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Chilled Mist as a Viable Alternative Method for Transporting Commercially Caught Crustacean and Mollusc Species

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Abstract:

Shellfish are often transported live to markets to ensure freshness upon arrival. Traditional transport methods involve large volumes of water (1:1 animal:water, vivier) hence reducing the mass of animals that can be transported with one journey, or they are transported dry, with both methods often inducing high stress levels. To assess the viability of an alternative method, the physiological stress of three commercially important species (*Buccinum undatum*, *Nephrops norvegicus*, and *Homarus gammarus*) was measured over time (24h – *N. norvegicus*, 72h – *H. gammarus*, and 96h – *B. undatum*) within an experimental re-circulating intermittent (IM) and continuous (CM) mist environment. Haemolymph stress parameters such as L-lactate, ammonia, D-glucose, total protein, pH, and behaviour were measured every 24h to determine the condition of the animals. The responses of animals in the misted environments were compared to the traditional method of transport for each species: vivier or dry. The mist was effective at reducing levels of haemolymph ammonia in the animals compared to simulated dry transport (3 and 2.4 fold lower ammonia concentration in *B. undatum* and *H. gammarus* haemolymph; at 96 hours and 72 hours; respectively). The IM group had 8.8 times lower ammonia concentration in the reservoir water compared to the CM group at 96 hours for the *B. undatum* trials, suggesting that IM may be a more efficient use of water for longer journeys. In its current form, misting is not suitable for the transport of *N. norvegicus*, as high mortalities were recorded in both IM – 30%, CM – 10%, compared to traditional vivier – 0%, however IM reduced mortality rates compared to traditional dry transport of *B. undatum* (IM - 5.25%, CM – 28.07%, dry - 22.8%). The efficacy of Accutrend handheld meter for L-lactate determination in decapod crustaceans is discussed in detail within this study. This study offers a novel, easily implemented method of transport with potential for replacing traditional methods, whilst maintaining animal health. This study can be used by fishers as a base for developing more efficient, cost-effective methods of live shellfish transport.

Contents:

List of Figures:

List of Tables:

1 Introduction

1.1 General Introduction:

The successful transport of live seafood for export, is an important practice to ensure food security is met, and the value of the product is enhanced (Bondad-Reantaso et al., 2012). Live Transport was developed in order to improve the quality of the product whilst being exported to other nations, as there is a demand for live organisms on show in high-end restaurants, and for the market in general, as they typically fetch a higher price when the organisms are sold alive (Beard & McGregor, 1991; Vijayakumaran & Radhakrishnan, 1997; Barrento, 2010; Smyth & Uglow, 2015). In addition to the higher prices paid for live seafood, it also means that the producer does not have to invest in equipment to kill and process the animals, and does not have to pay the associated labour costs (Myers et al., 2010). The live seafood trade may also be as a result of adopted cultural practices and values, as the consumption of live seafood is the norm in the south east coast of China, and many other Asian cultures (Chan, 1999; Liu, 1999; Fotedar & Evans, 2011).

The fisheries production (both aquaculture and capture) in the EU is valued at around €11.62 Billion (Eurostat, 2017). Eighty-five percent of households in 22 countries (primarily EU countries) admitted to regularly purchasing seafood in 2018 (GlobeScan, 2018). Much of the shellfish captured on UK coasts is destined for export to markets with high demand for the products, such as Spain and Portugal, both countries have little/no infrastructure for the commercial fishing of certain shellfish species including *Cancer pagurus* (Smyth & Uglow, 2015). There are also exports from the UK to central Asia (SFIA, 2016), although the long-haul transport of marine organisms can lead to high levels of physiological and biochemical stress, and then to the degradation of meat quality (Jacklin and Combes, 2007). The maintenance of marine organisms during transport in good condition is therefore vital, as this can influence the survival of the product, and hence the value of the stock at the end of the transport chain. If a large proportion of the stock being supplied consistently arrives in poor condition at the end of the transport chain, then this may influence the perception of the quality of product from that region, and therefore the foreign buyers from those regions may decrease (Uglow et al., 1986). The quantification of stress during transport is often done using multiple physiological and biochemical biomarkers of stress often including testing for

changes in haemolymph constituents such as: glucose, L-lactate, total protein, ammonia, and pH (Lorenzon et al., 2007; Bernasconi & Uglow, 2008a; Smyth & Uglow, 2015). These biomarkers offer insights into the metabolic, and homeostatic function of the animals. Behaviour is also often used to get an overall view of stress in the animals. Recently, handheld meters have been used to determine L-lactate concentrations in the haemolymph of decapod crustaceans (Albalat et al., 2010; Bakke & Woll, 2013), but the data for this equipment is lacking and larger data sets are required to determine the accuracy and reliability of such equipment.

Traditional methods of live transport of marine shellfish include vivier, and 'dry'. Vivier transport typically involves the animals being placed in large containers of water at a 1:1 ratio, and dry transport involves the packing of animals in polystyrene boxes with wetted materials to limit the effects of exposure (Hosie, 1993; Smyth & Uglow, 2015). The research of new methods for the improvement of conditions for live organisms during transport is essential, as current methods increase stress on the organisms, and/or require too much additional weight (weight other than the live organism e.g. water) to support the functioning of the product (Hosie, 1993; Woll et al., 2010; Smyth & Uglow, 2015). Novel methods of transport could offer an improvement in animal quality and a decrease in mortality, but it may also improve the volume of product that can be transported with each trip, improving the profitability of each export (Smyth & Uglow, 2015). To date, research focusing on novel transport practices of live, commercially caught shellfish has been carried out on *C. pagurus* (Smyth & Uglow, 2015), and in some cases other crustacean species (Barnett et al., 1973; Jacklin, 1996), but to a much lesser extent. Chilled misting is the novel transport method tested in this study, it involves the use of small (50 microns) water droplets to maintain high humidity within the transport environment. The mist can be controlled on a continuous or intermittent time frame, to allow for more efficient use of water, which may further reduce the volume of water that may need to transport. Current gaps in the knowledge surrounding transport methods remain regarding the use of chilled mist to transport marine crustaceans and molluscs, especially with long transport times up to 96 hours.

1.2 Current Transport Practices:

There are several methods that are currently applied to the transport of commercially caught crustacean and mollusc species. The primary aim of these transport methods is to ensure that the live products are transported long distances, alive and in good quality, with reduced stress. The two primary methods for transport of live organisms commonly adopted by the fishing industry are vivier and dry/Semi-dry (Hosie, 1993; Barrento, 2010; Smyth & Uglow, 2015; Table 1). The many disadvantages ranging from physico-chemical aspects such as hypoxia, to more economic issues, such as reduced volume of product, limit each of these methods from achieving desired economic yields, which results from an inability to maintain suitable conditions viable for ensuring minimal mortalities and stress of the product (Schmitt & Uglow, 1998; Smyth & Uglow, 2015). Typical transport via the road, also introduces a number of other stressors to the organisms being transported, including vibrations and noise (Regnault & Lagardere, 1983; Welsh et al., 2013).

Table 1 – The positives and negatives of each modern transport method (adapted from Smyth & Uglow, 2015)

Transport Method	Positives	Negatives
Vivier	Keeps the organisms immersed during transport	Large volumes of water are being transported - expensive
	Allows removal of some waste products from the animals	Cannot be stacked - limited storage space use
		Organisms cannot be stored in small groups and handled easily, meaning that there is risk of increased handling stress
		Requires the use of specialised vivier trucks
Dry	No water is being transported (100% water savings)	Organisms are emersed during the transport phase
	Can allow for an efficient use of storage space - stacking	No medium for waste transfer from the animals
	Simple to implement	Can be wasteful - use of single-use polystyrene boxes
	Relatively cheap to implement	
	No maintenance of equipment required	
	Can be used in any transport vehicle with chilling unit	
Mist	Relatively cheap to implement	Uses more water than dry system
	Uses less water (~80% water savings)	Still in the experimental phase, and there is no confidence from the industry in this method
	Can allow for an efficient use of storage space - stacking	Needs maintenance to work efficiently
	Can be used in any transport vehicle with chilling unit	
	Simple to install	
	Further cools the animals by evaporative cooling	
	Can allow for the efflux of metabolic waste products	

1.2.1 Vivier System: -

The vivier system for transport of marine organisms is the most commonly used method, this is due to its ability to maintain many crustaceans in a suitable condition for the duration of transport, as it is believed that this method simulates their natural environment (SFIA, 1990). The vivier system typically uses large containers/vessels often filled with 50% seawater, and 50% animals for transport (Figure 1; Hosie, 1993). This type of transport ensures that the animals are immersed in seawater, which acts as the life supporting medium. Transport using the vivier system improves efflux of waste products, including ammonia, nitrates, and nitrites, as well as providing oxygen, through sufficient aeration systems (Schmitt & Uglow, 1998). Using this system, organisms are typically transported long distances for up to 48 (Schmitt & Uglow, 1998; Barrento, 2010) or even in some cases 72 hours (Welsh, 2010; Smyth & Uglow, 2015). However, during times of severe delays (at ports for example) which may occur as a result of administrative errors, and the exit of the United Kingdom from the European union may also lead to more delays at ports, so it would not be unreasonable to assume that some journeys may take longer, sometimes up to 96 hours.

1.2.1.1 Limitations for Vivier Transport:

It must be highlighted that there are a number of limitations of this method for the transport for crustaceans. This method requires the use of a large volume of water to ensure the survival of the animals used, as there is often a 1:1 ratio (enough water to just cover large containers of animals; Smyth & Uglow, 2015), or in the case with the European lobster *Homarus gammarus* a 1:2 ratio of animal to water in terms of mass (Hosie, 1993; Jiménez-Ruiz et al.,2015; Smyth & Uglow, 2015). The considerable volumes of water mean that the ability to transport large numbers of live organisms is severely restricted (Smyth & Uglow, 2015), as a standard truck that is able to carry 20 tonnes, will only have the ability to carry 6.6 tonnes of *H. gammarus,* and the remainder (13.4 tonnes) will be water. There has also been evidence of the vivier system being a poor choice for long-distance transport of crustaceans to warm climates such as Portugal, as it had led to high mortalities (40-60%; Barrento et al., 2012). However another study which provided questionnaires to fishermen and dealers, suggested the figures for mortality is typically lower with 2/3 of the responses suggesting that mortalities are below 20%, and 1/3 of the responses suggested a higher mortality between 31-40% for

summer import into Portugal (Smyth & Uglow, 2015). This could allow for the over-exploitation of the fisheries, as more animals will be captured to replace the losses during transport (Barrento et al., 2009).

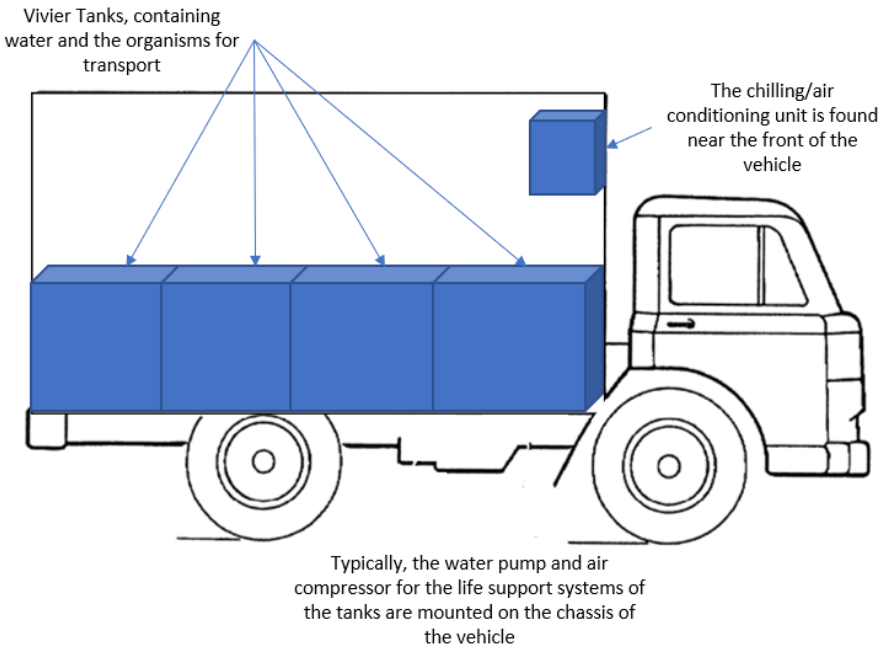


Figure 1 – A typical vivier set-up (modified from Jacklin 1993).

There is also the opportunity for a reduction in dissolved oxygen in the vivier tanks when storing live organisms (Hosie, 1993; Schmitt & Uglow, 1998; Barrento et al., 2009; Smyth & Uglow, 2015). This is likely to be as a result of unsuitable aeration and circulation of the water while there are high densities of live animals present, or even a failure in the systems that were previously suitable (Barrento, 2010). Although, previous studies claim that the success of this method is often linked to the temperature of the water (Uglow et al., 1986; Hosie, 1993; Smyth & Uglow, 2015), as lower temperatures often mean lower oxygen consumption (a decrease from 12°C to 8°C can lead to a 30-50% decrease in oxygen consumption in *Cancer pagurus*; Uglow et al., 1986), and slower production and therefore efflux of ammonia (SFIA, 1990; Hosie, 1993; Smyth & Uglow, 2015). However, there is no industry standard temperature for the storage of crustaceans during transport (Smyth & Uglow, 2015), and so current transport practices may not be representative of the optimal conditions that are recommended for vivier transport.

1.2.2 Dry/Semi-dry Transport:

This method is often used aboard small sea-going vessels, and in vans/lorries incapable of vivier storage (Hosie, 1993; Jacklin & Lart, 1995; Barrento, 2010), as its somewhat crude methodology is capable of keeping organisms alive, and its low initial cost (Smyth & Uglow, 2015) is convenient for smaller operators. The aim of this method is to keep the animals sufficiently moist as to prevent desiccation, and to protect them from the air, as to further reduce stress (Jacklin and Combes, 2007). This method is typically carried out using porous material that has been wetted, and then placed directly onto the catch, and the material is wetted on an ad hoc basis, to ensure it stays moist. The organisms are also often stored in insulated boxes/containers to ensure the temperature remains consistently low during transport (Smyth & Uglow, 2015). An adaptation of this method is commonly used for the transport of mussels in particular, as the mussels are packed in/on ice but the conditions remain as what the industry calls 'dry' (meaning not submerged in water; Barrento et al., 2013). This method offers significant savings in water usage when compared to the traditional vivier transport, and is often considered a more efficient use of space when compared to vivier transport, as the boxes of product can be stacked, and therefore the vertical space in transport vehicles can be utilised (Smyth & Uglow, 2015; Figure 2). The success of this method appears to also be linked very closely to temperature (Lorenzon et al., 2007; Smyth & Uglow, 2015), as the survivability of *C. pagurus* for example improved (from 57.1% to 87.5% survival) when the temperature was decreased from 12°C to 8°C (Barrento et al., 2011). The sub-individual biomarkers used to measure stress in the lobster *Homarus americanus* were also shown to be affected by temperature, as the concentrations of glucose, lactate, proteins, and cholesterol were all shown to be negatively affected by an increase in temperature from 6°C to 15°C, however, these differences appeared to diminish as the length of study increased (Lorenzon et al., 2007).

1.2.2.1 Limitations of Dry transport:

The primary issue with using the semi-dry method for the transport of organisms is the higher risk of desiccation, particularly the gills of crustaceans (SFIA, 1990). However, if the animals are maintained in a moist state using wetted materials, then desiccation is not likely to be an issue, as dry transport has been used for the transport of *Syclla spp.* (Mud-crab), and was successful in ensuring their survivability for 26 days (Dagoon, 1997;

Robson et al., 2007). Despite this, the stress levels of some organisms (shown through blood lactate levels) are much higher in organisms transported through this method, as opposed to vivier transport (Lorenzon et al., 2008). Dry transport also allows for the build-up of potentially harmful metabolic waste-products within the animal such as ammonia (which is often referred to as an increase in ammonia debt), as there is little/no external water available which is required for excretion of waste products from many marine shellfish (O'Donnell., 2011).

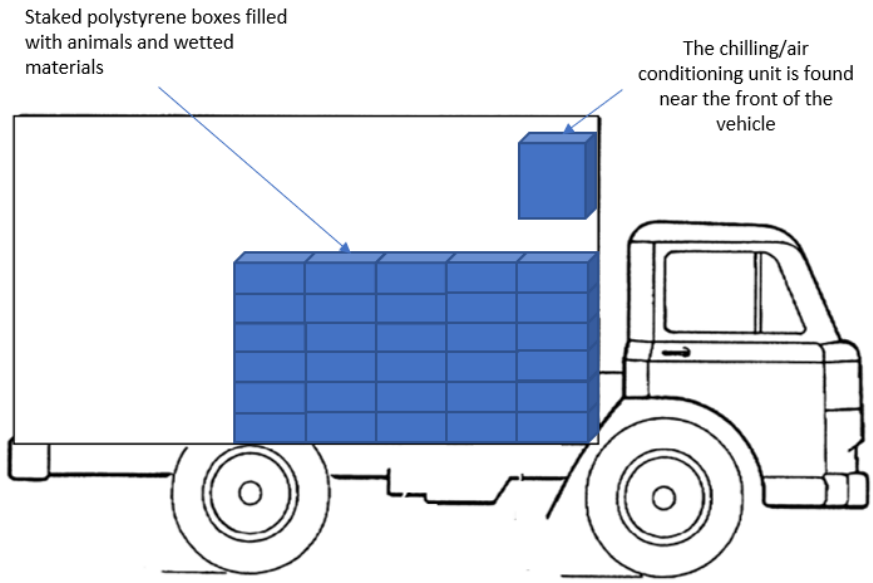


Figure 2 – A typical 'dry' set-up (modified from Jacklin, 1993)

1.3 Novel Transport Methods - Misting:

This method of transport for live organisms has been used in a limited number of tests, and even fewer applications in a commercial setting. This method has 2 main aspects which heavily influence its success: Cold temperatures (below 8°C, ideally), and the use of fine water particles suspended in the air, which ensure high humidity between 90-100% (Barnett et al., 1973; Jacklin, 1996; Smyth & Uglow, 2015). As to ensure the improved efficacy of this system, a mist-forming machine is used in conjunction with a chilling unit, which cools the room/area which will be saturated by the mist (Figure 3). The mist itself also improves the cooling qualities, as it leads to evaporative cooling on the animals which are subjected to it (Jussila et al., 2013; Smyth & Uglow, 2015). The

mist system has many alternative applications, such as maintaining the quality of decking/patio structures, reducing the spoilage of fruit and vegetables in supermarkets (Smyth & Uglow, 2015), also, on smaller scales being used to diffuse fragrances, and to generally improve the humidity in dry areas of the home. The many alternative applications provides a wealth of different misting systems available, and at a relatively low cost.

Previous studies of this method of transport have focused on the efficacy of the method on Crustacean species such as *Cancer pagurus* (Table 2)*, C. magister,* and *Nephrops norvegicus,* and the bivalve *Pecten maximus* with varying degrees of success (Barnett et al., 1973; Duncan, 1993; Jacklin, 1996; Smyth & Uglow, 2015). In one particular study there were high mortalities recorded during a 46-hour journey, which the accumulation of nitrite was believed to be a factor that resulted in the high mortalities (Hosie, 1993). Also, the mist system has in other cases been shown to be ineffective, as there was a 30% mortality in 96 hours of *Cancer magister* when using the mist system (Barnett et al., 1973). However, on the other hand, the system worked effectively for example in such cases as the signal crayfish (*Pacifastacus leniusculus*), whereby there was a 10% decrease in mortalities using a mist system, when compared to dry transport (Jussila et al., 2013). The differences in success could be as a result of discrepancies in methodologies, as some researchers had found an increase in nitrites within the reservoir of the mist system (Hosie, 1993), whereas other researchers claim, that the water is not recycled, and therefore a build-up of nitrites and other waste products should not be possible using this method (Smyth & Uglow, 2015). Comparisons have been made between the method described in this study, and the 'cascade' system, whereby the primary difference is that the cascade method uses larger water droplets, rather than a 'mist' to ensure the organisms are not subjected to desiccation (Barnett et al., 1973). The method used in the present study aims to improve on the current transport systems, through reducing the volume of water being transported, therefore allowing increased volume of profitable cargo, whilst also improving the transport conditions for the organisms and improving survival, which could in-turn reduce the exploitation of the fisheries.

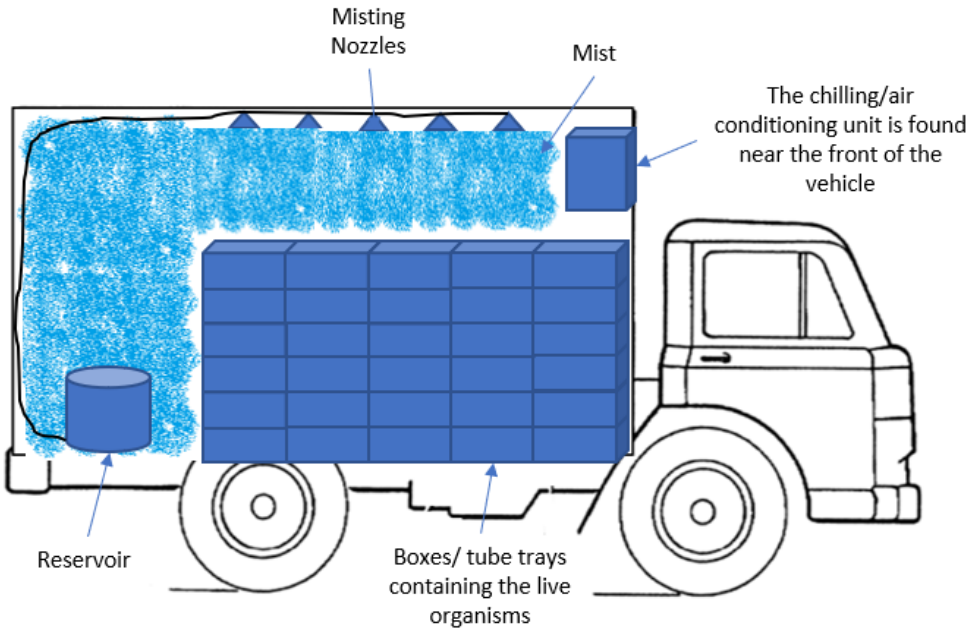


Figure 3 – The suggested set-up when using a mist system (modified from Jacklin, 1993).

