacheco-Aguilar, R. (2007). Quality parameters of spiny lobster (*Panulirus interruptus*) tails as affected by short-term emersion at two different air temperatures. Ciencias Marinas, 33(1), 73-82.

Marshall, R. D., & Dunham, A. (2013). Effects of culture media and stocking density on biofouling, shell shape, growth, and survival of the Pacific oyster (*Crassostrea gigas*) and the Manila clam (*Venerupis philippinarum*) in suspended culture. Aquaculture, 406, 68-78.

Meng, J., Wang, T., Li, L., & Zhang, G. (2018). Inducible variation in anaerobic energy metabolism reflects hypoxia tolerance across the intertidal and subtidal distribution of the Pacific oyster (*Crassostrea gigas*). Marine environmental research, 138, 135-143.

Michaelidis, B., Haas, D., & Grieshaber, M. K. (2005). Extracellular and intracellular acid-base status with regard to the energy metabolism in the oyster *Crassostrea gigas* during exposure to air. Physiological and Biochemical Zoology, 78(3), 373-383.

Miossec, L., Le Deuff, R. M., & Goulletquer, P. (2009). Alien species alert: *Crassostrea gigas* (Pacific oyster). International Council for the Exploration of the Sea Cooperative Research Report No. 299, Copenhagen.

Moana New Zealand. (2018) Integrated Annual Report 2018. Auckland, New Zealand

Mondol, M. R., Kim, C. W., Kang, C. K., Park, S. R., Noseworthy, R. G., & Choi, K. S. (2016). Growth and reproduction of early grow-out hardened juvenile Pacific oysters, *Crassostrea gigas* in Gamakman Bay, off the south coast of Korea. Aquaculture, 463, 224-233.

Moore, L. E., Smith, D. M., & Loneragan, N. R. (2000). Blood refractive index and whole-body lipid content as indicators of nutritional condition for penaeid prawns (*Decapoda: Penaeidae*). Journal of Experimental Marine Biology and Ecology, 244(1), 131-143.

Nell, J. A. (2002). Farming triploid oysters. Aquaculture, 210(1-4), 69–88.

Newell, R. I. (2004). Ecosystem influences of natural and cultivated populations of suspension-feeding bivalve molluscs: a review. Journal of Shellfish Research, 23(1), 51-62.

O'Meley, C. M. (1995). Effects of shell abrasion and aerial exposure on the performance of Pacific Oysters *Crassostrea gigas* (Thunberg, 1793) cultured in Tasmania, Australia. Unpublished Masters thesis, University of Tasmania, Australia.

Parry, H. E., & Pipe, R. K. (2004). Interactive effects of temperature and copper on immunocompetence and disease susceptibility in mussels (*Mytilus edulis*). Aquatic toxicology, 69(4), 311-325.

Paul-Pont, I., Dhand, N. K., & Whittington, R. J. (2013). Influence of husbandry practices on OsHV-1 associated mortality of Pacific oysters *Crassostrea gigas*. Aquaculture, 412, 202-214.

Percy, J. A., Aldrich, F. A., & Marcus, T. R. (1971). Influence of environmental factors on respiration of excised tissues of American oysters, *Crassostrea virginica* (Gmelin). Canadian Journal of Zoology, 49(3), 353–360

Pickwell, G. V., & Steinert, S. A. (1984). Serum biochemical and cellular responses to experimental cupric ion challenge in mussels. Marine Environmental Research, 14(1-4), 245-265.

Pipe, R. K., Coles, J. A., Carissan, F. M. M., & Ramanathan, K. (1999). Copper induced immunomodulation in the marine mussel, *Mytilus edulis*. Aquatic Toxicology, 46(1), 43-54.

Quayle, D. B. (1988). Pacific oyster culture in British Columbia. Canadian Bulletin of Fisheries and Aquatic Science 218, 1-241.

Robert, R., & Gerard, A. (1999). Bivalve hatchery technology: The current situation for the Pacific oyster and the scallop in France. Aquatic Living Resources, 12(2), 121–130.

Samain, J. F. (2011). Review and perspectives of physiological mechanisms underlying genetically-based resistance of the Pacific oyster *Crassostrea gigas* to summer mortality. Aquatic Living Resources, 24(3), 227-236.

Samain, J. F., & McCombie, H. (2008). Summer mortality of Pacific oyster *Crassostrea gigas*: the Morest project. Versailles: Editions Quae.

Schmitt, P., Duperthuy, M., Montagnani, C., Bachère, E., & Destoumieux-Garzón, D. (2012). Immune responses in the Pacific oyster *Crassostrea gigas* an overview with focus on summer mortalities. In: Oysters: Physiology, Ecological Distribution and Mortality (ed. J. G. Qin), New York: Nova Science Publishers, 227-273.

Seafood New Zealand (2017) Seafood New Zealand Export Information. https://www.seafoodnewzealand.org.nz/fileadmin/documents/Export_data/17.12.10a.pdf . Retrieved 7th of January 2020.