Table 2 - The different percentage mortalities for 3 different live transport methods for *Cancer pagurus* and *C. magister*

System Used	Time (Hours)	Temperature (°C)	Species	Percentage Mortality	References
Vivier	36-40h	…	*Cancer pagurus*	19.1%	Hosie, 1993
	48h	…	*C. pagurus*	50-70%	Uglow et al., 1986
	48h	12	*C. pagurus*	25%	Barrento et al., 2012
	48h	8	*C. pagurus*	22.2%	Barrento, 2010
	48h	12	*C. pagurus*	0%	Barrento, 2010
	48h	16	*C. pagurus*	100%	Barrento, 2010
Dry	48h	4	*C. pagurus*	4%	Barrento et al., 2012
	72h	2	*C. pagurus*	0%	Woll et al., 2010
	72h	5	*C. pagurus*	4.8%	Woll et al., 2010
	96h	5	*C. pagurus*	25%	Woll et al., 2010
	36h	15	*C. pagurus*	9%	Woll et al., 2010
	18h	20	*C. pagurus*	40%	Woll et al., 2010
	48h	8	*C. pagurus*	12.5%	Barrento, 2010
	48h	12	*C. pagurus*	42.9%	Barrento, 2010
	48h	16	*C. pagurus*	50%	Barrento, 2010
Mist	72h	4	*C. pagurus*	6-8%	Smyth & Uglow, 2015
	96h	6	*Cancer Magister*	30%	Barnett et al., 1973

1.4 Biology, Ecology, & Commercial Considerations of Test Organisms:

Two commercially important crustacean species (*Homarus gammarus* and *Nephrops norvegicus*) and one commercially important Gastropod mollusc (the common whelk, *Buccinum undatum*) were used in this study. These animals were selected based on their commercial importance, and because the performance of the mist system had not been tested on these animals previously, with the exception of *N. norvegicus*.

1.4.1 Crustaceans:

There are approximately 67,000 known species in the sub-phylum Crustacea, most of which are aquatic species (Hickman et al., 2012). The order that will be of focus in this subphylum will be decapoda, which are often defined by 10 appendages, and a carapace that covers the entire body (Hickman et al., 2012). Many crustaceans (with few exceptions, whiteleg shrimp for example; FAO, 2018b,c) are not common in the aquaculture market when compared to other marine species such as molluscs, and teleost fishes, however they have high wild fishery capture rates: Brown Crab – 53,728 tonnes, Norway Lobster – 59,033 tonnes, European Lobster – 4,713 tonnes (in 2016; FAO, 2018a). The aquaculture market of crustaceans as a whole is expected to increase in the near future, as between 1970-2008 crustaceans had the biggest average increase per year of aquaculture production at around 18% (Bondad-Reantaso et al., 2012). However, the growth of aquaculture primarily for lobsters has been limited in recent years, as a result of biological factors, such as cannibalism, and territorialism (Jeffs, 2010).

1.4.1.1 *Homarus gammarus*:

1.4.1.1.1 Biology and Distribution:

The European lobster (*Homarus gammarus)* is widely distributed along the coasts of NW Europe, including the British Isles, although they are absent from the Baltic sea, this is likely to be as a result of a higher temperature range and lower salinity as a result of its semi-enclosed nature (Triantafyllidis et al., 2005; Ashton et al., 2017). Cobb & Castro, (2006) claim that lobsters in the genus *Homarus*, are only exclusively found in the Atlantic Ocean, but there has been captures of *H. gammarus* in the Mediterranean Sea, albeit with lower densities (Holthuis, 1991; FAO, 2018a). *H. gammarus* is typically found

no deeper than 150m, but more commonly around 50m on coastal shelves (Holthuis, 1991; Prodöhl et al., 2007). They are territorial organisms (Holthuis, 1991; Childress and Jury, 2006), meaning that they often cause damage to other individuals of the same species when found in a small area with high densities, such as holding tanks; however, this is often mitigated in the fishing industry, through applying durable bands to the chelae to prevent them from causing damage to other lobster (Jacklin & Combes, 2007; BIM, 2015; Fotedar & Evans, 2011).

1.4.1.1.2 Capture, Transport, and market:

The European lobster is a commercially important species, with approximately 4,713 tonnes captured in 2016 (FAO, 2018a). The largest markets for lobster catches are found in the UK and France, with 3281 and 586 tonnes in 2016 respectively (FAO, 2018a); the UK capture production quantity saw a 6.5% increase from 2012 to 2016 (Caveen & Green, 2013; FAO, 2018a). The price of lobster in Europe as of March 2019 was €38 per kg, which is an overall increase from March 2012 when it was approx. €20 (FAO, 2019b), although prices fluctuate throughout the year based on the fishing season and general availability. There is not a significant aquaculture industry that focuses on the production of *H. gammarus,* although it has been suggested that there is likely to be a growth in this sector in the coming years (Prodöhl et al., 2007); however, there are no current data on the production of European Lobster (FAO, 2018b,c). Lobsters are typically caught through 'potting', where baited pots are set up on the seabed, and time is allowed for the lobster to enter the pots, from which they are unable to escape (Spence, 1989).

1.4.1.2 *Nephrops norvegicus*:

1.4.1.2.1 Biology and Distribution:

N. norvegicus, the scampi or Norwegian lobster is typically found along the north-east Atlantic coasts, notably around the coastal regions of the British Isles (Johnson et al., 2013; Ashton et al., 2017). There is evidence that the southern limit is the Canary Islands (Barquin et al., 1998; Johnson et al., 2013), however the southern limit may be the North-West coast of Morocco (Holthuis, 1991). Further disagreements occur regarding the depth limits of the Norway Lobster, as some researchers believe the depth range of this species is 4 – 754m (Johnson et al., 2013), although the authors acknowledge that the species may be found outside this range, and therefore a more conservative

estimate was given by Holthuis (1991) indicating a maximum depth of 800m. *N. norvegicus* is an ecologically important species, as its habit of forming burrows changes the abiotic aspects of the sediment within the ecosystem. *N. norvegicus* creates burrows within its environment, meaning that it can be formally classified as a bioturbator (Atkinson, 1986; Hughes & Atkinson, 1997; Johnson et al., 2013); the burrows can be over 1m in length, with wide openings larger than 10cm (Hughes & Atkinson, 1997). The burrows increase the surface area of the sediment, thus increasing the oxygen content within the upper layers of the sediment, and these sediments are linked to a higher diversity of benthic organisms (Thrush et al., 2001; Shull et al., 2009; Johnson et al., 2013). The Norway Lobster is also important for the nutrient cycling processes within the marine ecosystem, as the burrowing activities have been known to resuspend carbon and nitrogen compounds into the water column, that were previously sequestered in the sediments (Johnson et al., 2013).

1.4.1.2.2 Capture, Transport, and market:

In addition to its ecological importance, the *Nephrops* is important as a commercial fishery. Of the 59,033 tonnes annually captured of *Nephrops* worldwide in 2016, 53.34% was captured in the UK (FAO, 2018a). When sold as whole and/or live, this species can fetch up to €28 kg^{-1} in France (Josupeit et al., 2018), although the price of this species can vary greatly depending on which country it is being sold. Typically, the organisms that have been captured via creel netting bring a higher price, as these organisms are typically sold on the whole and/or live market (Leocádio et al., 2012; Johnson et al., 2013; Russell & Mardle, 2017). Mostly, *N. norvegicus* is captured by trawling as opposed to creel or potting, but this may be as a result of the environments being exploited, and the preferences of the business owners (Leocádio et al., 2012; Russell & Mardle, 2017).

1.4.2 Molluscs:

There are approximately 50,000 Mollusc species most of which are found in marine environments; molluscs are typically soft-bodied organisms, with a protective calcareous shell (Gosling, 2015). They are a commercially important group of organisms, with an aquaculture production in the EU approx. 587,600 tonnes in 2014, and with an aquaculture production value in the EU worth approx. €902,712,000 in 2014, which is 47% of the total aquaculture production of all species (Eurostat, 2017).

1.4.2.1 *Buccinum undatum*

1.4.2.1.1 Biology and Distribution:

The common whelk (*Buccinum undatum*) is distributed exclusively throughout the northern hemisphere, at both the eastern and western parts of the Atlantic Ocean (Allcock et al., 2017; FAO, 2019a). This is a subtidal species of whelk but is sometimes found on the lower shore during spring tides (despite this often being lethal for the animal) down to 1,200m depth (Gowanloch, 1925; Hancock; 1967; Allcock et al., 2017). When the organism is exposed to the air, this species does not take approaches to minimise water loss, by retracting into its shell, but instead the behaviour appears to be deleterious, as it has been observed to extend the foot allowing for further water loss (Gowanloch, 1925; Hancock, 1967; pers. obs. this study). *B. undatum* is a carnivorous carrion feeder, which feeds on a number of live organisms including polychaetes, bivalves, and urchins, as well as dead organisms (Hancock, 1967; Himmelman & Hamel, 1993). This species has been affected by overfishing and the presence of the endocrine disruptor Tributyltin (TBT), especially in the waters around the Dutch Wadden Sea, where their numbers fell markedly, leading to its local extinction in the early 1990's (Cadée et al., 1995).

1.4.2.2.2 Capture and Market:

Whelks are a commercially important gastropod species, with 41,092 tonnes captured in 2016, and a small decrease to 38,115 tonnes in 2017 (FAO, 2018a). The UK has the largest capture statistics for this species, with 51% being from the UK, and France accounting for 36% of captures in 2017 (FAO, 2018a). From January to May 2018 the value of the Whelk landings into the UK by UK vessels was estimated to be £9,419,000, which is just under 10% of the shellfish value between these months (MMO, 2018). This species is often confused with the Red Whelk (*Neptunea antiqua*), although the Red Whelk does not have the coarse ribs that the common whelk does, and the Red whelk is not edible (FAO, 2019a). Some markets use dredging – though this has been found to be ineffective in the UK, because of the low densities, and because whelks are able to burrow below the surface of soft substrata (Hancock, 1967). It has also been shown that dredging for these animals can also reduce their population size, as it may increase the predation upon them by the common starfish (*Asterias rubens*), as rolling the animal was shown to increase the time it took for them to 'right' themselves, increasing the

opportunity for predation (Ramsay & Kaiser; 1998). Baited pots are more commonly used, using dead marine animals as bait, such as crab, herring, and whiting (Hancock, 1967). Baited pots are often preferred as this allows for the organisms to be captured in good condition, and transported live to varying parts of the UK. Much of the product captured off the coast of the British Isles is exported to far east countries such as Japan and South Korea (Fahy et al., 2000; Hollyman et al., 2018). This export could be set to increase if the UK exits the European Union.

1.5 Biomarkers of Stress:

Defining stress is complex and there are many different fields of study adopting alternative definitions (Friend, 1980); the definition of stress can also be extended from the cellular level of biological organisation, to the community, or even environment and ecosystem levels (Bayne, 1975; Widdows, 1978; Rapport et al., 1985; Menge & Sutherland, 1987; Solan & Whiteley, 2016). The current study considers stress at the individual and sub-individual level and defines it as: the change in the internal physiological conditions of the organisms as a result of stressors, resulting in a reduction in the fitness for survival which may lead to further risk from changes in the environment (Bayne, 1975; Stoner, 2012). Stress can be regarded as a result of the environmental conditions exceeding a tolerance threshold (or deviation from an optimal range) of the individual organism, and therefore the organism is unable to regulate its internal environment optimally by physiological homeostasis (Koolhaas et al., 2011). Stress can arise as a result of a number of factors, both biotic and abiotic (Menge & Sutherland, 1987). Within the scope of this study, abiotic factors of stress during the transport of organisms will be monitored, stressors including: emersion/desiccation, temperature fluxes, and hypoxic conditions (Uglow et al., 1986; Smyth & Uglow, 2015). The incidence of stress is important for the commercial trade of seafood, as this can lead to the degradation, or even death of the organisms, which can reduce the value of the individual catch and the export product as a whole (Uglow et al., 1986; Jacklin, 1996; Powell et al., 2017; Figure 4).

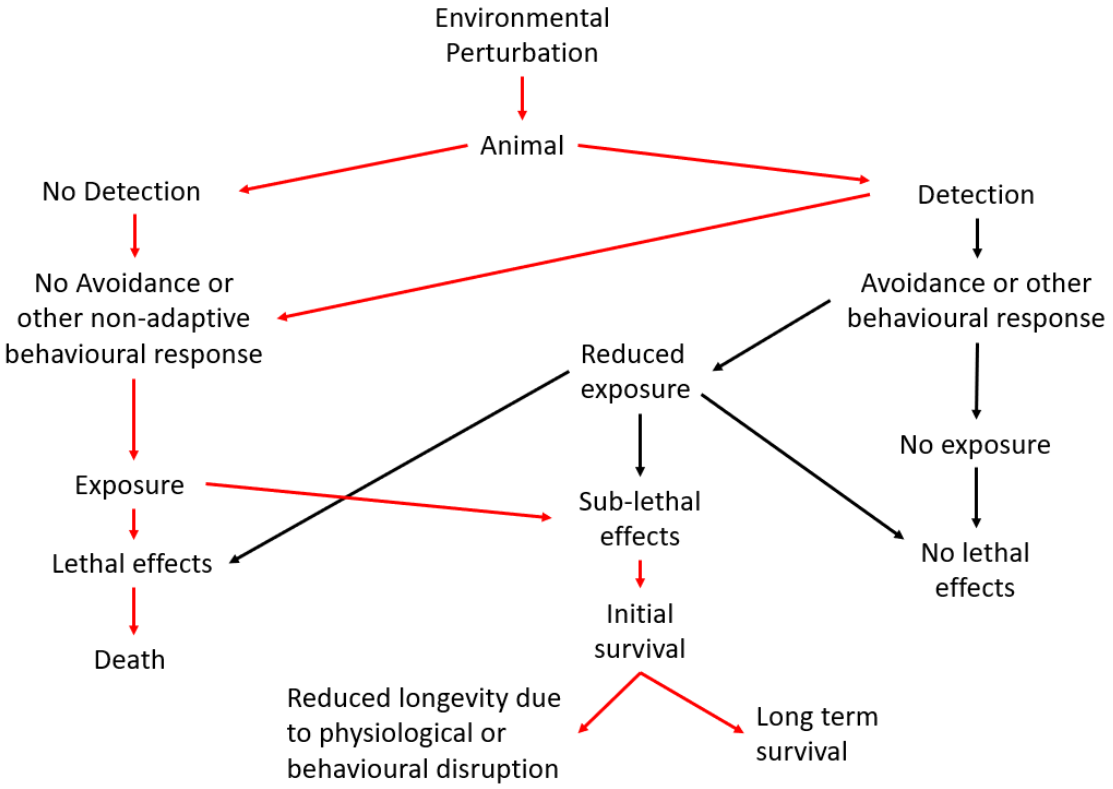


Figure 4 – Possible responses to environmental perturbation and the consequences for the individual animal. The red lines indicate the mechanism believed to be appropriate for this study (modified from Olla et al., 1979a).

Stress at the sub-individual, and individual level is generally monitored in two ways; biomarkers and behavioural responses (Friend, 1980; Paterson et al., 1999; Spanoghe & Bourne, 1999; Rato, 2016). Testing the behavioural or vigour aspects of an animal consists of observing individuals that are not stressed, and their responses to certain stimuli, and using such results as a basal level of health. Any deviation from this standard group of behaviours, suggests the organism may be stressed (Barrento et al., 2011; Smyth & Uglow, 2015). Alternatively, the behavioural responses measured may be adaptive, and may increase the fitness of the animal (Kittredge et al., 1979; Olla et al., 1979a; Riedel et al., 2016; Figure 4). The use of behavioural parameters is also species dependent, and therefore requires the observation of the individual species, as not all behaviours occur throughout all species. The use of behaviours to test the health and stress levels of an organism is often described as a subjective parameter, and therefore will not be suitable for use independently; it is often more appropriate to use these parameters in conjunction with objective, quantitative, biomarker data (Friend, 1980; Barrento et al., 2009). A biomarker has many definitions, with most focussing on the fact

that a biomarker must be testable and measurable (WHO, 1993; Strimbu & Tavel, 2010). But more specifically, the definition provided for this study will encompass many definitions: *a measurable and quantifiable sub-individual physiological parameter, that is a response to external stimuli which is acting as a stressor on the organisms, and is able to detect a change against a background of inherent variability* (WHO, 1993; Hannam et al., 2010; Strimbu & Tavel, 2010). Examples of objective, quantitive biomarker data that could be used for the determination of stress in organisms include the concentration of ammonia, lactate, glucose, pH and protein in the blood/haemolymph of the individuals (Lorenzon et al., 2008; Barrento, 2010; Smyth & Uglow, 2015). Each of these biomarkers offer insights into the metabolic and homeostatic function of the animals. These biomarkers can also be tested as efflux products in the medium that the organisms are stored in (Hosie, 1993; Schmitt & Uglow, 1998; Smyth & Uglow, 2015).

1.6 Measurement of L-lactate Concentrations

The methods for measuring stress biomarkers such as L-lactate has recently come into question, as newer novel methods are being developed for marine decapods. Traditionally, L-lactate is measured by an enzymatic colorimetric method (Albalat et al., 2010; Woll et al., 2010; Smyth & Uglow, 2015). Despite the enzymatic colorimetric method's proven accuracy, it requires the use of large and expensive equipment which is not suitable for all labs and experimental designs (Bakke & Woll, 2014). Recently however, handheld meters are being tested to determine their suitability for measuring L-lactate concentrations in the haemolymph of marine decapods (Albalat et al., 2010; Bakke & Woll, 2014). This method may offer reduced measurement response times, and opportunities to carry out direct measurements in the field. But the data surrounding these methods is lacking, and therefore more work needs to be carried out to determine if these methods are suitable for stress measurements in marine invertebrates.

1.7 Mitigation of Stress during the Live Transport of Organisms

Despite the numerous and varied causes of stress in marine organisms during live transport, there are many practices and methods that are applied to mitigate the stress effects. For example, there has been numerous studies into the efficacy of various

anaesthetics at reducing the stress of marine organisms during transport, examples of the most common anaesthetics include: AQUI-S®, MS222, and clove oil (Coyle et al., 2005; Barrento et al., 2008; Barrento et al., 2011). The aim of using anaesthetics during transport, is to reduce the activity of the organisms, which in turn reduces the production of waste products of the organisms, therefore reducing overall stress (Barrrento et al., 2008). However, the adoption of anaesthetics for live organisms destined for consumption has been low, as no anaesthetics are sanctioned for use within the EU (Barrento, 2010), because there are concerns that the anaesthetics may enter the human food chain, and impact human health (Marking & Meyer, 1985; Soto & Burhanuddin, 1995; Akbulut et al., 2011). It appears that the use of anaesthetics to reduce stress during transport is a promising prospect, but with many legal requirements that must be met, it will likely be a long time before widespread adoption. In addition to the use of anaesthetics, there are many physical measures that influence the stresses organisms face during transport. This includes the careful handling of the live organisms, to reduce the incidence of mechanical damage and/or death of the organisms (Uglow et al., 1986), hence the practice of banding/nicking of crustacean claws in order to prevent organisms from damaging each other (Jacklin & Combes, 2007; Fotedar & Evans, 2011). Selection is also employed as a method for ensuring the survival of organisms for long-duration travel, meaning that only the healthiest organisms (determined by vigour) are chosen for transport, and the rest are processed immediately, as they would likely die as a result of the stresses imposed during transport (Uglow et al., 1986; Barrento, 2010; Smyth & Uglow, 2015). However, selection does not reduce the stressors upon the organisms, but rather a means of increasing the stress threshold of the organisms used, in order to reduce mortality.

The control of numerous physical parameters such as temperature, salinity, humidity, and the efflux of nitrogenous waste products, is vital for the survival and maintenance of low stress levels in marine organisms during transport (Uglow et al., 1986). Temperature is widely considered as one of the most important parameters to be controlled during transport, together with humidity (Barrento, 2010; Smyth & Uglow, 2015). The controls for these physical parameters are usually implemented through the different methods of transport (see section 2.1), which control each parameter differently. During transport, temperature is generally lowered in order to reduce the

metabolic rate of the organisms, which in turn lowers the production of nitrogenous wastes from the organism, this process is termed cold anaesthesia (Smyth & Uglow, 2015; Robertson et al., 2018). However, the overall effectiveness of cold anaesthesia has been brought into question with studies on the Southern Rock lobster *Jasus edwardsii*, with some researchers claiming that it only delays the stress response, and does not contribute to an overall reduction in stress in the long-term, and the possibilities of reduced survivability during emersion (Forgan et al., 2014; Robertson et al., 2018). Despite this, the use of cold temperatures during transport of live organisms has been used successfully in the past, with many studies showing that lower temperatures (around 5°C) are more suitable for reducing stress, and mortality in *C. pagurus* and *P. leniusculus* (Robson et al., 2007; Jussila et al., 2013; Smyth & Uglow, 2015). Therefore, the success of using a cold temperature for the transport of seafood is species specific, as thermal tolerances may differ, and the temperature must be selected to ensure optimal survivability. All of the above practices may mitigate stress in marine organisms during live transport, and all aspects must be considered to ensure a good quality product, as when used synergistically this may reduce stress levels to a minimum.

1.8 Aim, Objectives, and Hypotheses:

1.8.1 Aim:

To determine the efficacy of chilled mist at reducing physiological and biochemical stress indicators of live commercially caught crustaceans and molluscs, when compared to the primary traditional transport method for each species – vivier or dry.

To determine whether the Accutrend handheld lactate meter provides an accurate method of L-lactate when compared to the traditional enzymatic method of L-lactate determination.

1.8.2 Objectives:

- To determine which stress indicators (behavioural and biochemical) are the most suitable for determining condition during transport of live shellfish
- To develop a suitable mist system for testing this mode of transport
- To determine a suitable stress indicators (behavioural and biochemical) to carry out further tests

- To use stress indicators (ammonia, pH, total protein, glucose, and lactate haemolymph concentrations) of commercially caught crustaceans and molluscs to determine the efficacy of the chilled mist machine at reducing stress and improving mortality rates
- To subjectively measure the vigour for the crustacean and molluscan species to determine the condition of the organisms
- To validate the use of new equipment (Roche AccuTrend) for determining the sub-lethal biomarker concentrations

1.8.3 Hypotheses:

1. Crustaceans kept under the mist conditions will have a lower overall stress level and lower mortality, when compared to the traditional means of transport
2. Molluscs kept under the mist conditions will have a lower overall stress level and lower mortality, when compared to the traditional means of transport
3. The Accutrend handheld lactate meter will not produce statistically different lactate measurements compared to the traditional method of L-lactate determination

2 Methods:

2.1 Animals Used:

The whelks used in this study (shell size 51 – 107mm) were obtained from Independent Shellfishermen's Co-operative (Bridlington) Ltd. courtesy of MacDuff Shellfish (Scotland) Ltd. The Whelks were collected on 3 separate occasions during late winter/early spring (February to March) from day boats. The animals were stored either in large vats of seawater or stored dry in a chilled storage room upon collection. After collection the animals were transported dry to the University of Hull by car, and upon arrival at the university were placed directly into tanks of seawater (35 salinity) at 10°C and stored for 4 days prior to testing. Fifty-seven animals (N=57) were used for each experiment, so 171 individuals were used over the 3 treatments. The animals were also fed a small amount of pellet after 2 days, and the water was changed based on when the water became discoloured and had a weak ammonia smell (approximately every 1.5-2 days). Going into the tests, the animals were checked for responses to minor stimuli (pressure on foot, and siphon), if no response was recorded, then the animal was excluded from the tests.

The *N. norvegicus* (carapace length 42 - 60mm) used in this study were obtained from Sutherland Game & Shellfish (Lairg, Scotland). These individuals were caught by creel pots. Three batches of animals were obtained during April and May. These individuals were caught off the coast of Scotland and transported by vivier to Manchester, where they were transferred to car and surrounded by gel ice packs, then transported dry to the University of Hull. Upon arrival at the university the animals were transferred into tanks of seawater (35 salinity) at 10°C. Individuals with signs of damage and/or ecdysis were excluded from the study. Fifty animals (N=50) were used for each experiment, and so 150 individuals were used over the course of the 3 tests. Both male and non-ovigerous females were used in this study. The *Nephrops* were stored in seawater for 12 hours to allow for the efflux of waste materials, and temperature acclimation before the start of the experiments.

The *H. gammarus* used for this study (carapace length 88 – 115mm) were obtained from Independent Shellfishermen's Co-operative (Bridlington) Ltd. courtesy of MacDuff Shellfish (Scotland) Ltd. Thirty individuals (N=30) were used for each treatment, so a

total of 90 individuals were used. The animals were stored in large storage pools in a chilled room prior to collection. The lobsters were collected on two separate occasions during June. After collection the animals were transported dry to the University of Hull by Car, and upon arrival at the university were placed directly into aquaria of seawater (35 salinity) and stored for 12 hours prior to testing at 8-10°C. Before the tests commenced, the animals were checked for injury, and their vigour (see section 3.2.5.6) was measured to ensure that the animals used were of good health.

2.2 Laboratory Testing

Three trials were carried out for three species (one neogastropod mollusc: *Buccinum undatum* and two decapod crustaceans: *Nephrops norvegicus* and *Homarus gammarus*) to determine the efficacy of a mist system, against traditional transport methods on the survival and stress of the animals. Analysis was carried out between, and within the transport types (Continuous misting, Intermittent misting, and the traditional method of transport for each species), to determine how the stress parameters change over time in each of the test environments, and to compare those changes to each of the transport methods.

The aim of the study was to determine if the use of a chilled mist (both intermittent and continuous misting) was suitable for transporting live marine organisms, when compared to the traditional method of transport for each species (dry for both *H. gammarus* and *B. undatum*, but vivier for *N. norvegicus*). The tests were carried out in a temperature-controlled room, which maintained the temperature of the test area at 5°C (±2°C). Seven *N. norvegicus* and *B. undatum* individuals, and five *H. gammarus* individuals were sampled for haemolymph and behavioural biomarkers every 24 hours: from 0 hours to 24 hours (*N. norvegicus*), 72 hours (plus 24 hour recovery; *H. gammarus*) or to 96 hours (*B. undatum*). Recovery of the animals after experimental treatment was only done for *H. gammarus* because the high death rates of the other two species meant that insufficient unsampled individuals remained in all tests to allow for a meaningful comparison. Each individual was only sampled once in order to minimise the effect haemolymph sampling may have on stress. After sampling the animals were marked with pen to ensure pseudoreplication did not occur. The organisms were tested in dark

conditions and were only subjected to light during periods of sampling (i.e. every 24 hours) for a short period <90 minutes. The equipment used for the experiments was all thoroughly cleaned and disinfected before use, to ensure that bacterial growth and pathogens were not a factor that may reduce the survivability of organisms. Any deaths that occurred during testing were recorded for mortality analysis, and the deceased organisms were removed from the test environment. Samples of the water in the reservoir/sump were also taken at regular 24-hour intervals to determine whether there was any efflux of waste by the organisms into the surrounding environment. The water samples were tested for ammonia, and pH (except for the *Nephrops* trial, where there was no change in pH over 24 hours).

2.2.2 Misting System

The mist system (Figure 5) is the novel experimental treatment and approach of this study. It consists of a 24 Volt DC pump that is capable of producing a pressure of up to 75 PSI (Available from Mistking.com – Standard diaphragm misting pump). The activity of the pump was controlled by an electronic digital seconds timer, and differed based on the treatment group. For the intermittent mist system, the timer was set so the system operated for one minute every three hours, meaning that the system turns on eight times in a 24 hour period using approximately 8.8L of water in the 24 hour period. For the continuous misting group, the system was allowed to operate for 11 hours, with a one hour break following each period of operation to allow for the pump to cool. This means that for a 96 hour period, the system was operating for 88 hours with 8 hours of 'breaks'. This system uses approximately 24.2L of water in a 24 hour period. Before the experiments commenced for both mist systems, the mist machine was operated for 30 minutes to ensure a sufficiently humid environment. The pump is gravity fed by a reservoir (15L for the *B. undatum* and *N. norvegicus* trials, and 25L for the *H. gammarus* trials) of recirculated seawater (which also acted as a sump, by collecting the mist as it falls), through a series of 6.3mm diameter tubes. A full water change was carried out during the *H. gammarus* trial at 48 hours, as the build-up of ammonia was determined to be too high, and this may have caused high death rates in the animals if left unchanged. The water from the pumps creates water droplets 50 µm in size through the misting nozzles. There were 3 misting nozzles, all positioned above the organisms being tested, so the mist was predominantly falling onto the organisms vertically (Figure 5). In

order to contain the mist a greenhouse structure was used (69cm x 200cm x 49cm; Figure 5), which had 5 shelves, and a polyester membrane to limit mist migration away from the test location. Polyethylene bags were used to cover the exposed areas of reservoir/sump, in order to prevent foreign bodies from entering the water supply and impacting the health of the animals. The animals used for this test were placed into the mist system similarly to how they are typically transported: *Nephrops* were placed in tube-trays, Lobsters and whelks were stacked on each other in industry standard mesh boxes (plastic boxes with regular holes to allow water to drain).

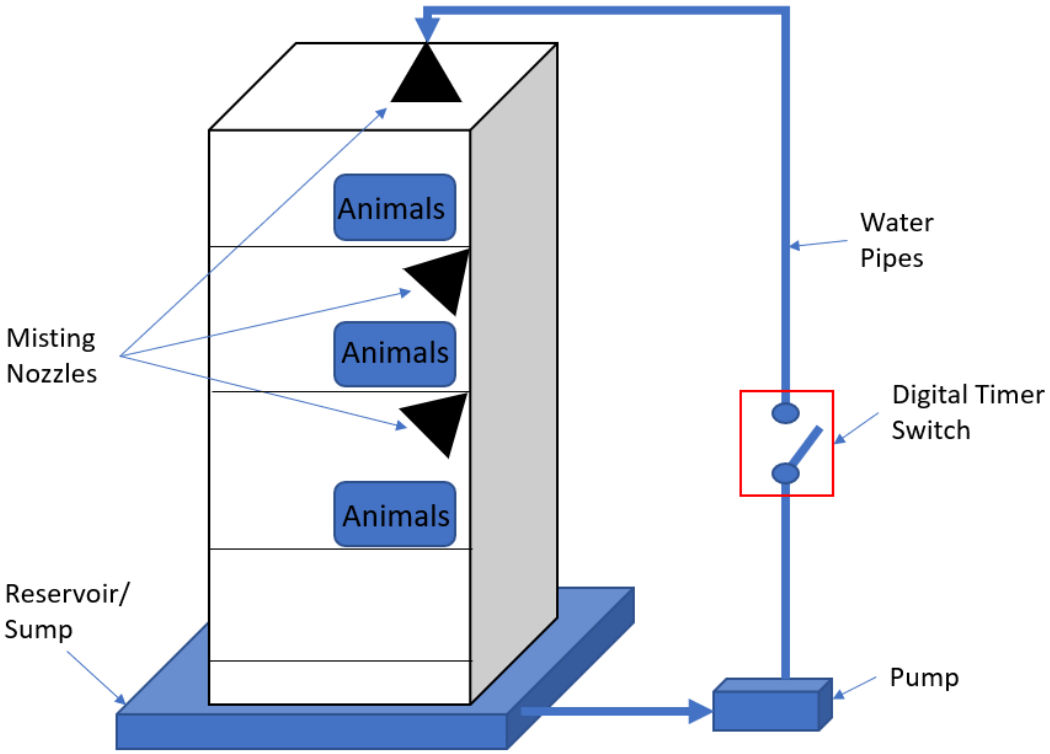


Figure 5 - The recirculating mist system used (created by Cherry & Smyth)

2.2.3 Traditional Transport Methods:

Traditionally, whelks are transported in large nylon bags, and stacked on top of each other. For this study this treatment was reproduced as closely as possible. It was determined that the survival of this species during transport may be based upon the maintenance of a humid environment around the high densities of animals (this was observed as the humidity around the animals was seen to be very high >90%). The

animals for this treatment were stored in the greenhouse structure used for the misting system to replicate a situation where the densities of whelks are higher, allowing the humidity surrounding the animals to increase. The nylon bag typically used in the live transport of these species could not be used in this study as they do not allow for easy access to the animals for observations and haemolymph sampling, which would therefore lead to an increase in handling stress on the animals. The industry standard mesh boxes were used as an alternative to the nylon bags as the plastic used did not allow for any water retention (the same as the nylon bags), but the rigidity allowed for easier access to the animals for sampling. The greenhouse also helped to minimise the water-loss through evaporation, as this ensured that the area surrounding the whelks was maintained in high humidity. The reservoir remained in place below the greenhouse to collect any water run-off from the whelks. Mantle cavity water was also tested for ammonia, from four individuals at 96 hours in this group, it was not done throughout the experiment as it may have influenced the mortality of this simulated transport group.

Nephrops are traditionally transported via vivier throughout the UK and to EU countries. In order to replicate this, 50 individuals were placed into tube trays and stored in a 43cmx38cmx29cm clear crate, with 30 litres of seawater. Several air-stones were used in order to aerate and circulate the water. This closely replicates the traditional method of transport of *Nephrops*, as they are typically transported at a 1:1 ratio of water to animal, so the minimum volume of water was used to cover the whole of the animals, and this was estimated to be 30L.

The control experiment for the trials for *H. gammarus* consisted of storing the individuals in a dry state. The animals were stored in a polystyrene box within the cold-room at a temperature of 5°C ±2°C, with wetted paper covering the animals, and some below the animals in order to collect any moisture. Gel ice packs were also placed into the polystyrene box in order to reduce any temperature variability. The fishing industry usually creates holes in the polystyrene boxes to allow for gaseous exchange, however this study omitted this process in favour of maintaining a highly humid environment.

2.2.4 Haemolymph Withdrawal

Haemolymph collection from live whelks has only been done a limited number of times previously (Andrews, 2010). Therefore, a small sub-group were used (N=8) to find a suitable sinus from which to extract haemolymph, whilst minimising stress on the animals. After a number of trials, it was determined that the most consistent place to collect haemolymph from was the cephalopedal sinus (pers. obs.), through the foot of the whelk using a 25-gauge hypodermic needle and a 1ml syringe. It was found that the best practice was to hold the operculum against the shell of the individual in order to prevent the animal from retracting too far into the shell where haemolymph collection is impossible. The needle was inserted approximately 5mm into the foot, whereby it was slowly drawn back whilst pulling on the plunger of the syringe, and the sinus was usually located around 1-2mm below the surface of the epidermis. Slight movements of the needles depth (1 or 2mm) was shown to be effective at increasing the haemolymph withdrawal rate as this process appeared to allow the sinus to refill with haemolymph and ease the collection process. From each individual, 0.8ml of haemolymph was extracted, although this was not possible for all animals, and so the small amount of haemolymph was diluted using distilled water to make 0.8ml of solution. The 0.8ml volume of haemolymph collected was calculated to be the amount required to carry out all necessary analyses. All haemolymph sampling of this species was carried out at room temperature. There did not appear to be a significant difference between the mortality of the individuals sampled for haemolymph, and those that had not been sampled (ad-hoc obs.). If haemolymph collection was not possible from the individual, they were marked and not used for further haemolymph collection. This process had a 70-80% success rate for the removal of at least 0.2ml of haemolymph.

Haemolymph withdrawal from the crustaceans in this study was carried out through the arthrodial membrane of the 5th pereiopod using a 25-gauge needle and a 1ml syringe. There was a faster rate of haemolymph coagulation in the crustacean species tested, and so after extraction at 10°C the haemolymph was placed directly on ice, and transferred immediately into two 1.5ml Eppendorf tubes, one containing perchloric acid (for deproteinisation), and the other deionised water (both were 1:1 dilutions), and centrifuged at 7200 RPM for 5 minutes to prevent coagulation.

2.2.4.1 Haemolymph Preparation

After a suitable amount of haemolymph was collected (minimum 0.2ml) the haemolymph was then diluted to 0.8ml (Figure 6). This haemolymph was then transferred into 2 separate Eppendorf tubes: 400µl into one, and 350µl into the other (Figure 6). The Eppendorf containing 400µl of haemolymph was diluted 1:1 with 0.6M Perchloric Acid in order to deproteinise the samples, and the Eppendorf containing 350µl of haemolymph was diluted 1:1 with deionised water. The Eppendorf tube which had perchloric acid was centrifuged at 7200RPM for 5 minutes and the supernatant removed, 500µl was decanted into another Eppendorf for Glucose analysis, and the remaining 300µl was decanted into a separate Eppendorf tube for lactate analysis. The Eppendorf tube with distilled water dilution, was centrifuged at 3500RPM for 10 minutes (*B. undatum)* or 7200RPM for 5 minutes (*N. norvegicus* & *H. gammarus*), and the resulting supernatant was removed. 250µl of the suprnatant was decanted into an Eppendorf tube for ammonia analysis, and finally 300µl was pipetted into an Eppendorf tube for protein analysis. Any remaining haemolymph from any of the stages were stored along-side the processed haemolymph. The resulting haemolymph samples were then frozen at -30°C for analysis at a later time.

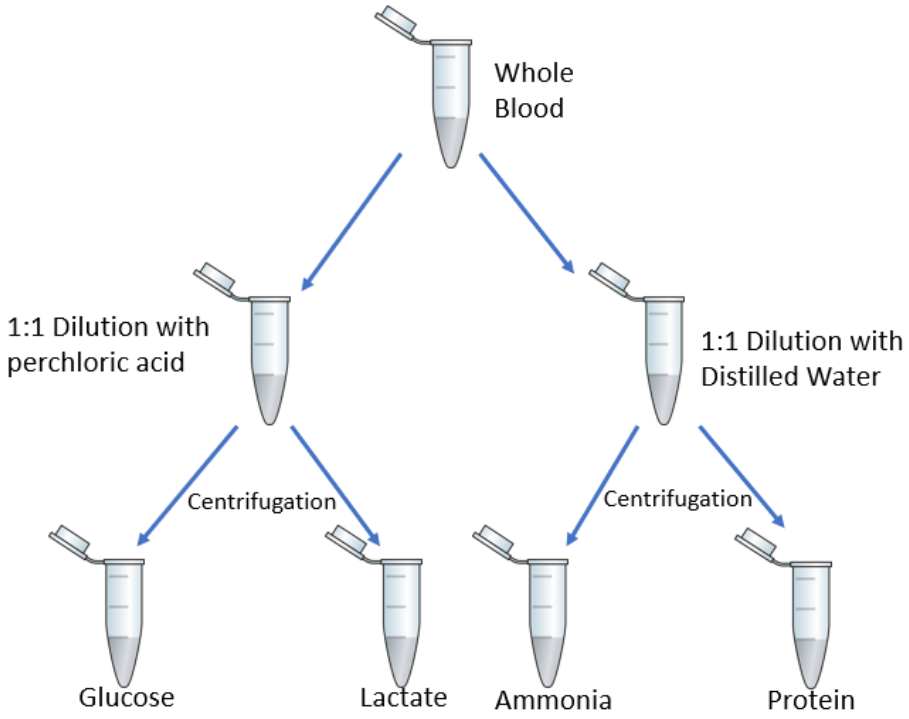


Figure 6 - A The means of separating haemolymph into different Eppendorf tubes after extraction from the animals (OCAL, 2007)

2.2.5 Stress Parameters

2.2.5.1 L-Lactate

L-lactate in deproteinised haemolymph was determined using an enzymatic colorimetric kit (Trinity Biotech, kit 735-10), and a spectrophotometer to measure changes in absorbance at 540nm. The enzymatic reaction in the kit reflects the lactic acid being converted to pyruvate and hydrogen peroxide by lactate oxidase. The hydrogen peroxide then undergoes a condensation reaction with the chromogen precursors to form a purple coloured dye (Smyth et al., 2011).

The process for using this test kit was firstly to establish a solvent blank, and a standard of a known lactate concentration. The solvent was made up of 1ml of the lactate reagent, and 10µl of haemolymph or lactate standard was added to this to begin the reaction. The absorption from the standard solution was used to convert the absorption readings of test solutions into usable lactate concentrations (see equation below). After

the haemolymph samples (or lactate standards) were added to the lactate reagent, the solutions were incubated for 5 minutes at 25°C, then placed into the spectrophotometer whereby the absorbency was measured. The equation used to convert the absorbency measurement into lactate concentration was (where 40 is the concentration of standard):

$$\text{Lactate (mg/dl)} = \frac{(A_{540} \text{ of test}) * 40}{A_{540} \text{ of standard}}$$

The corresponding lactate result is then multiplied by 0.111 as per the kit's leaflet instructions, in order to convert the concentration to mmol/L.

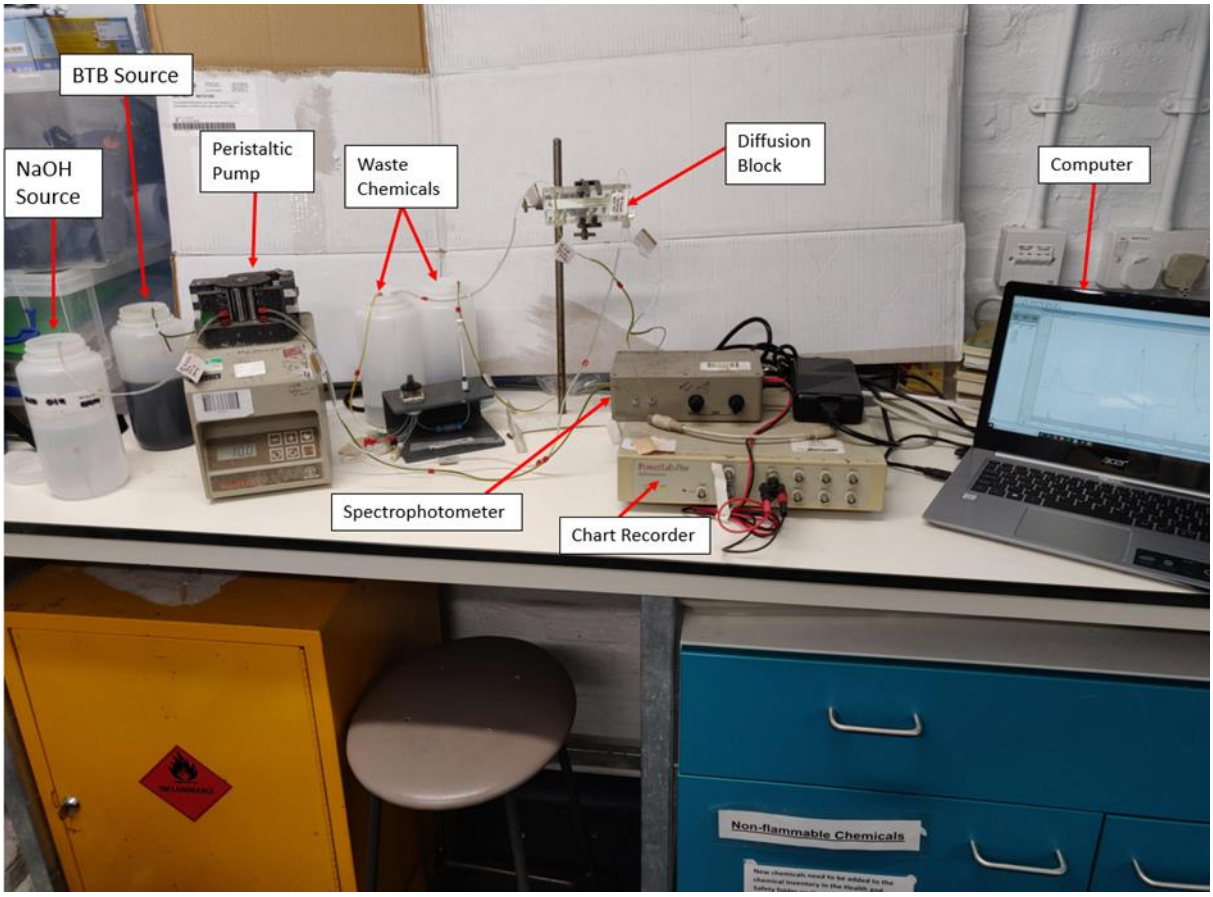
L-lactate was not measured in *B. undatum* haemolymph as the concentrations measured were often below the detection range of the kit (0.02mmol/L).

2.2.5.2 Ammonia

Ammonia was determined using Flow-Injection Analysis (FIA) equipment (Hunter & Uglow, 1993). The general premise of this method was that a 0.1M sodium hydroxide solution was used as a carrier solution for haemolymph samples to a PTFE membrane, whereby any ammonia in the haemolymph would then diffuse down a concentration gradient into the 0.6M bromomethyl blue indicator solution, which was running parallel to the carrier solution (Figure 7). Each sample required ≥250µl of haemolymph, as the 'loop' system ensured that this was the exact amount used, even if excess was added. The influx of ammonia into the indicator solution would then initiate a change in colour which was then detected as spectrophotometer peaks (AD Instruments Chart 5 peak analysis software). A digital filter of 10Hz, and a low pass of 50Hz was required in order to reduce the noise on the spectrum, and allow signals to be seen more clearly. The software was calibrated using a series of ammonium chloride solutions of different concentrations to form a linear calibration regression equation (y = mx + c; which was determined by dividing the known concentration of the standard solutions, in µmol/l, by the corresponding voltage output for the standards, in volts) based on the peaks shown and the known concentrations of the standard solutions. The regression equation

allowed for the determination of the ammonia values present in the haemolymph, and the conversion from a value in Volts to µmol/l of ammonia. Ammonia standards of 20, 50, 100, 200, 300, 500 µmol/l were used for most haemolymph samples, although particularly for samples with higher ammonia levels, 800, 1000, 3000, and 5000µmol/l standards were also used. Calibration equations were formed for every 20-30 samples, or if there was a change in sensitivity of the equipment which was identified by a baseline shift over-time. The PTFE membrane was replaced when precipitates were noticed on the membrane, and the system was flushed with 10% HCl and distilled water. This method has a lower detection limit in the region of 0.02 µmol/l and precision of between 0.9-3.3% (Hunter and Uglow,1993; Smyth, 2011).