Seaman, M. N. (1991). Survival and aspects of metabolism in oysters, *Crassostrea gigas*, during and after prolonged air storage. Aquaculture, 93(4), 389-395.

Shamseldin, A., Clegg, J. S., Friedman, C. S., Cherr, G. N., & Pillai, M. (1997). Induced thermotolerance in the Pacific oyster, *Crassostrea gigas*. Journal of Shellfish Research, 16(2), 487-491.

Shatkin, G., Shumway, S. E., & Hawes, R. (1998). Considerations regarding the possible introduction of the pacific oyster (*Crassostrea gigas*) to the Gulf of Maine: A review of global experience. Journal of Shellfish Research, 16, 463-478.

Shumway, S.E., Davis, C., Downey, R., Karney, R., Kraeuter, J., Parsons, J., Rheault, R., Wikfors, G., (2003). Shellfish aquaculture—in praise of sustainable economies and environments. World Aquaculture, 34, 15–17.

Song, L., Li, X., Clarke, S., Wang, T., & Bott, K. (2007). The effect of size on the response of Pacific oysters (*Crassostrea gigas*) to changes in water temperature and air exposure. Aquaculture International, 15(5), 351-362.

Spencer, B. E., Key, D., Millican, P. F., & Thomas, M. J. (1978). The effect of intertidal exposure on the growth and survival of hatchery-reared Pacific oysters (*Crassostrea gigas* Thunberg) kept in trays during their first ongrowing season. Aquaculture, 13(3), 191-203.

Spencer, B. E. (1990). Cultivation of Pacific oysters. Laboratory Leaflet no. 63, Ministry of Agriculture, Fisheries and Food, Directorate of Fisheries Research, Lowestoft.

Spencer, B. E., Edwards, D. B., & Millican, P. F. (1992). Growing hatchery-reared Pacific oysters (*Crassostrea gigas* Thunberg) to marketable size in trays — observations on costed small-scale culture methods and rough-handling trials. Aquaculture, 106(3-4), 261–274.

Spiga, I., Caldwell, G. S., & Bruintjes, R. (2016). Influence of pile driving on the clearance rate of the blue mussel, *Mytilus* edulis (L.). In Proceedings of Meetings on Acoustics 27, (1), Acoustical Society of America.

Strohmeier, T., Duinker, A., Strand, Ø., & Aure, J. (2008). Temporal and spatial variation in food availability and meat ratio in a longline mussel farm (*Mytilus edulis*). Aquaculture, 276(1-4), 83–90.

Sumner, C. E. (1981). Growth of Pacific oysters, *Crassostrea gigas* Thunberg, cultivated in Tasmania. II. Subtidal culture. Marine and Freshwater Research, 32(3), 411-416.

Takeo, I. M. A. I., & Sakai, S. (1961). Study of breeding of Japanese oyster, *Crassostrea gigas*. Tohoku Journal of Agricultural Research, 12(2), 125-171.

Thompson, R. J., Bayne, C. J., Moore, M. N., & Carefoot, T. H. (1978). Haemolymph volume, changes in the biochemical composition of the blood, and cytological responses of the digestive cells in *Mytilus californianus* Conrad, induced by nutritional, thermal and exposure stress. Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology, 127(4), 287-298.

Wang J.C.C., Amiro E.R.. (1977) Cold Storage of the American Oyster (*Crassostrea Virginica*) in the Shell. Fisheries and Marine Service Industry Report no. 97, Department of Fisheries and the Environment, Halifax, Nova Scotia.

Webb, S. C. (1999). Aspects of stress with particular reference to mytilid mussels and their parasites. Unpublished PHD thesis, University of Cape Town, South Africa.

Wells, R. M., & Pankhurst, N. W. (1999). Evaluation of simple instruments for the measurement of blood glucose and lactate, and plasma protein as stress indicators in fish. Journal of the World Aquaculture Society, 30(2), 276-284.

Wijsman, J. W. M., Troost, K., Fang, J., & Roncarati, A. (2019). Global production of marine bivalves. Trends and challenges. In Goods and Services of Marine Bivalves, Cham: Springer, 7-26.

Wingerter, K., Mujica, B., Miller-Morgan, T., & Grant, O. S. (2013). Development of Live Shellfish Export Capacity in Oregon. Report by Sea Grant Oregon, Oregon State University, USA.

Zamora, L. N., Ragg, N. L., Hilton, Z., Webb, S. C., King, N., & Adams, S. (2019). Emersion survival manipulation in Greenshell™ mussels (*Perna canaliculus*): Implications for the extension of live mussels' shelf-life. Aquaculture, 500, 597-606.

Zhang, Z., Li, X., Vandepeer, M., & Zhao, W. (2006). Effects of water temperature and air exposure on the lysosomal membrane stability of hemocytes in pacific oysters, *Crassostrea gigas* (Thunberg). Aquaculture, 256(1-4), 502–509.