A)



B)

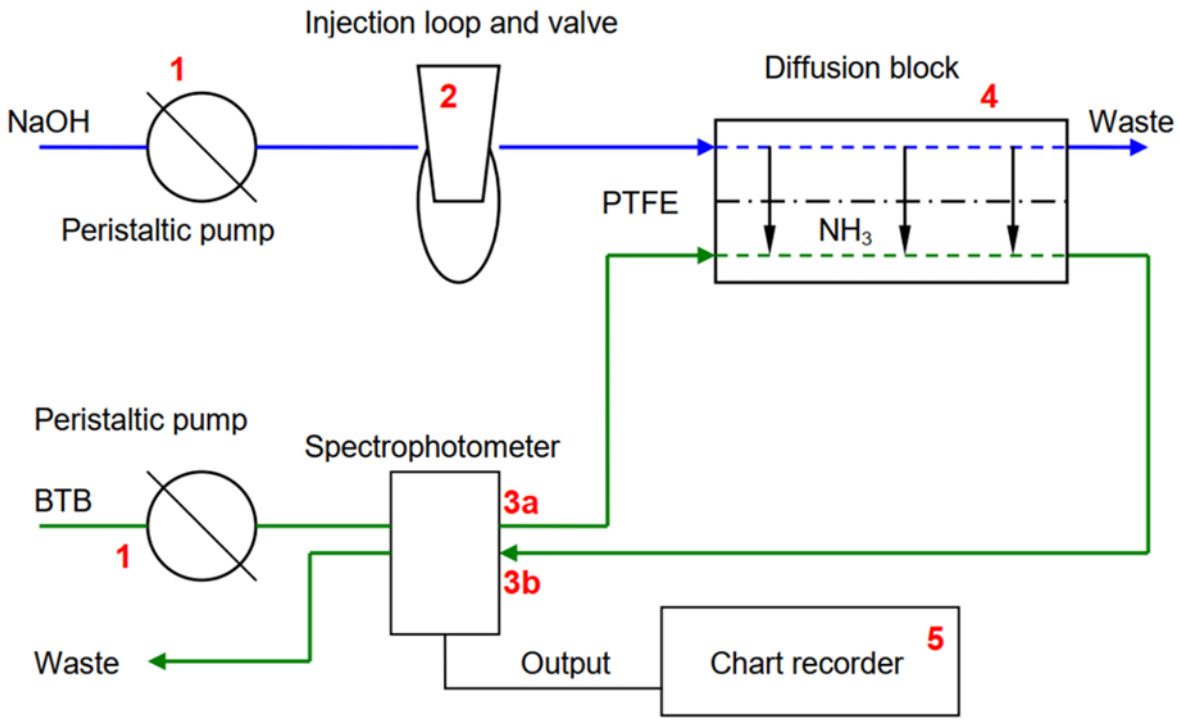


Figure 7 – A) The flow injection analysis equipment used; B) a schematic diagram showing how the flow injection analysis equipment works (Taken from Smyth, 2011)

2.2.5.3 D-Glucose

An enzymatic colorimetric assay kit was used to determine the concentration of glucose in the haemolymph samples (Sigma-Aldrich – Product Code GAGO-20). A spectrophotometer was used to determine the absorbance change at 540nm. The general principle of this enzymatic kit is that glucose oxidase catalyses the reaction between D-glucose, water, and oxygen, to then produce D-Gluconic acid and hydrogen peroxide. The hydrogen peroxide then is involved in an oxidation reaction of o-dianisidine which is catalysed by peroxidase causes a colour change to a light brown, though this colour is deemed to be unstable, and so the sulphuric acid is added to change the colour to pink, which is more easily measured and is much more stable.

A reagent blank was created using deionised water, and 0.05mg/L D-glucose standard was used to determine the unknown concentrations of D-glucose in the haemolymph samples. The reactions were started by adding 250µl of haemolymph sample to a test-tube and adding 500ul of Assay reagent (this is a mixture of glucose oxidase/peroxidase and o-Dianisidine reagents) to the test-tube and incubating the reactions in a water bath at 37°C for exactly 30 minutes. The reactions were then stopped by the addition of 0.5ml of 12M of sulphuric acid. The resulting solution was transferred to a plastic cuvette, and the colour change was measured at 540nm in a spectrophotometer. The absorbency value was then used in the following equation in order to determine the actual concentration of D-glucose (in mg/L) in the sample:

$$\frac{(\boldsymbol{\Delta A_{540} of\ Test})(\boldsymbol{mg\ Glucose\ in\ Standard})}{(\boldsymbol{\Delta A^{540} of\ Standard})}$$

D-Glucose was not measured in the haemolymph of *B. undatum*, as the concentrations were below the limits of detection of this kit (20-80µg/ml).

2.2.5.4 Total Protein

Haemolymph total protein concentrations were determined using a handheld protein refractometer (ATAGO Clinical refractometer SUR-NE Cat. No. 2734). Prior to sample testing, the meter was calibrated by adding 150µl of distilled water to the lower prism slide and adjusting the calibration screw as necessary. After adding 150µl of haemolymph sample to the lower prism slide, the protein concentration was read from where the blue and white portions meet through the viewfinder using the $g.l^{-1}$ scale,

and this result was multiplied by the dilution factor used for the specific haemolymph sample, and then the result was converted to mg/L for consistency. This method is accurate from 0.0 to 12.0 g/100ml.

2.2.5.5 pH

pH was measured immediately following the extraction of the haemolymph from the animal at room temperature. A pH meter (Oakton Waterproof pH Spear Pocket pH Tester) was used to determine the pH of haemolymph and water samples. Calibration was achieved by placing the meter sensor into pH 4, pH 7, and pH 10 buffers at room temperature. Measurements were taken when the pH shown by the meter had stabilised, which had normally occurred by one minute from initial contact to the sample.

2.2.5.6 Behavioural Parameters

Behavioural parameters were derived during this study in order to determine the relative stress levels of the organisms. Different behavioural scales were formed for each species. The parameters were decided based upon previous studies on this topic, and through personal observations of the organisms. The behaviour responses were placed into a response table (Tables 4, 6 & 7) whereby each behavioural response was used to create a scale from 0 – 4 (5 individual categories; *H. gammarus;* Table 6), or 0 – 3 (four individual categories; *B. undatum* and *N. norvegicus*; Table 4 & 7) where an increasing number shows a healthier animal (0-Dead, 3/4- very healthy). The behavioural responses that determined individuals were healthy were formed by comparisons to published data (Spanoghe & Bourne, 1997; Barrento et al., 2009; Woll et al., 2010; Smyth & Uglow, 2015). The behavioural assays for the smaller animals of the study (*B. undatum* and *N. norvegicus*) had one less category, as it was more difficult to observe minor differences in behaviour in these animals, and so the study used a coarser separation in categories. This process somewhat resembles the process used within the industry to determine individuals that are suitable for long-distance transport (Barrento et al., 2011). The behavioural responses were measured every 24 hours, at the same time as haemolymph was extracted from the individuals, in order to reduce stress by unnecessary handling of the organisms.

The behavioural parameters for whelks (Tables 3 & 4) were determined based on the observations made in this study, and loosely adapted from the observations made by Boulter (1999). When emersed the organisms did not attach to any surfaces, or attempt to move considerable distances, and so this was not an option for the behavioural assay. From the observations made in this study, it was determined that siphon movement, and foot reaction to stimulus should be the main indicators of health/stress in the organisms, as these were easily accessible areas of the whelks, and there appeared to be a deterioration in the responses as the animals became more stressed.

Table 3 – Descriptions of different behavioural parameters used to determine the health of *B. undatum* individuals

Behavioural Response	Description
Siphon	A stimulus was applied upon the siphon using an elongated 'pointer', and the strength and speed of the retraction reaction was noted
Body	A stimulus was applied upon the foot and general body surface of the individual using an elongated 'pointer'
Colouration	The animal was removed from the misting environment, and the colouration was observed under bright, uniform lighting conditions

Table 4 – Responses used to determine the health of *B. undatum*

		Health Scale			
		0 (Dead)	**1 (Moribund)**	**2 (Good)**	**3 (Healthy)**
Behaviour	**Siphon**	No reaction to stimulus	Weak reaction to stimulus	Strong reaction to stimulus	Strong reaction to stimulus
	Body	No reaction to stimulus	Little/no reaction to stimulus	Reacts to stimulus, but reaction may not be strong enough to cover shell aperture	Strong reaction to stimulus
	Colouration	More brown	Body colouration has become browner	Body colouration light yellow brown	Body colouration light yellow brown

N. norvegicus and *H. gammarus* share much of the same anatomy, and so share many of the same behavioural responses. The parameters used to form the behavioural assay for the crustaceans were derived both from personal observation and combining the previous assays found in the literature (Spanoghe & Bourne, 1997; Barrento et al., 2009; Woll et al., 2010; Smyth & Uglow, 2015). Much of the behavioural assay for the crustaceans relied upon the appendage movements (Tables 5, 6 , &**Table 7**), as this proved to be the most reliable form of vigour determination.

Table 5 - Descriptions of different behavioural parameters used to determine the health of *H. gammarus* & *N. norvegicus* individuals

Behavioural Response	Description
Tail Flipping	The movement of the tail, paying particular attention to the vigour of the movement, and duration
Appendage Movements	The movement of the appendages (i.e. claws, legs) paying particular attention to the vigour of the movement, and duration
Blood removal	The ease of blood removal with use of a hypodermic needle
Eyestalk Response	The response of the animal's eyestalk when exposed to external stimuli
Mouthpart Closure	The mouthpart closure response of the organisms, and if the organism is unable to close them, aid will be given by manually closing them

Table 6 - Responses used to determine the health of *H. gammarus*

		Health Scale				
		0 (Dead)	**1 (Moribund)**	**2 (Weak)**	**3 (Healthy)**	**4 (Very Healthy)**
Behaviour	**Tail Flipping Behaviour**	No tail flipping	No tail flipping	No tail Flipping	Some tail flipping	Rigorous and sustained tail flipping
	Appendage Movements	No Movement	No movement	Slow movement	Some/rapid movement	Rapid
	Blood removal	Not Possible	Possible, but with slow flow	Possible	Possible	Possible
	Eyestalk Response	No Response	Limited Response	Response	Response	Response
	Mouthpart Closure	No Closure	No closure	Closed with aid	Slow closure	Rapid Closure

Table 7 - Responses used to determine the health of *N. norvegicus*

Behaviour	Health Scale: 0 (Dead)	1 (Moribund)	2 (Good)	3 (Healthy)
Tail Flipping Behaviour	No tail flipping	No tail flipping	No tail Flipping	Rigorous and sustained tail flipping
Appendage Movements	No Movement	No movement	Slow movement	Rapid
Blood removal	Not Possible	Possible, but with slow flow	Possible	Possible
Eyestalk Response	No Response	Limited Response	Response	Response
Mouthpart Closure	No Closure	No closure	Closed with aid	Rapid Closure

2.2.6 Evaluation of AccuTrend Plus Lactate meter

Following extraction of haemolymph from the animals and the centrifugation of the different fractions, the haemolymph fraction that was used for protein analysis was used also for this procedure. The haemolymph sample of 50µl was applied to the test strip that had been placed into the machine as per the included instructions, the lid of the meter was closed, and 60 seconds was allowed to pass before a reading was recorded. This process was carried out for all of the samples from the *Nephrops* and *Homarus* trials. All the tests using this meter were carried out at room temperature. This method of L-lactate determination has a detection range of 0.8-21.7mmol/L and a working temperature range of 15-35°C.

2.3 Statistical Analysis

The statistical analysis for this project was carried out using SPSS statistics software 25, and Microsoft Excel. The tests for normality was carried out on the data using the Shapiro-Wilk test, and showed that many of the data were not normally distributed for the mist experiments, but the data were shown to be normal for the Accutrend tests ($p<0.05$). Therefore, a non-parametric Kruskal-Wallis test with a Bonferroni post-hoc test, and Mann-Whitney U test was used to determine if there were any differences in the stress biomarkers between and within transport methods. PROBIT analysis was used to determine the LT_{10}, LT_{20}, and LT_{50} (the time at which 10, 20, and 50% of animals had died) of each transport method for each species, as a method of comparing mortality data between transport methods. For comparisons between the Accutrend handheld meter and the traditional method for measuring L-Lactate concentration, a Pearson correlation coefficient was calculated, as well as Lin's Concordance Correlation Coefficient (CCC; Lin, 1989) to determine the agreement between the methods. The value calculated by Lin's CCC was then compared against the scale agreed upon by McBride (2005), which then allowed for the classification of the relationship to be either poor (90), moderate (90-95), substantial (95-99), and perfect (>99).

3 Results

3.1 *Buccinum undatum*

The *B. undatum* trials consisted of 3 transport methods: Continuous Misting, Intermittent Misting, and Dry (traditional method of transport for this species), and each trial lasted 96 hours in total.

3.1.1 General Observations:

During transport, this species appears able to survive a wide range of conditions for a short period of time (Table 8). During all three transport trials, it was observed that the male whelks would extend the penis far beyond the aperture of the shell. During both the intermittent and dry simulated transport trials the haemolymph withdrawal became progressively more difficult as the trials progressed . As shown exclusively during the dry trials there were pooling of haemolymph under the skin of some individuals, clearly visible by blue 'mounds' on the skin, commonly on and around the foot. Upon release back into tanks after the tests, the efflux of waste products was apparent as within 1 hour of placement into the environment, the water colour had changed into a yellow/green.

Table 8 - Summary of the mortalities (and proportion of the whole cohort that has died) in each transport trial after every 24 hours for *B. undatum*

Trial	**0h**	**24h**	**48h**	**72h**	**96h**
Continuous misting	0	0	4 (7%)	5 (8.77%)	16 (28.07%)
Intermittent misting	0	0	1 (1.75%)	1 (1.75%)	3 (5.26%)
Dry	0	1 (1.7%)	3 (5.3%)	6 (10.5%)	13 (22.8%)

3.1.2 Between transport method comparisons

A Shapiro-Wilk test for normality, indicated that the data were not normally distributed, so non-parametric Kruskal-Wallis and Bonferroni Post-Hoc tests were used to test for differences between transport methods. Generally, the mean pH was observed to be higher in both misting trails when compared to the dry transport trial. During the experiments, it was observed that haemolymph pH was significantly higher in the continuous misting trial when compared to the dry method at all time intervals ($p<0.05$). At 96 hours the animals had a mean haemolymph pH value of 8.08 in the continuous misting trial, compared to 7.8 in the dry trial. The haemolymph ammonia was also shown to be significantly higher in the dry trial compared to the continuous misting trial at 24, 48, & 72 hours ($\chi^2=8.627, df=2, p<0.05$; $\chi^2=12.045, df=2, p<0.05$; $\chi^2=11.273, df=2, p<0.05$; respectively), but only the intermittent misting treatment was significantly lower than the dry treatment at 96 hours (374 µmol/L compared to 1632µmol/L; $\chi^2 =10.36$, df=2, $p<0.05$). The intermittent mist transport method also had a significantly higher haemolymph ammonia concentration at 48 hours ($p<0.05$). The mean haemolymph total protein concentration was 7571mg/L higher in the continuous misting treatment compared to the intermittent misting trial at 24 hours, and this result was statistically significant ($\chi^2 =6.218$, df=2, $p<0.05$). The mean behavioural score was significantly higher in the continuous misting trial, when compared to the intermittent mist trial at 24, 48, and 72 hours ($p<0.05$), and was also higher than the dry trials at 24, 72, and 96 hours ($p<0.05$). Combined, the patterns in the results show that the hypothesis can be accepted as the mist systems appear to improve the stress levels of the marine mollusc.

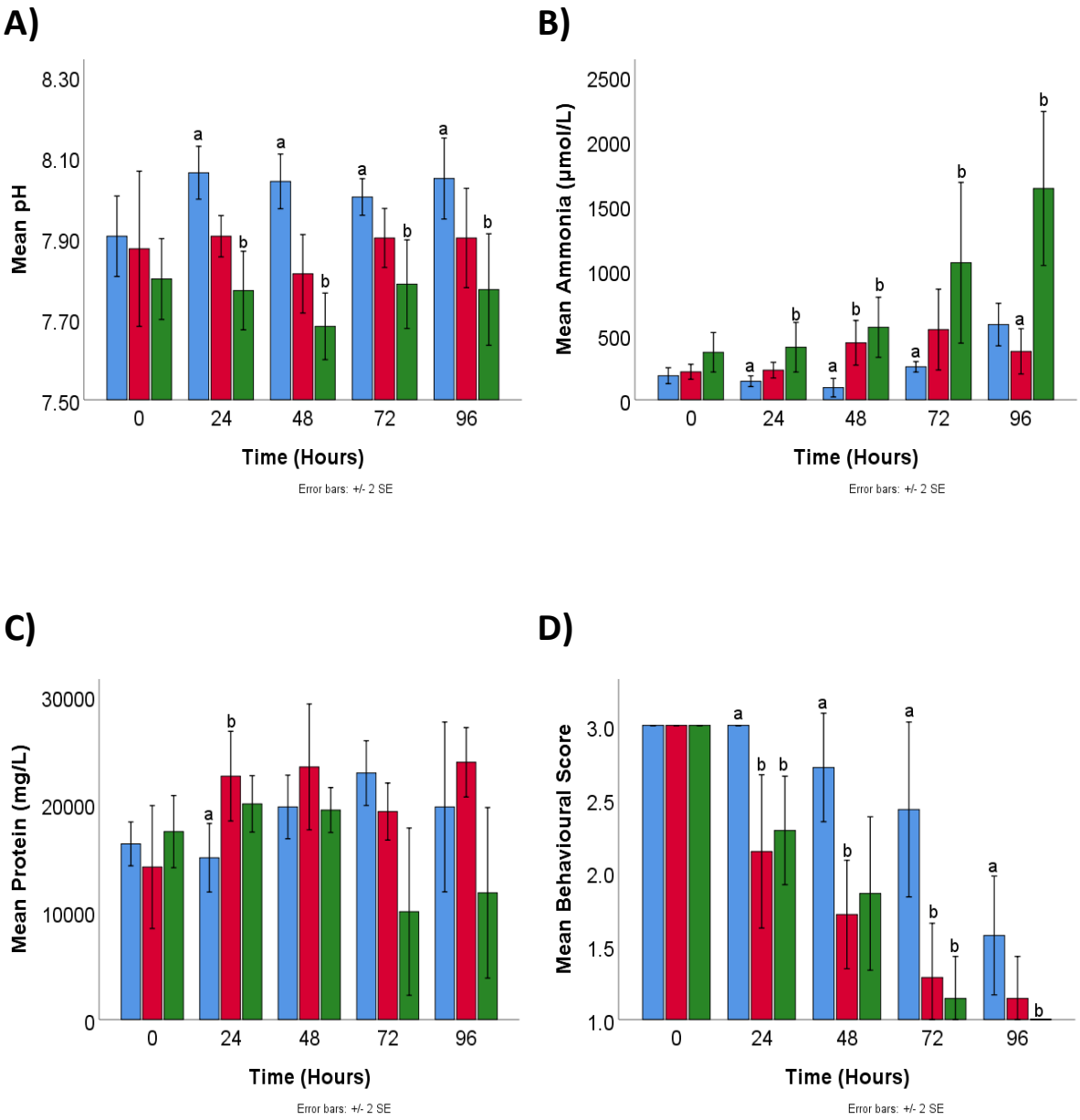


Figure 8 - The differences in measured stress parameters of *B. undatum*, between 3 simulated transport treatments: Continuous Misting – Blue; Intermittent Misting – Red; Dry – Green. The different letters represent a significant difference between treatment groups of the same time period based on post hoc testing. A) The mean haemolymph pH of all 3 treatments every 24 hours from 0 to 96 hours. B) The mean haemolymph ammonia concentration of all 3 treatments every 24 hours from 0 to 96 hours. C) The mean protein concentration of all 3 treatments every 24 hours from 0 to 96 hours. D) The mean behavioural score (a higher number represents a better behavioural response) of all 3 treatments every 24 hours from 0 to 96 hours.

3.1.3 Mortality Rates

The time at which 50, 20, and 10% of individuals would die (LT_{50}, LT_{20}, & LT_{10}, respectively) during transport using each treatment group was determined using PROBIT analysis (Table 9). The LT_{50} for each treatment group is as follows: Continuous Misting 127.3 hours, Intermittent Misting 167.37 hours, and Dry 129.2 hours. LT_{20} Continuous Misting 31.2 hours, Intermittent Misting 71.3, and Dry 33.1 hours. Finally, LT_{10} Continuous Misting 18.4 hours, Intermittent Misting 58.5 hours, and Dry 20.3 hours. Overall, the results show that the intermittent misting method offers a small decrease in mortality rates when the LT_{20} results were compared to the continuous misting, and dry transport methods. However, when the LT_{50} and LT_{10} results observe an overlap in 95% confidence limits between all three groups and so this difference can be considered to be small.

Table 9 - The PROBIT results for each transport method during the *B. undatum* trials

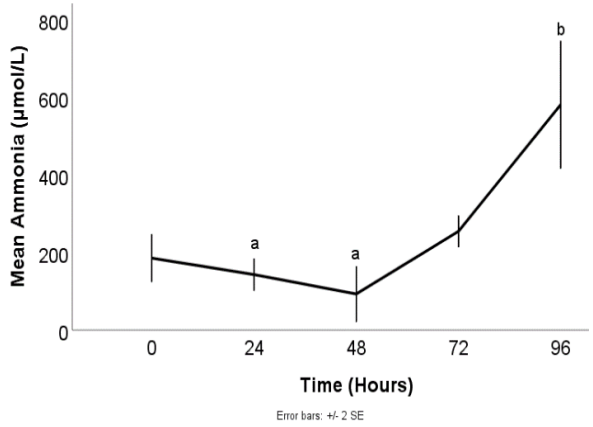
Transport Method	PROBIT ± 95% confidence limit (hours)		
	LT10	**LT20**	**LT50**
Continuous Mist	18.436 (-7.987-34.338)	31.19 (9.317 - 44.975)	127.278(111.633-153.164)
Intermittent Mist	58.526 (35.910-76.821)	71.28 (50.969 - 89.702)	167.368 (141.811-209.366)
Dry	20.313(-5.453-35.953)	33.067 (11.781-46.660)	129.155 (113.048-155.898)

3.1.4 Within transport method comparisons

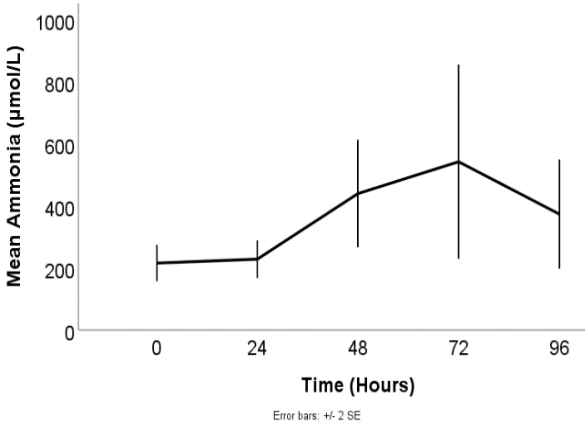
Observations were also made within transport types over the 96 hours, in order to determine any trends in stress parameters over time (Figure 9). Over the course of each trial there was no observed change in haemolymph pH over time ($p>0.05$). However, mean total protein was also only observed to have increased significantly between 24 and 72 hours by 7857mg/L in the continuous misting trial ($\chi^2 = 14.46$, df=2, $p=0.006$), and did not show any statistically significant differences for the other transport types. Behavioural response and ammonia on the other hand were observed to decrease and increase significantly (respectively) within the three transport types over time: behaviour (continuous Misting - $\chi^2 = 21.083$, df=4, $p<0.001$; intermittent misting - $\chi^2 = 23.083$, df=4, $p<0.001$; Dry - $\chi^2 = 27.864$, df=4, $p<0.001$) and ammonia (continuous

misting - χ^2 = 22.714, df=4, p<0.001; intermittent misting - χ^2 = 11.053, df=4, p=0.026; Dry - χ^2 = 14.234, df=4, p=0.007). The Bonferroni Post-Hoc test (Figure 9) shows that the mean haemolymph ammonia was significantly higher after 96 hours, compared to 24 and 48 hours (an increase of 438 µmol/L and 488 µmol/L respectively) of the continuous misting, and compared to 0 and 24 hours (an increase of 1266 µmol/L and 1227 µmol/L, respectively) for the dry trial (p<0.05). The mean behaviour score was significantly less at 96 hours compared to 0, 24, and 48 hours for the continuous misting trial, the behavioural score was lower at both 72 and 96 hours compared to 0 hours for the intermittent misting trials, and finally the behavioural score was significantly lower at both 72 and 96 hours when compared to 0 and 24 hours for the dry trials (p<0.05). Finally, the mean total protein increased from 15,000mg/L at 24 hours to 22,857mg/L 72 hours (p<0.05). These results generally conform with the hypothesis which suggests that the misting environment will reduce stress on marine molluscs, and this may also suggest that the intermittent mist system may not reduce stress in marine molluscs as effectively as the continuous mist system.

A)


Mean Ammonia (µmol/L)
800
600
400
200
0
a
a
b
0
24
48
72
96
Time (Hours)
Error bars: +/- 2 SE

B)


Mean Ammonia (µmol/L)
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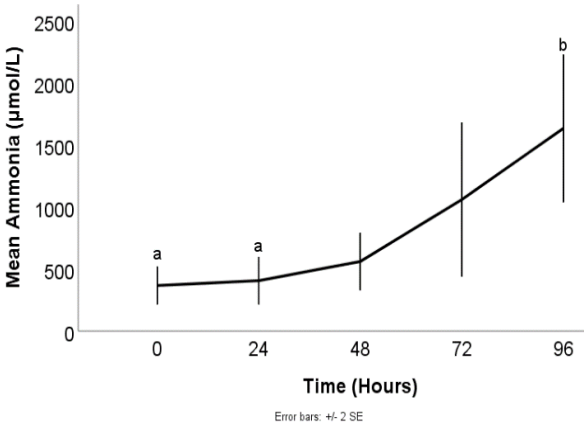
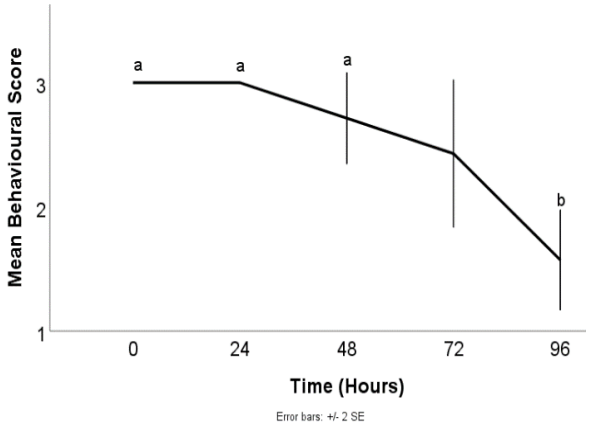
800
600
400
200
0
0
24
48
72
96
Time (Hours)
Error bars: +/- 2 SE

C)


Mean Ammonia (µmol/L)
2500
2000
1500
1000
500
0
a
a
b
0
24
48
72
96
Time (Hours)
Error bars: +/- 2 SE

D)


Mean Behavioural Score
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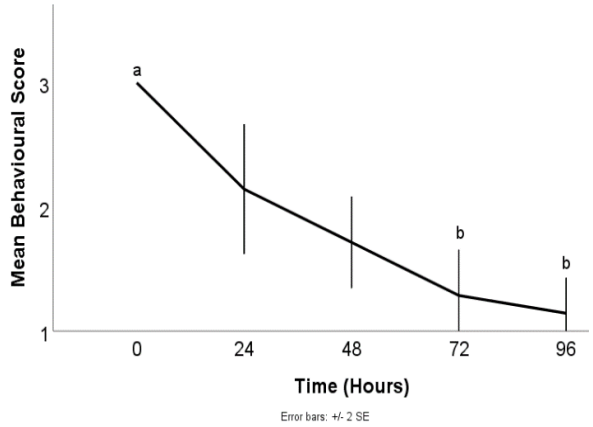
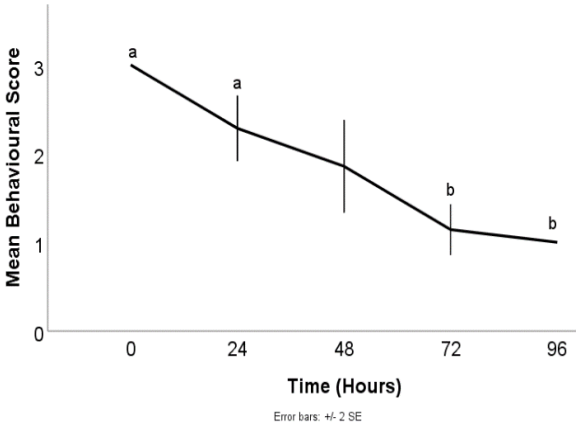
2
1
a
a
a
b
0
24
48
72
96
Time (Hours)
Error bars: +/- 2 SE

E)


Mean Behavioural Score
3
2
1
a
b
b
0
24
48
72
96
Time (Hours)
Error bars: +/- 2 SE

F)


Mean Behavioural Score
3
2
1
0
a
a
b
b
0
24
48
72
96
Time (Hours)
Error bars: +/- 2 SE

G)

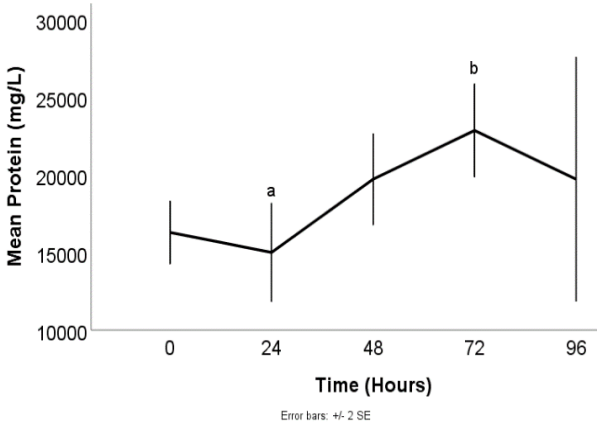


Figure 9 - The change in haemolymph ammonia over the course of the *B. undatum* trials; Continuous mist, Intermittent Mist, and Dry treatments respectively (A – C). The change in behaviour over time during the course of each treatment period; Continuous mist, Intermittent Mist, and Dry treatments respectively (D - F). The change in mean protein over time during the continuous misting trial (G). Differences in each lower case letter shows significant differences between each time period (p<0.05).

3.1.5 Water parameters

The water used in the trials was also tested for changes in pH and ammonia over the course of the tests (Figure 10). The baseline reading for ammonia in the continuous and intermittent trials was 0 µmol/L of ammonia. The ammonia levels increased in both trials, the concentration was 95.74 µmol/L after 24 hours, 505.96 µmol/L after 48 hours, 1016.45 µmol/L after 72 hours, and 1641.58 µmol/L after 96 hours for the continuous misting trials. The concentration of ammonia in the intermittent misting trial was 17.24 µmol/L after 24 hours, 118.89 µmol/L after 48 hours, 127.1 µmol/L after 72 hours, and 186.58 µmol/L after 96 hours. In addition, there was a measured decrease in the pH of the water during the two trials from the baseline of 8.07 for the continuous misting trial, and 7.8 for the intermittent misting trial. During the continuous misting trial the pH was 8.2 after 24 hours, 8.03 after 48 hours, 7.94 after 72 hours, and finally 7.92 after 96 hours. For the intermittent misting trial the pH was 7.7 after 24 hours, 7.6 after 48 hours, 7.52 after 72 hours, and 7.44 after 96 hours (Figure 10). The cavity water was also taken from 4 individuals in the dry simulated transport at 96 hours, and the mean ammonia concentration was 1658.6 µmol/L.

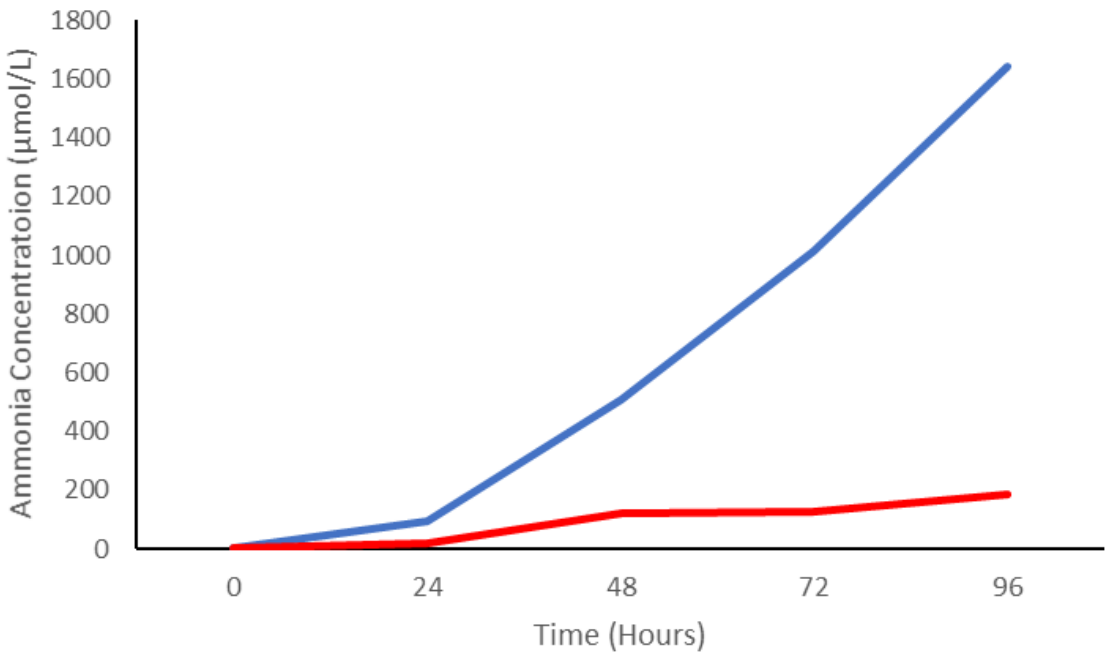


Figure 10 - The change in ammonia concentration in the reservoir for continuous mist (blue) and intermittent mist (red) transport methods of the *B. undatum* trials

3.2 *Nephrops norvegicus*

The *Nephrops* trials consisted of 3 treatment groups: Continuous Misting, Intermittent Misting, and Vivier (traditional method of transport). The intermittent trial was discontinued at 24 hours due-to the high mortalities, whereas the continuous mist and vivier experiments were carried out for 48 hours.

3.2.1 General Observations

There were many cast-off claws during transport and experiments, especially when transferring the individuals from the car to temporary laboratory storage, but the individuals were in good health as shown by vigour analysis, and were deemed suitable to be used in the trials. Generally, the *Nephrops* were very sensitive to perturbations, which was evident in mortalities (Table 10). Smaller trials (N=20 & 10, respectively) were carried out where the animals were placed horizontally and diagonally in the mist system, but this still yielded high mortalities after 24 hours (25%). A small dry trial was carried out, whereby the individuals were placed in tube trays in a polystyrene box surrounded by gel ice packs, but this also yielded a high mortality after 24 hours (20%), and the surviving individuals were described as moribund. Five individuals were placed directly into the reservoir water from the misting trials, in order to determine if there was contamination as a result of contact with the misting system i.e. pathogens, metals etc. All individuals survived this contact, suggesting that the misting system was not polluting the reservoir water, and therefore was not a causative factor in the deaths.

Table 10 - Summary of the mortalities (and proportion of the whole cohort that has died) in each transport trial after every 24 hours for *N. norvegicus*

Trial	**0h**	**24h**	**48h**
Continuous misting	0	5 (10%)	49 (98%)
Intermittent misting	0	15 (30%)	N/A
Vivier	0	0	3 (6%)

3.2.2 Between transport method comparisons

Over the 24 hour test period for this species, there were differences in some stress parameters between the 3 treatment groups. Generally, there was no observable difference between the 3 treatment groups in haemolymph pH, total protein concentrations, and glucose concentrations. Haemolymph ammonia was observed to only increase in the intermittent mist transport method, as this result was 1044 µmol/L higher than the vivier transport method (χ^2 = 17.462, df=2, $p<0.001$). Haemolymph L-lactate concentrations were shown to only increase from 0 mmol/L in the misting trials and the increase in the vivier trial was minimal. L-lactate concentrations was observed to be significantly higher (χ^2 = 13.677, df=2, $p<0.001$) in both the continuous (an increase of 16.33mmol/L; $p<0.05$) and intermittent (an increase of 12.63 mmol/L; $p<0.05$) mist transport method when compared to the vivier transport. The mean behavioural score was shown to be lower in both the misting trial when compared to the vivier transport, but only was the intermittent transport group significantly lower than the vivier group (χ^2 = 8.362, df=2, $p<0.05$). Overall, the patterns in the results suggest that both mist methods are not suitable for reducing stress in *N. norvegicus* when compared to the traditional method of transport (vivier), so for this species the hypothesis must be rejected.

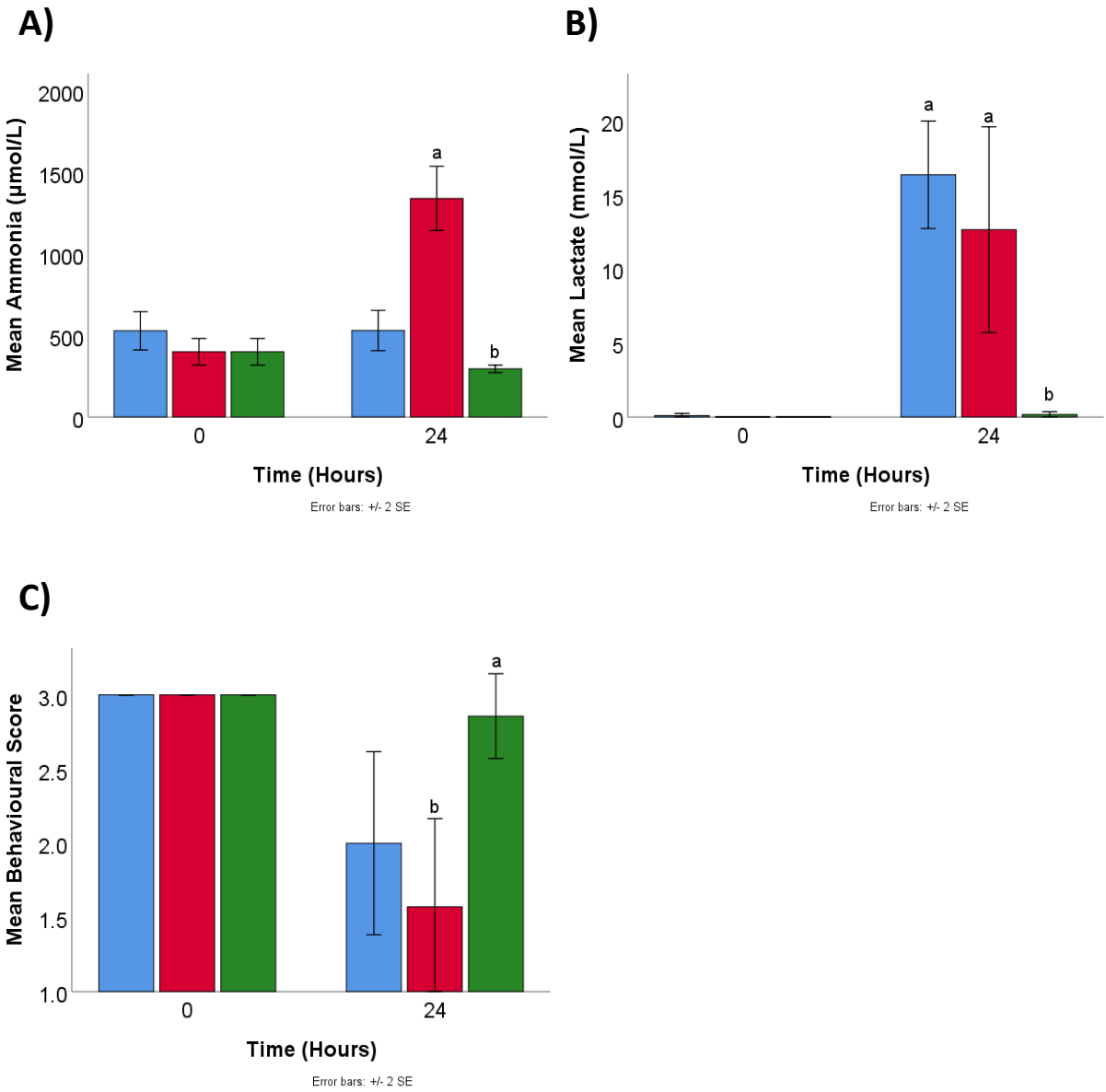


Figure 11 - Stress parameter measurements for *N. norvegicus* between three simulated transport treatments: Continuous Misting – Blue; Intermittent Misting – Red; Vivier – Green. A difference in letters represents a significant differences between measured stress parameters at the same time period based on post hoc testing . A) The mean haemolymph ammonia concentration of all 3 treatments at 0 and 24 hours. B) The mean haemolymph L-Lactate concentration of all 3 treatments at 0 and 24 hours. C) The mean behavioural score (a higher number represents a better behavioural response) of all 3 treatments at 0 and 24 hours.

3.2.3 Mortality rates

The mortality results for the *Nephrops* trials were used to carry out PROBIT analysis, to determine if the rate of mortality differed between tests, which would be signified by no overlap in the 95% confidence limits (Table 11). The LT_{50} for the continuous & intermittent misting, and vivier treatment was 33.42 hours, 27.84 hours, and 64.32 hours respectively. The LT_{20} for each group was: Continuous Misting 18.014 hours; Intermittent Misting 12.427 hours; Vivier 48.92 hours. Finally, LT_{10} Continuous Misting 15.97 hours; Intermittent Misting 10.38 hours; Vivier 46.869 hours. Overall, the results show that both mist systems have higher mortality rates when compared to the traditional vivier transport, but neither mist systems show any significant differences of mortality when compared to each other. These results suggest that the hypothesis must be rejected as the mist systems induce a high level of stress on the crustacean *N. norvegicus* when compared to vivier transport.

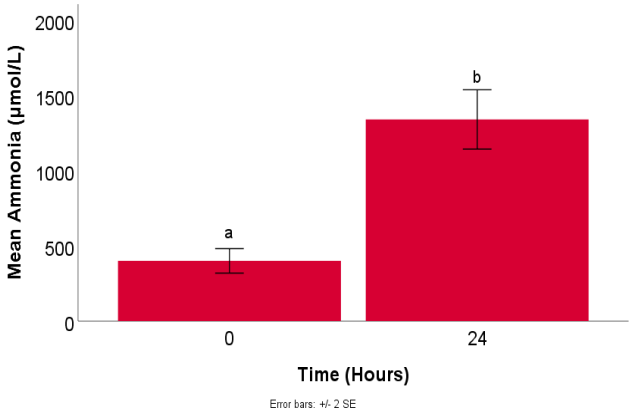
Table 11 - The PROBIT results for each transport method during the *N. norvegicus* trials

Transport Method	PROBIT ± 95% confidence limit (hours)		
	LT10	**LT20**	**LT50**
Continuous Mist	15.97(9.41-20.14)	18.014 (12.1-21.868)	33.42 (30.45-36.817)
Intermittent Mist	10.38(4.403-14.19)	12.427(7.088-15.92)	27.84 (25.09-31.22)
Dry	46.869(39.94-53.613)	48.92(42.16-55.82)	64.32(57.29-73.98)

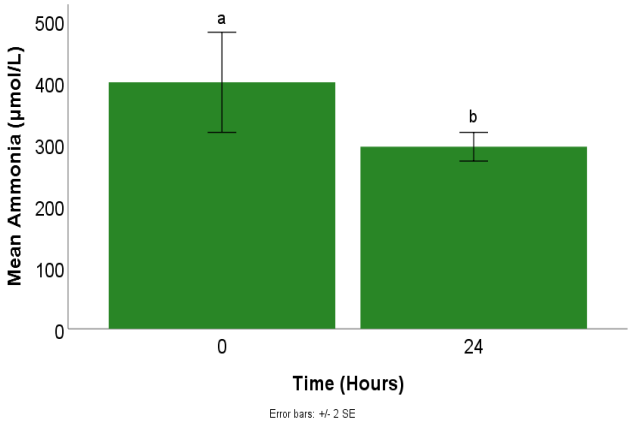
3.2.4 Within transport method comparisons

As there were only two time intervals at which the haemolymph was sampled for this species (0 and 24 hours), a Mann-Whitney U test was used to test for differences between stress parameters within each treatment group (Figure 12). For the continuous misting trial, the behaviour score significantly decreased, whereas concentrations of total protein, and lactate in haemolymph increased significantly over the course of the test ($W=7$, $df=1$, $p=0.026$; $W=40$, $df=1$, $p=0.005$; $W=49$, $df=1$, $p<0.001$). The mean behaviour score decreased from 3 to 2, and mean haemolymph lactate also significantly increased from 0.1mmol/L to 16.3mmol/L, during the intermittent mist trial ($W=3.5$, $df=1$, $p=0.004$; $W=49$, $df=1$, $p<0.001$), but there was no difference in protein concentration during this trial ($p>0.05$). Mean haemolymph ammonia was also observed to be significantly higher at 24 hours (1240 µmol/L) when compared to 0 hours (400 µmol/L) during the Intermittent Mist Trial ($W=49$, $df=1$, $p<0.001$), and haemolymph ammonia concentration was the only stress parameter that was observed to have changed in the vivier transport group, whereby it was significantly lower at 24 hours (295 µmol/L) compared to 0 hours (400 µmol/L; $W=7$, $df=1$, $p=0.026$). Overall, the results suggest that the mist systems showed significant increases in stress parameters over 24 hours, however the vivier transport method showed a decrease in haemolymph ammonia therefore suggesting a lowered level of stress in the animals. Given the patterns in the results, it suggests that the mist systems are not suitable for lowering the stress levels in the crustaceans during transport, and so the hypothesis must be rejected.

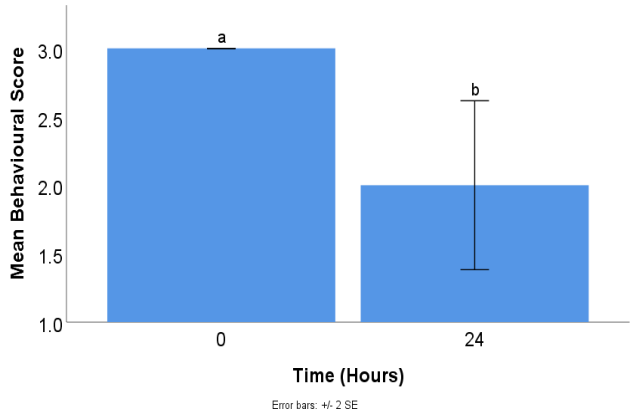
A)


Mean Ammonia (µmol/L)
2000
1500
1000
500
0
a
b
0
24
Time (Hours)
Error bars: +/- 2 SE

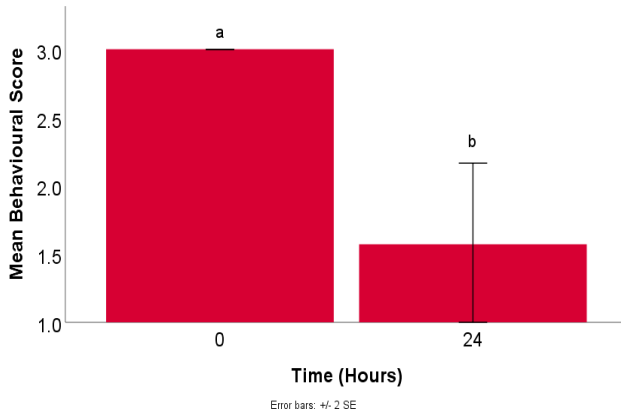
B)


Mean Ammonia (µmol/L)
500
400
300
200
100
0
a
b
0
24
Time (Hours)
Error bars: +/- 2 SE

C)


Mean Behavioural Score
3.0
2.5
2.0
1.5
1.0
a
b
0
24
Time (Hours)
Error bars: +/- 2 SE

D)


Mean Behavioural Score
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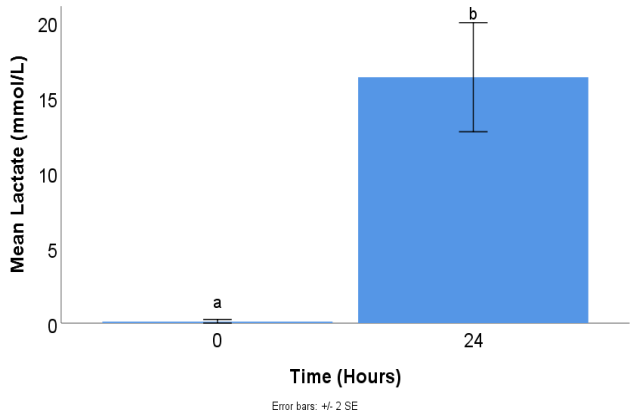
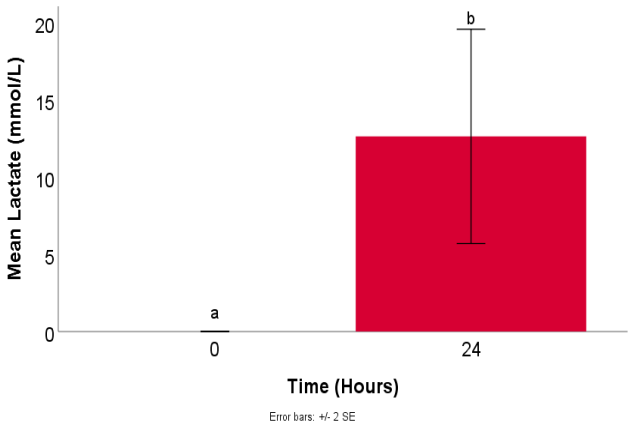
2.5
2.0
1.5
1.0
a
b
0
24
Time (Hours)
Error bars: +/- 2 SE

E)


Mean Lactate (mmol/L)
20
15
10
5
0
a
b
0
24
Time (Hours)
Error bars: +/- 2 SE

F)


Mean Lactate (mmol/L)
20
15
10
5
0
a
b
0
24
Time (Hours)
Error bars: +/- 2 SE

G)

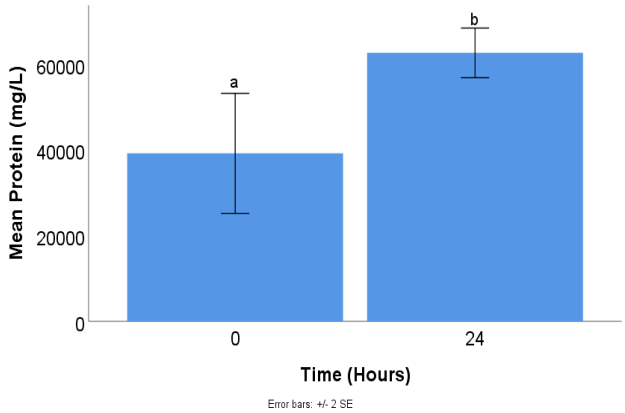


Figure 12 - Changes in mean haemolymph ammonia concentrations over time during the *N. norvegicus* trials; changes in mean haemolymph ammonia during the continuous and dry trials (A & B); changes in mean behavioural score during the continuous and intermittent mist trials (C & D); changes in mean haemolymph L-lactate during continuous and intermittent misting trials (E & F); changes in mean haemolymph total protein during the continuous misting trials (G). Lower case letters show significant differences between different time periods. Red – Continuous misting, Blue – intermittent misting, Green – vivier.

3.2.5 Water parameters

Ammonia measurements were taken for the water from the three treatments. All three treatments showed an increase in ammonia concentration. 46.02 µmol/L was the baseline ammonia concentration for the 3 *Nephrops* trials. The continuous misting test saw an increase in ammonia to 617.04 µmol/L at 24 hours, and the intermittent misting trial increased to 200.72 µmol/L at 24 hours. The ammonia in the vivier transport increased to 466.44 µmol/L at 24 hours.

3.3 *Homarus gammarus*

The *Homarus* trials consisted of 3 treatment groups: Continuous Misting, Intermittent Misting, and Dry (traditional method of transport). The trials lasted 72 hours in total, but also included a 24-hour recovery period.

3.3.1 General observations

H. gammarus appears to be quite well adapted to emersed conditions, which is seen by the low death rates of both the intermittent and dry simulated transport trials (Table 12). There was also a large build-up of waste products in the sump of the continuous misting trial, which was observed when the water changed from clear and relatively colourless, to more translucent dark yellow with a smell that was described as "fishy with a yeast undertone". The extracted haemolymph varied quite greatly in colour from coral pink, to dark blue. Upon re-immersion of the animals, it was also observed that the animals removed detritus from the gill surface/branchial chamber after the trials, which was observed by plumes of brown particulate matter coming from around the mouthparts of the animal; which was observed in most animals upon re-immersion.

Table 12 - Summary of the mortalities (and proportion of the whole cohort that has died) in each transport trial after every 24 hours for *H. gammarus*

Trial	**0h**	**24h**	**48h**	**72h**	**24h recovery**
Continuous misting	0	0	2 (6.66%)	6 (19.98%)	8 (26.66%)
Intermittent misting	0	0	1 (3.33%)	1 (3.33%)	1 (3.33%)
Dry	0	0	0	2 (6.66%)	2 (6.66%)

3.3.2 Between transport method comparisons

During the *Homarus* trials, there were no significant differences in the behaviour, protein, and glucose levels between any of the simulated transport types ($p<0.05$). Generally, the mean haemolymph pH was measured to be lower in the dry transport group when compared to both misting groups. However, only the intermittent group showed a statistically significant increase in pH when compared to the dry group

($p<0.05$), the Bonferroni Post-Hoc test shows that this difference occurred at 24 (an increase of 0.18; $\chi^2 = 8.811$, df=2, $p<0.05$) and 48 hours (an increase of 0.28; $\chi^2 = 11.281$, df=2, p=<0.05). Haemolymph ammonia was shown to be higher in the dry transport group when compared to both of the mist methods, but it was shown to decrease in the recovery period for all 3 treatment groups. The mean haemolymph ammonia was significantly higher in the dry method when compared to the intermittent misting transport group ($p<0.05$), and the increases were observed at 24, 48, and 72 hours ($\chi^2 = 8.24$, df=2, p=<0.05; $\chi^2 = 8.06$, df=2, p=<0.05; $\chi^2 = 7.28$, df=2, p=<0.05; respectively). The dry transport group however, was shown to have a lower haemolymph ammonia concentration of 231.6 µmol/L when compared to the intermittent transport method which had a concentration of 336.8 µmol/L during 24 hour recovery ($p<0.05$). Generally, haemolymph L-lactate was observed to be higher throughout the tests in both the misting groups when compared to the dry transport method. However, there was only a significant increase in L-lactate from 2.7mmol/L in the dry treatment to 26.7 mmol/L in the continuous misting group ($\chi^2 = 9.98$, df=2, $p<0.05$) and this difference was observed at 72 hours ($p<0.05$). Generally, the patterns in the results suggest that the intermittent mist system offers significant reduction in stress when compared to the dry transport method, however the reductions provided by the continuous misting group appear to be quite minor. Given these results, it is likely that the hypothesis can be accepted for the intermittent mist system as it allowed for a reduction in stress parameters throughout the trials when compared to the dry treatment group.

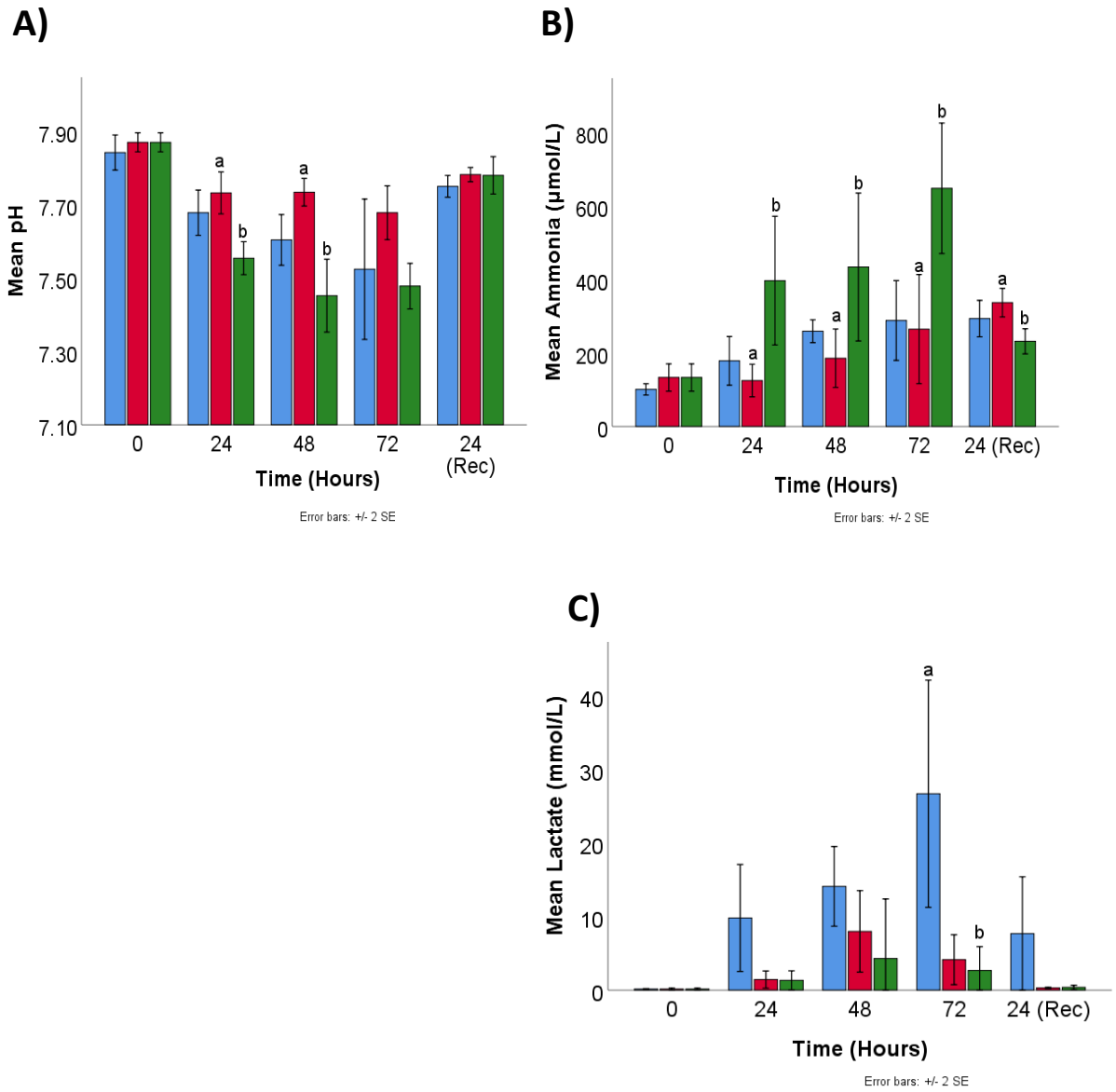


Figure 13 - Stress parameter measurements for *H. gammarus* between three simulated transport treatments: Continuous Misting – Blue; Intermittent Misting – Red; Dry – Green. A difference in letters represents significant differences between measured stress parameters at the same time period based on post hoc testing. A) The mean haemolymph pH of all 3 treatments every 24 hours, between 0 and 72 hours, and after a 24 hour recovery period. B) The mean haemolymph ammonia concentration of all 3 treatments every 24 hours, between 0 and 72 hours, and after a 24 hour recovery period. C) The mean haemolymph L-Lactate concentrations of all 3 treatments every 24 hours, between 0 and 72 hours, and after a 24 hour recovery period.

3.3.3 Mortality rates

The trials for *Homarus gammarus* resulted in the mortalities of some individuals (Table 12), which allowed for the PROBIT analysis for each trial (Table 13). The LT_{50} for the 3 trials was: Continuous Misting: 97.8 hours; Intermittent Misting 119.7 hours; Dry 121.542 hours. The LT_{20} was carried out for continuous misting - 71.8 hours, Intermittent Misting - 93.7 hours, and Dry 95.6 hours. Finally, the LT_{10} was analysed for the 3 trials: Continuous Misting 58.2 hours, Intermittent Misting 80.1 hours, and Dry 82 hours. Overall, the results show that the continuous mist method of transport has a higher mortality rate when compared to the other methods of transport, however because there is overlap between the 95% confidence limits they cannot be regarded as significantly higher mortality rates. These results suggest that the continuous mist environment may increase stress when compared to the dry transport method, but the lack of a significant difference means that the hypothesis cannot be accepted.

Table 13 - The PROBIT results for each transport method during the *H. gammarus* trials

	PROBIT ± 95% confidence limit (hours)		
Transport Method	**LT10**	**LT20**	**LT50**
Continuous Mist	58.2 (40.7 to 72.8)	71.8 (59.4 to 98.7)	97.8 (80.3 to 163.3)
Intermittent Mist	80.1 (61.1 to 120)	93.7 (73.8 to 152)	119.7 (93.3 to 218)
Dry	82 (62 to 122.4)	95.6 (75 to 154.1)	121.542 (94.8 to 219.7)

3.3.4 Within transport method comparisons

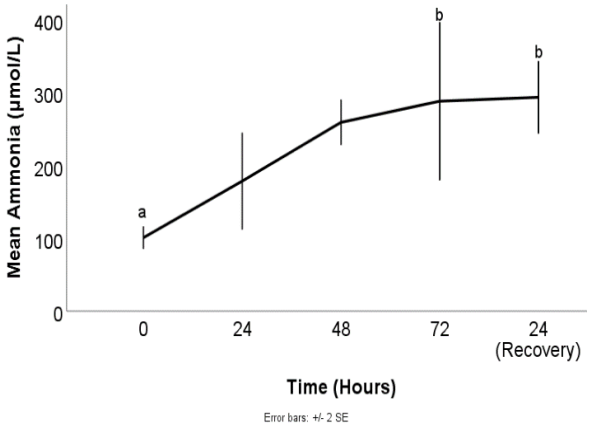
There was no significant change in protein and glucose concentrations over time for any of the treatment groups ($P>0.05$), but there were significant differences measured between the remaining stress parameters. There was a significant decrease in pH over time for all 3 tests: Continuous Misting ($\chi^2 = 18.465$, df=4, $p<0.001$), Intermittent Misting ($\chi^2 = 15.623$, df=4, $p=0.004$), and Dry ($\chi^2 = 19.634$, df=4, $p<0.001$). Haemolymph ammonia concentration had also significantly increased over time for all 3 test environments: Continuous Misting ($\chi^2 = 14.385$, df=4, $p=0.006$), Intermittent Misting ($\chi^2 = 14.171$, df=4, $p=0.007$), and Dry ($\chi^2 = 18.971$, df=4, $p<0.001$). Over time for Continuous

Misting and Intermittent Misting, L-lactate significantly increased (χ^2 = 14.385, df=4, p=0.006; χ^2 = 15.157, df=4, p=0.004), but there was not a significant change in L-lactate over time within the Dry treatment group (p>0.05). There was a significant decrease in behavioural responses in both Continuous misting (χ^2 = 11.669, df=4, p=0.02) and Dry treatment groups (χ^2 = 10.559, df=4, p=0.032), but the exact time at which this significant reduction in behavioural responses occurred during the dry trial is unclear, as the Bonferroni Pairwise test did not show any significance between times (p>0.05). The Bonfferoni Post-Hoc test (Figure 14) shows that there was a significant increase in mean haemolymph ammonia at the 24 hour recovery time period which had a concentration of 293.4µmol/L when compared to 0 hours which had a concentration of 100.7µmol/L for the continuous misting trial (p<0.05). At 0 and 24 hours the mean haemolymph ammonia concentration decreased significantly from 113µmol/L and 124.8µmol/L, respectively, in the intermittent mist trial when compared to 336.8µmol/L the 24 hour recovery period. There was also a significant increase in mean haemolymph ammonia at 48 and 72 hours of 300.5µmol/L and 647.8µmol/L respectively, when compared to 0 hours for the dry trial (p<0.05). pH was significantly lower at 48 and 72 hours compared to 0 hours in all three transport trials (p<0.05). The mean behavioural score was shown to be significantly lower at 72 hours, compared to 0 hours for the continuous misting trials, and despite the Kruskal Wallis test showing a significant difference between the mean behavioural score, the exact causes of difference could not be identified using the Bonfferoni post-hoc test. The mean haemolymph lactate concentration was shown to be significantly higher at 72 hours compared to 0 hours (an increase of 26.6mmol/L) for the continuous misting trial, and significantly higher at 48 hours compared to 0 hours (an increase of 7.8mmol/L) for the intermittent misting trial (p<0.05).

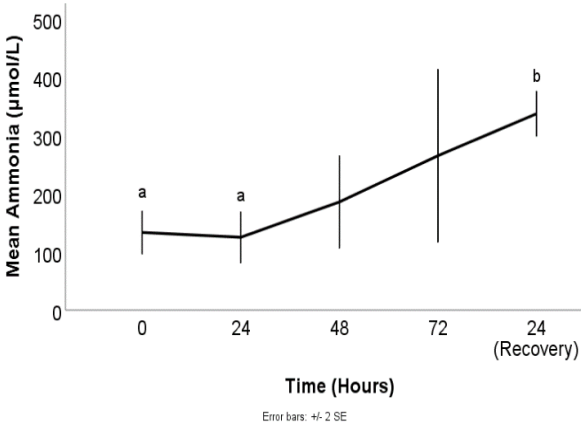
During the *Homarus* trials there were no observed changes in haemolymph glucose or pH (p>0.05). Haemolymph pH was observed to have decreased over time for each of the transport methods, and then an increase was observed after the 24 hour recovery period. The decrease in pH for all three transport methods was significant (continuous mist - χ^2 = 18.465, df=4, p<0.001; Intermittent mist - χ^2 = 15.623, df=4, p=0.004; dry - χ^2 = 19.634, df=4, p<0.001), with the Bonferroni Post-Hoc test showing that the decreases in pH were all observed at 48 and 72 hours (p<0.05) from the beginning of the tests. There was an observed increase in haemolymph ammonia concentration for all three

transport methods over the period of the tests, and it only decreased in the recovery period for the dry transport group. The haemolymph ammonia increased significantly for all 3 transport methods (continuous mist - $\chi^2 = 14.385$, df=4, p=0.006; intermittent mist - $\chi^2 = 14.171$, df=4, p=0.007; dry - $\chi^2 = 18.971$, df=4, p<0.001), the Bonferroni Post-Hoc test showed the increases occurred between 0 hour, and the 72 hour and 24 hour recovery period (p<0.05) for the continuous mist, as there was an increase of 187.4µmol/L and 192.7µmol/L respectively. The intermittent mist transport method showed a significant increase in haemolymph ammonia after the 24 hour recovery period when compared to 0 and 24 hour time intervals (an increase of 203.7µmol/L and 212µmol/L, respectively; p<0.05). The dry treatment method showed a significant increase in haemolymph ammonia from 133.1µmol/L at 0 hours to 433.6µmol/L at 48 and 647.8µmol/L at 72 hours (p<0.05). Mean L-lactate was shown to increase over the period of the experiments for all 3 transport methods, and decrease after the 24 hour recovery period. The significant increases in L-lactate concentrations were only observed in the continuous ($\chi^2 = 14.385$, df=4, p=0.006) and intermittent misting groups ($\chi^2 = 15.157$, df=4, p=0.004), the increases occurred at 72 hours in the continuous misting trial (an increase of 14mmol/L) and at 48 hours from the beginning of the tests in the intermittent misting trial (an increase of 7.7mmol/L ;p<0.05). The mean behavioural score generally decreased over the period of each transport trial, and increased after the 24 hour recovery period. Both the continuous mist and dry transport methods showed a significant decrease in behavioural response scores ($\chi^2 = 11.669$, df=4, p=0.02; $\chi^2 = 10.559$, df=4, p=0.032; respectively), but there was no significant statistical change over time in the intermittent mist transport group (p>0.05). The Bonferroni post-hoc test could not determine the time intervals at which the decrease in behavioural score occurred for the dry transport group, but showed that the significant decrease of 1.8 occurred between 0 and 72 hours for the continuous mist transport method (p<0.05). Overall, the results suggest that the intermittent mist method is sufficient at maintaining a low stress level in the *H. gammarus* individuals during transport, but the continuous mist method may not be as effective when compared to the dry treatment. Therefore, the hypothesis can be accepted as the intermitted mist system did not allow for an increase in stress parameters when compared to the dry system.

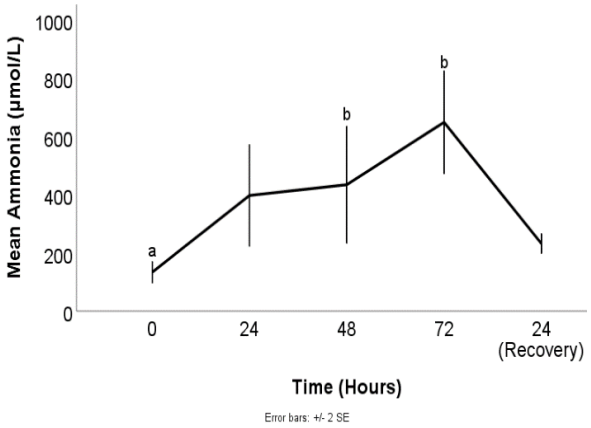
A)


Mean Ammonia (µmol/L)
400
300
200
100
0
a
b
b
0
24
48
72
24
(Recovery)
Time (Hours)
Error bars: +/- 2 SE

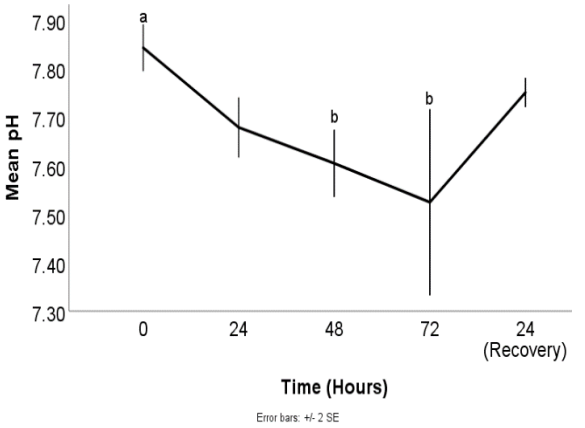
B)


Mean Ammonia (µmol/L)
500
400
300
200
100
0
a
a
b
0
24
48
72
24
(Recovery)
Time (Hours)
Error bars: +/- 2 SE

C)


Mean Ammonia (µmol/L)
1000
800
600
400
200
0
a
b
b
0
24
48
72
24
(Recovery)
Time (Hours)
Error bars: +/- 2 SE

D)


Mean pH
7.90
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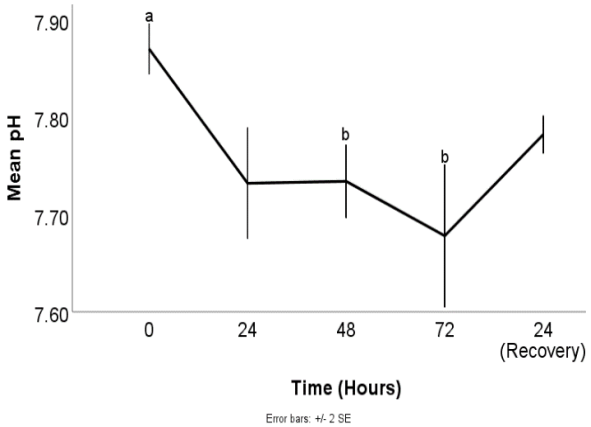
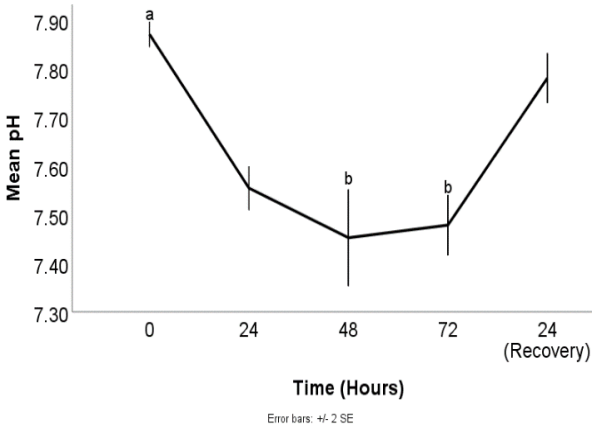
7.70
7.60
7.50
7.40
7.30
a
b
b
0
24
48
72
24
(Recovery)
Time (Hours)
Error bars: +/- 2 SE

E)


Mean pH
7.90
7.80
7.70
7.60
a
b
b
0
24
48
72
24
(Recovery)
Time (Hours)
Error bars: +/- 2 SE

F)


Mean pH
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7.70
7.60
7.50
7.40
7.30
a
b
b
0
24
48
72
24
(Recovery)
Time (Hours)
Error bars: +/- 2 SE

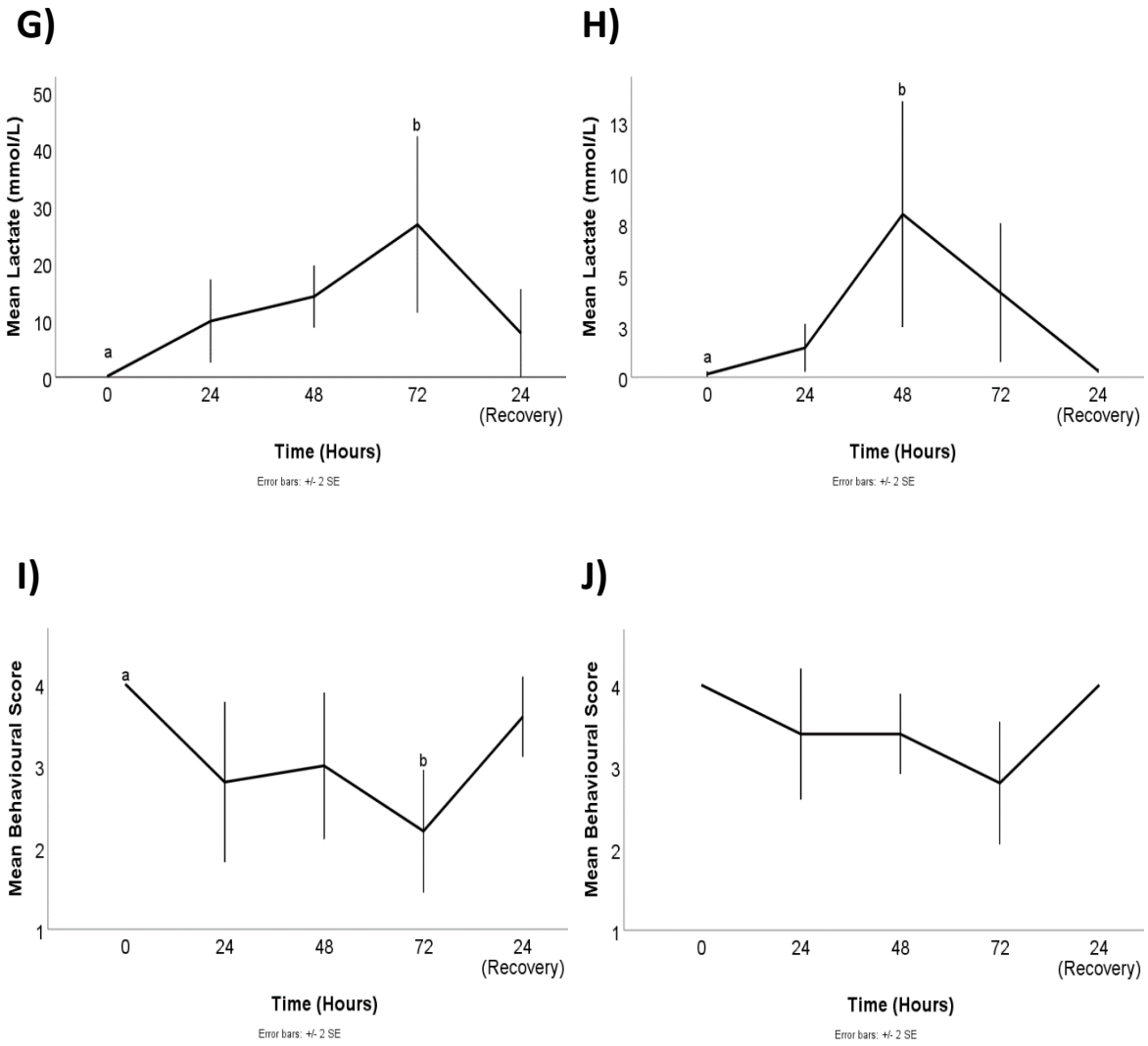


Figure 14 - Changes in mean haemolymph ammonia concentrations over time during the *H. gammarus* trial; Continuous mist, Intermittent mist, and Dry respectively (A-C). Changes in mean pH over time for each trial; Continuous mist, Intermittent mist, and Dry respectively (D-F). Changes in haemolymph L-lactate over time for each trial; Continuous mist, and Intermittent mist respectively (G-H). Changes in behaviour over time for each trial; Continuous misting, and dry trials, respectively (I-J). Differences in each lower case letter shows significant differences between each time period (p<0.05).

3.3.5 Water parameters

Both pH and ammonia were measured as reservoir water quality parameters for both of the misting trials for this species. The baseline ammonia concentration for both trials was 0 µmol/L, and both trials saw an increase in ammonia concentration overall (Figure 15). At 24 hours during the continuous misting trial, the ammonia concentration was 760.07 µmol/L, 1085.48 µmol/L at 48 hours, and after a full water change at 72 hours, the ammonia was measured at 1309.65 µmol/L. The intermittent misting trial saw an increase to 186.23 µmol/L at 24 hours, 169.11 µmol/L at 48 hours, and 429.3 µmol/L at

72 hours. The pH was measured to have decreased over the course of both misting trials, whereby the baseline pH was measured at 8.1 for the continuous and 8.05 for the intermittent trials. For the continuous misting trial the pH was 7.85 at 24 hours, and 7.75 for both the 48 and 72 hour time intervals, even after the water change at 48 hours. The pH for the intermittent trial was 7.35 at 24 hours, and 7.3 for both the 48, and 72 hour time intervals.

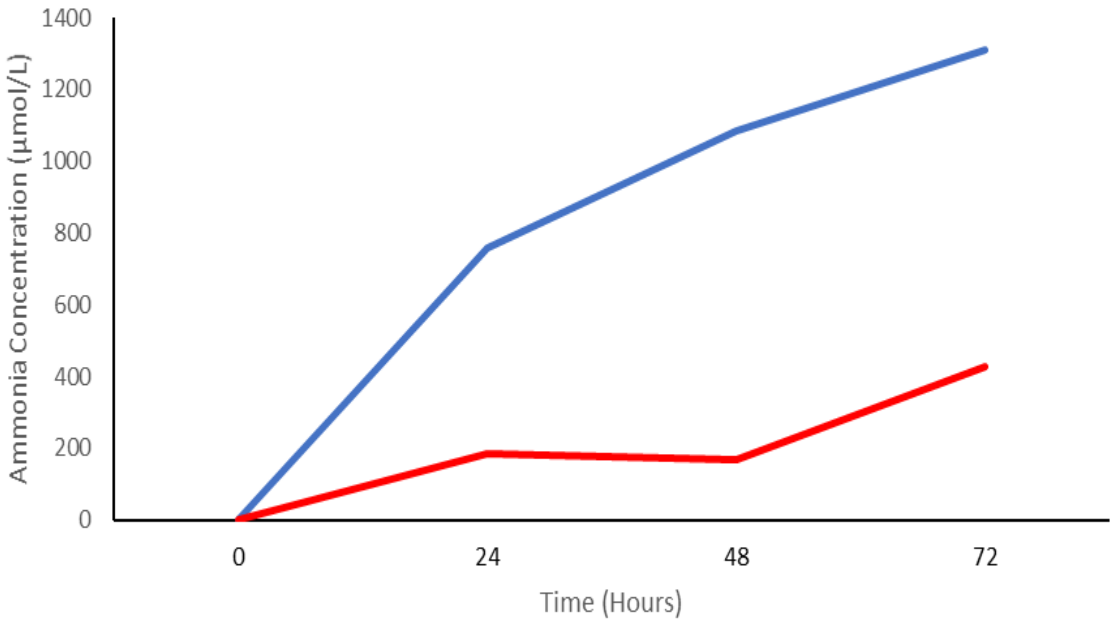


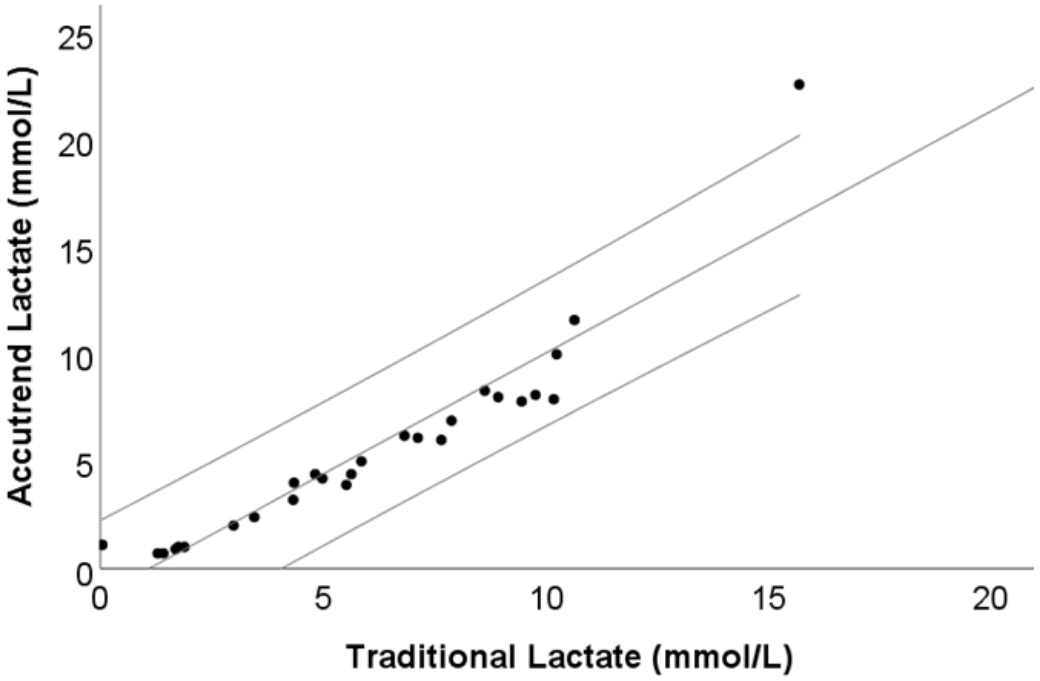
Figure 15 - The change in ammonia concentration over time in the reservoir of the continuous mist (blue) and intermittent mist (red) transport methods for the *H. gammarus* trials

3.4 Accutrend Handheld Meter Validation

The lactate measurements from this study were also used to determine the reliability and accuracy of the Accutrend Plus handheld meter for measuring haemolymph Lactate against the traditional method of using an enzyme assay kit. Initial tests of the current study showed that the meter may not be suitable for some gastropod and bivalve species (*Buccinum undatum* & *Mytilus edulis*), as the levels of L-Lactate appears to be below the meter range of detection, as D-Lactate may be the principle product of anaerobic respiration in these species (Baldwin et al., 1981; O'Omolo et al., 2003). The

concentrations of L-lactate in the whelks were below the detection limit of the handheld meter, and D-Lactate was likely the prevailing metabolic end product. However, the handheld kit was able to measure the haemolymph lactate levels of the 2 decapod crustaceans (*N. norvegicus* & *H. gammarus*) in this study after a period of emersion. Due to the size of the animals, it was not always possible to draw sufficient whole blood to use for all of the analyses required in this thesis, so the haemolymph was often diluted 1:1 with deionised water in order enable all the required tests. Unfortunately, it was later found that the 1:1 dilution with deionised water meant that the levels of lactate were often lower than the detection range of the meter, hence the handheld meter was not able to measure all the samples collected. Correlations were calculated between the Accutrend meter and the traditional method for 28 *H. gammarus* (N=28) individuals and 13 *N. norvegicus* (N=13) individuals for 1:1 diluted blood (no dilution correction was used for either method). The results for the *H. gammarus* trial shows a highly significant positive correlation ($r=0.94$, $p<0.001$; Figure 16) and a moderate Concordance Correlation Coefficient (CCC; 0.92). However, the *N. norvegicus* results yielded a weaker but still statistically significant correlation ($r= 0.86$, $p<0.001$; Figure 16), and a poor CCC (0.9).

A)



B)

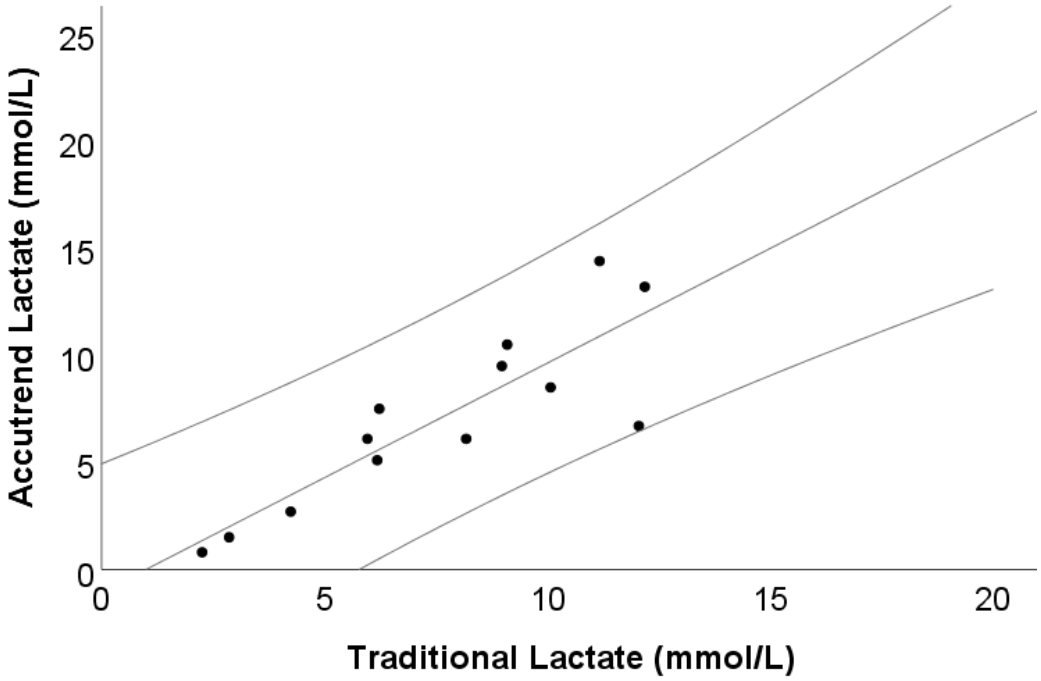


Figure 16 – A) The correlation between the traditional method of L-Lactate determination, and the handheld Accutrend Lactate meter for *H. gammarus*. 95% confidence intervals are displayed. B) The correlation between the traditional method of L-Lactate determination, and the handheld Accutrend Lactate meter for *N. norvegicus*. 95% confidence intervals are displayed.

4 Discussion:

4.1 Buccinum undatum:

The present study is the first (to the best of the authors knowledge) to test the effects of emersion on *Buccinum undatum*. Other studies on the emersion of gastropods (*Littorina spp.,* and *Monodonta turbinata*) have been limited to osmotic regulation, ionic regulation, and oxygen consumption (Micallef, 1967; Rumsey, 1973). Therefore, no meaningful comparisons can be made with the literature for *B. undatum* as the present study has a focus on metabolic end-products and acid-base status. During the *Buccinum* trials, the mean haemolymph pH was significantly higher in the continuous misting trial (at pH 8.05) compared to the dry transport method (at pH 7.8). This may be explained by arise in haemolymph $ppCO_2$ and D-Lactate within the haemolymph of the animals during dry transport. H^+, HCO_3^-, and CO_3^{2-} are formed when CO_2 is in solution, and at a higher $ppCO_2$ (hypercapnia) the concentration of H^+ ions within the haemolymph increases (Whiteley & Taylor, 1990). This reduces the pH as the haemolymph becomes more acidic, as was also seen in the gastropod *Littorina littorea* (Rumsey, 1973). Knowledge of this chemical reaction would suggest that a lowering of pH would occur over time within the transport methods, which was not the case for this study. This may be explained by a robust compensatory mechanism to maintain a stable pH, such as the mobilisation of $CaCO_3$ which raises levels of HCO_3^- therefore reacting with H^+ ions in the haemolymph to increase pH which is believed to be the case in marine decapods (Whiteley & Taylor, 1990). This process has also been hypothesised to occur in marine gastropods (Rumsey, 1973). More CO_3^{2-} ions are formed through the mobilisation of $CaCO_3$ in the haemolymph, which may react with the H^+ ions formed from a high $ppCO_2$, therefore reducing the concentration of H^+ ions (Whiteley & Taylor, 1990; Taylor & Waldron, 1997). This mechanism is similar to that observed over-time in species affected by ocean acidification (Duquette et al., 2017), but the effects of ocean acidification are over a longer period of time, and therefore not comparable to this study. It has been suggested that marine gastropods resist haemolymph pH changes in relation to environmental changes when compared to other animal groups (Mangum and Lykkeboe, 1979; Magnum and Polites, 1980). Magnum and Lykkeboe, (1979) also suggest that below 6°C the heart of some gastropods stop beating, and the metabolic and oxidative rates drop significantly which may explain the lack of change in pH. This

hypothesis suggests that the levels of ammonia would be minimal as a result of reduced production/metabolism, especially in the external water environment during the misting trials but this was not the case, with levels of external ammonia increasing in both misting transport simulations. The higher pH in the continuous mist trial compared to the dry transport simulation may however have resulted from the free mixing of water and haemolymph in the cephalopedal sinus (Mangum, 1979). Despite this the internal pH would be expected to closely resemble the pH of the external water in this case, which was not wholly true as the haemolymph ammonia was lower than in the external environment. The haemolymph pH was more acidic in the intermittent mist group where the external water was observed to be more acidic, so this suggests that water mixing may occur in the foot of *B. undatum,* and to some extent influence the pH of the haemolymph extracted from the cephalopedal sinus. This finding may have implications for the haemolymph withdrawal sites in future studies, as the high fluctuations in haemolymph stress parameters found in this species, may have been as a result of this mixing.

During the *B. undatum* trials, there were also significant differences between haemolymph ammonia concentrations. The continuous misting system appears to be more efficient for the removal of ammonia from the whelks when compared to the dry transport system for the short term (<72 hours), but it may be that the intermittent system is a more efficient method that allows for ammonia removal over longer term (96 hours), when compared to the dry transport method. The longer-term benefits of the intermittent system may result from the lower external ammonia levels in the reservoir water, when compared to the continuous misting trial, as the water is in contact with the animals for a much shorter period during the trials to allow for ammonia efflux. This study suggests that the efflux of ammonia is dependent on the presence of water in the external environment, as the dry transport group consistently had the highest haemolymph ammonia levels, which allows for the increase in the ammonia debt (the increase/storage of ammonia within the body) associated with the emersion of marine molluscs (Shick et al., 1988; O'Donnell, 2011). Both mist systems reduced the ammonia debt to differing degrees during this study when compared to the dry trial. It is thought that the primary excretion site in marine gastropods is the gill (Mangum & Polites, 1980; Taylor & Andrews 1988). The current study also suggests that

marine molluscs are at least partly able to remove ammonia from the body, to the external environment against a concentration gradient, as the external water ammonia levels often exceeded those found in the haemolymph. It may be that ammonia is still removed by passive diffusion in the presence of high external ammonia concentrations, as it is believed that the Na^+/K^+ ATPase found on the baso-lateral (it is unlikely to be found on the apical portion; Hunter & Kirschner, 1986) portion of the epithelial cells (Regnault, 1987; Towle & Holleland, 1987; Weihrauch et al., 2009) actively pump ammonium ions into the intracellular environment of the epithelial cells from the haemolymph, as NH_4^+ replaces K^+ in the ATPase complex (Towle & Holland, 1987; Weihrauch et al., 1999; Weihrauch et al., 2009). The need for water in the external environment of the whelk for ammonia excretion may be to form a concentration gradient between the internal environment of the whelk, and the external environment. An active mechanism for ammonia excretion from the apical portion of the cell has been suggested, through the replacement of protons with ammonium ions in the Na^+/H^+ ATPase complex (Weirauch et al., 2009). This agrees with this study which observed that the whelks are able to excrete ammonia against a concentration gradient into the external environment. However, this remains uncertain, as the results from Hunter & Kirschner (1986) did not provide any evidence for the presence of the membrane-spanning pump on the apical portion of the epithelium of *Mytilus californianus* and *Nephtys caecoides.* The excretion pathways of molluscs are much less understood when compared to that of crustaceans, whereby most of the believed excretion mechanisms for this phylum has been extrapolated from the known structures of marine crustaceans. More work needs to be done to verify whether there are differences between marine crustacean and mollusc ammonia excretion pathways, and to explain how *B. undatum* can excrete ammonia seemingly against a concentration gradient.

There was also a difference in the level of mortality during each trial. The intermittent misting trial saw the lowest mortality of *B. undatum*, which was confirmed by the LT_{10} and LT_{20} results which showed no overlap between the intermittent misting and continuous misting, and there was no overlap of the LT_{20} between the dry and intermittent transport groups. This difference could not be explained solely by the tested parameters of this study, as the stress parameters, including behaviour, all suggest that continuous misting is good at reducing stress when compared to the dry

treatment. This inconsistency in the results suggests that more stress parameters may be required to gain a holistic view of what may have influenced the deaths in this study. D-Lactate is believed to be the principle metabolic end-product in some molluscs (N*assarius coronatus* & *Haliotis Midae*; Baldwin et al., 1981; O'Omolo et al., 2003) and this also appears to be the case in *B. undatum*, as little/no L-lactate could be measured in this species. D-Lactate would give a better understanding of the metabolic function within the animal, and would suggest whether anaerobic, or aerobic respiration prevails in the misted environment. However, high external ammonia concentrations: 1641.58 µmol/L at 96 hours in the continuous mist reservoir, and 1659 µmol/L at 96 hours in the cavity water of the dry transport group may be a stressor that induced deaths in both transport trials. Definitive causal relationships regarding the high mortality of the continuous misting and dry simulated transport conditions (28.07% and 22.8%; respectively), and the stressors cannot yet be made without further investigations. The high death rates do not seem to be linked to the behavioural score for this species, as the behavioural score was the highest in the continuous misting environment, which also had the highest death rate. This raises the question of the efficacy of using a behavioural assay to determine the health of *B. undatum* and marine gastropods in general. The behavioural assay used in this study is similar to that used by Boulter (1999) which did appear to be sufficient at determining the organoleptical (the taste of the whelk when cooked) aspects of the animals. The inaccuracy of the behavioural parameters at determining the health of the animals may have been due-to the subjective nature of the assay, and the limited number of ways that the behaviour could be measured in an emersed environment due-to the lack of observable appendages.

In conclusion for the whelk trials, it appears that based on mortality results alone intermittent misting offers an increased survival over the traditional method of transport which is dry in net bags. In turn, this may offer potential increases in profit to the industry, purely due to a lower mortality rate. However, the benefits for pH and reduced ammonia debt over the short-term for the continuous misting trials should not be overlooked. Further investigation may offer improvements in animal health, condition, and welfare during both short and long term holding and transport. In order to derive the benefits from this system, the cause of death of the animals in the continuous mist must be determined by further investigations into different stress

parameters. It is suggested that the intermittent misting system be implemented into transport systems in its present condition and this is likely to see improved survivability over long transport durations in a commercial setting.

4.2 Nephrops norvegicus:

The survivability of *Nephrops* was very low during the misting trials when compared to the simulated vivier control group. It is known that *N. norvegicus* are more sensitive to disturbance when compared to more 'robust' decapod crustaceans such as *H. gammarus* and *C. pagurus*, as when the animals are transported dry via air freight, they are often sold as 'fresh' rather than 'alive' because of their poor condition upon arrival at the destination market (Bernasconi, 2006). This observation is in line with the condition of the animals after 24 hours in the misting system, however other studies have shown that *N. norvegicus* is able to survive during emersed conditions for up to 72 hours in chilled polystyrene boxes (Spicer et al., 1990; Bernasconi & Uglow; 2008a, 2011). Survival beyond 24 hours in emersed conditions was not possible during this study even when the methods of the studies were replicated on a much smaller scale, with the only observable difference in experimental design being that the animals were stored in tube trays. The PROBIT analysis of the mortalities observed in this study suggest that both misting treatments have a significantly higher mortality than the simulated vivier transport group, as there was no overlap between the LT_{50}, LT_{20}, and LT_{10} between the vivier control transport trial, and both misting treatments. A previous misting trial on this species (Jacklin, 1996) also showed similar mortality results (88% survival after 23 hours and 9% survival after 33 hours following storage for 8 hours in dry conditions), but this study also included stressors in the form of vehicular movement which have recently been shown to have an influence on stress biomarkers, and post-transport survivability (Powell et al., 2017). The results from Jacklin (1996) may suggest that the mist system may not be suitable for long term (>24 hours) storage or transport, based on the mortality results when the animals were in the mist. The mortality results from both misting trials of the current study are also comparable to that of Spicer et al., (1990). In the latter, *Nephrops* were stored emersed at 10°C. This temperature is twice that used in the current study, and because current literature on *Nephrops* suggests that mortality is reduced at lower temperature (Spicer et al., 1990; Ridgeway et al., 2006) it was expected that a better survival would occur in the current trial with the lower

temperature. This was not, however, the case. The mortality results from this study when compared to those in the literature suggests 2 possible causes of death: 1) the positioning in tube trays may induce a physiological response that may reduce survivability whilst emersed, and/or 2) the mist may induce an increase in metabolism, which may cause an increased mortality, when compared to dry storage/transport. There have been no previous studies that have tested the effects of tube trays or the vertical positioning of *Nephrops* on mortality during emersion, and so conclusions cannot be made regarding the effects tube trays may have on the survivability of *Nephrops* during emersion.

With regard to possible metabolic changes the stress parameters measured for the *Nephrops* trial suggest that there is an efflux of ammonia during the continuous misting trial, which is comparable to that of the vivier transport trial. Both the continuous and intermittent misting trials showed evidence of ammonia efflux, as the levels of ammonia in the external water increased for both trials, but there was a much larger increase in the continuous misting trial. This suggests that the increased volume of water being sprayed resulted in an increase in ammonia efflux, which is also evident in the mean haemolymph ammonia concentrations. The increase in haemolymph ammonia concentration from 0 to 24 hours in the intermittent mist treatment was very high, and the concentration change from 0 to 24 hours was also much higher (approximately 700µmol/L) than the ammonia change observed in the literature for dry emersion during the same time period (Bernasconi & Uglow, 2008a); this is despite the evidence of an ammonia efflux in the reservoir water in the present study. This result may also suggest that the metabolic rate of the animals in the present study were much higher than those in the Bernasconi & Uglow (2008a) trial, suggesting that the mist may have a direct effect on the metabolic rate of the animals. Jacklin (1996) suggests that the misting system allows for the efflux of ammonia as a decrease in haemolymph ammonia was observed when the animals were transferred to a misted environment from a dry environment. The finding that the mist allows for the efflux of ammonia is supported by the present study, where levels of ammonia in the haemolymph were comparable during the continuous misting trial to those found by Jacklin (1996), and high levels of ammonia were found in the reservoir of the current study. The efflux of ammonia may be linked to the droplet size of the mist, as this study and the Jacklin (1996) study have relatively

coarse droplet sizes (50 and 150 µm, respectively). However, previous misting trials, for example on the brown crab, *C. pagurus,* were performed with smaller droplet sizes (Dr. K Smyth, pers. Comm; Dr. RF Uglow, pers. comm.) where the mist was often described as a 'fog' or 'dry mist', and this treatment did not show any efflux of ammonia (Smyth & Uglow, 2015). Further, a study is needed to determine whether ammonia efflux, as well as survival rate and other metabolic markers, are affected by mist droplet size.

The haemolymph L-lactate concentration was also measured to be very high in both misted environments compared to the simulated vivier transport in this study. This may suggest that the flux of oxygen into the animals is minimal, and anaerobic respiration is the prevailing metabolic function in misted animals. This result is expected, as the studies on emersion in this species all show an increase in L-lactate with time (Spicer et al., 1990; Ridgeway et al., 2006; Bernasconi & Uglow, 2008a, b). When the haemolymph L-lactate concentrations from the animals in the mist experiments were compared to results obtained from emersion experiments in the literature, it showed that over 24 hours this current experiment yielded very high L-lactate concentrations which over 200 times higher than those found in the literature for emersion in *Nephrops* (Bernasconi & Uglow, 2008a). This is further evidence of an increase in metabolic rate in the misting trials of this study compared to the complete emersion trial of Bernasconi & Uglow (2008a), where the only differences are that the animals in the present study were misted and in tube trays. In Ridgway et al., (2006) high mortalities were recorded at L-lactate concentrations of around 17mmol/L, which also agrees with the findings of the present study. However, Ridgeway et al., (2006) did not mention any controls for desiccation, and so desiccation may have had a significant influence on the findings of that study, whereas it is unlikely to have had an influence in the present study. It is important that the gill structures remain moist as this has been shown to improve gas exchange across this surface (Danford et al., 2001). The high levels of L-lactate (metabolic acidosis) seen at 24 hours and an assumed associated build-up of CO_2 in the haemolymph (which is normally associated with L-lactate increases; respiratory acidosis) for both the misting transport groups in the present study would be expected to induce low haemolymph pH (acidosis). This was not the case and which contrasts the findings in the literature for the same and related species (Taylor & Whiteley, 1989; Schmitt & Uglow, 1997; Ridgeway et al., 2006).

In conclusion, the causal factors of the high mortalities observed in this species during the misting trials remain unclear, especially when the *H. gammarus* trials (discussed below) yielded such contrasting results despite the species being reasonably closely related (family Nephropidae). Comparisons to the literature suggest that the mist may induce an increased metabolic response to emersion, when compared to dry transport. The present study also suggests that tube trays may also induce a physiological response in *Nephrops* that may increase mortality rates whilst emersed; this also needs to be investigated in more detail. Similarly, more work is needed with this species to determine whether misting could be a viable transport method and aim to reduce the deaths observed in this study. Currently, therefore, misting of *Nephrops* is not recommended.

4.3 *Homarus gammarus:*

The effects of emersion on this species and the closely related *Homarus americanus* has previously been examined in detail (Taylor & Whiteley, 1989; Whiteley & Taylor, 1990; 1992; Whiteley et al., 1990; Lorenzon et al., 2007), but there remains no study on whether a mist would be suitable at maintaining the animals in a good condition for commercial transport and holding. The results from the current study show that the intermittent mist system and the continuous mist system (to a lesser extent, as no statistical significance was measured between continuous mist and the dry transport treatment) allows for increased efflux of ammonia at all times. The increased excretion of ammonia in the misted systems is likely to be related to the volume of water passing across the gills, as the gills have been identified as the primary location of ammonia excretion in crustaceans (Regnault, 1987). The ammonia is believed to be transported into the intracellular environment by facilitated diffusion of NH_3 using Caesium ion (Cs^+) sensitive channels or Na^+/K^+ ATPase located on the basolateral portion of the gill epithelial cells, whereby NH_4^+ is believed to displace the potassium ion (Regnault, 1987; Weihrauch et al., 1999; Romano & Zeng, 2007; Weihrauch et al., 2009). Then from the intracellular environment the ammonia is transported to the external environment by either vesicular action, or through Na^+/H^+ ATPase (Hunter & Kirschner, 1986; Weihrauch et al., 2009; figure 17). This mechanism is in line with the findings of this study, as this species appears to be able to somewhat actively transport ammonia against a concentration gradient to the external environment, as there was an approximate 4-fold

increase in ammonia in the external environment compared to the haemolymph (~1300µmol/L – external water; 300µmol/L -haemolymph at 72 hours). The ability to excrete ammonia against a concentration gradient is in line with the findings from Weihrauch et al., (1999) who saw that *C. pagurus* and *Carcinus maenas* were able to excrete ammonia against an 8-fold and 4-fold concentration gradient, respectively. It must also be noted that a water change was required at 48 hours for the continuous misting treatment, but it was not required for the intermittent mist treatment, which suggests that the intermittent misting system is a more efficient use of water, and may be more suitable for longer journey times. Despite the increased rate of water circulation in the continuous misting system and the increased efflux of ammonia into the external environment, as shown by the increased ammonia concentration in the water, it was not significantly lower than the haemolymph concentration in the dry treatment whereas it was the case in the intermittent mist trial. This finding may suggest a few hypotheses: that there is an increase in metabolic rate in the continuous mist trial. Alternatively, the increased ammonia in the external environment may induce an increased concentration of ammonia in the haemolymph either by diffusion back into the animal or a positive feedback mechanism whereby the increased external ammonia induces an increased production of ammonia within the animal. However, for the latter point it would be likely that there would be an increase in the rate of ammonia increase within the haemolymph and reservoir water as the test progressed, which was not the case here. More work needs to be done specifically on H. gammarus to determine if these mechanisms also apply for this species.

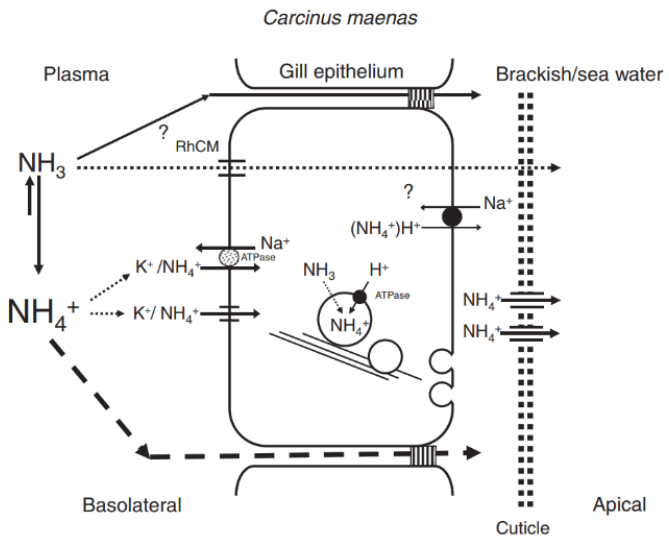


Figure 17 – A schematic diagram with a suggested hypothetical ammonia excretion model for *Carcinus maenas.* Taken from Weihrauch et al. (2009).

Differences in L-lactate were also measured between the transport trials throughout this study. The concentration of haemolymph L-lactate in Smyth & Uglow (2015) was lower than in the present study when the organisms were misted, this difference between the two species may be related to the difference in gill physiology, which in *C. pagurus* the phyllobranchiate gill structure allows for more efficient gaseous exchange in emersed conditions, compared to the trichobranchiate gill structure of *H. gammarus* which is known to be more prone to collapse during periods of emersion (Taylor & Whiteley, 1989; McMahon, 1995). An interesting comparison within the results of this study would be the concentration of L-lactate in the haemolymph of the animals in the continuous mist trial compared to the remaining two trials. The continuous mist appeared to induce the highest concentrations of haemolymph L-lactate, suggesting that the animals in this study were respiring more with the anaerobic pathway, despite a higher volume of water passing over the animals and likely into the branchial cavity. The deaths during this study were also the highest during the continuous misting trial (26.66% mortality at the end of the trial; although the overlap in the confidence limits of the LT_{10}, LT_{20}, and LT_{50} between the 3 transport groups suggest that this difference is not significant) which is consistent with the high lactate levels experienced in this transport group. A possible explanation for this result could be that the higher volume of water that comes in contact with the gills in this system is collecting on the gill surface, and after the initial

gaseous exchange has occurred, the water then acts as a barrier, furthering the diffusion distance of the gills, thus making them less efficient for gaseous exchange during emersion. It is unlikely to be that the water has become hypoxic, as the act of misting should be sufficient to oxygenate the re-circulating system. However, there is nothing in the literature to suggest that this may be the case, due-to the novel nature of this experiment. It would also be expected that there would be an increase rate L-lactate concentration increase with time during the continuous misting trial, as the water become more hypoxic, but this was not the case. The pH of the water in both the intermittent and continuous misting trials was shown to decrease overtime, suggesting that some gaseous exchange occurred meaning there wasa higher concentration of CO_2 in the reservoir. Despite the lower L-lactate concentrations in the haemolymph of the animals in the dry trials, a lower pH was measured when compared to the intermittent mist and to a lesser extent (and not statistically significant) the continuous misting environment. This reduction in pH was therefore unlikely to be result of increased L-Lactate concentrations, but instead may indicate respiratory acidosis or another mechanism. The decrease in pH, but with a small change in L-lactate may indicate that the metabolism in the dry treatment individuals was lower, but there was still a build-up of CO_2 in the haemolymph of the animals, which contributed to a significant reduction in pH. A possible reduction in pH could be as a result of the presence of gel ice packs in the polystyrene box, which were not responsible for a decrease in temperature overall in the transport environment, but it may have decreased temperature fluctuations, which may have contributed to higher metabolic rates in the misted environments.

A recovery period of 24 hours was implemented for this species. Haemolymph ammonia during the recovery period decreased from the levels seen during the trial in the group under dry transport conditions, but in the continuously misted animals remained approximately the same during recovery as during the trial. The animals from the intermittent misting treatment showed that there was an increase in haemolymph ammonia during recovery compared to during the trial; the reason for this is unknown as there is no evidence to support this in the literature. At the start of recovery, there was a significantly larger concentration gradient between the recovery water and the internal environment of the animals in the dry transport group (compared to the

recovery water and the continuously misted animals, and the recovery water and the intermittently misted lobsters). This would explain why the rate of ammonia excretion would be higher in this group. The greater gradient would allow for an increased rate of passive diffusion. However, the available studies do not explain the increase in concentration for the intermittent group. Hence, more work needs to be done to determine why this would occur. The removal of L-lactate from the haemolymph was observed in all treatments upon re-immersion. It has previously been suggested that gluconeogenesis (the generation of glucose from non-carbohydrate sources) may be a primary method of L-lactate removal following re-immersion and a return to aerobic respiration (Phillips et al., 1977; Schmitt & Uglow, 1997). The results from the current study suggest that this may not be true, as despite a significant decrease in haemolymph L-Lactate over the 24 hour re-immersion period, there was not a change in haemolymph glucose concentration. Conversely, it may even be that the rate of gluconeogenesis is so slow, that the metabolic rate in these animals are using the glucose as it is produced, as was suggested in Phillips et al., 1977). Other studies also suggest that some L-lactate may be excreted from the animal into the external environment (Spoek, 1974), but there was no detectable concentration of lactate in the external water (testing kit is sensitive to 0.02 mmol/L) suggesting that this may not be the case, which is also in line with other findings in the literature (Bridges & Brand, 1990). It is likely that the primary mechanism of L-lactate removal in *H. gammarus* is oxidation (Ellington, 1983; Schmitt & Uglow, 1997), but more work needs to be done to confirm this prediction. The rate by which this species removes L-lactate upon re-immersion has been described as slow in previous studies (Phillips et al., 1977; Whiteley & Taylor, 1990; Taylor & Whiteley, 1992), and that observation is supported by the results presented here, given that the continuous misting group has not returned to pre-trial concentrations of L-lactate following 24 hours of immersion in clean seawater.

In conclusion, the mist system appears to allow for some gaseous exchange across the gill epithelium, and the higher pH and lower haemolymph ammonia (compared to dry lobsters) is evidence for this. However, this study suggests a possible increase in metabolism induced by the continuous mist which is evident by the increased L-lactate concentrations at 72 hours. Throughout the recovery period, the mean haemolymph ammonia concentration was significantly higher for the intermittent misting group

when compared to the dry group, and the intermittent misting group saw a slight increase (not significant) when placed into the recovery tanks from the test environment. This cannot yet be explained and more work is needed. In general the intermittent mist system appears to be suitable for maintaining a minimally stressed environment for *H. gammarus* when being transported commercially, however many of the results are comparable to the dry transport environment so more work would need to be done to improve the efficiency of this novel system, and determine whether the benefits outweigh the costs of using such a system in a commercial environment. It is likely, however, to offer significant cost savings over vivier transport where 50:50 water/product is used. This would allow significantly more lobsters to be transported as water would recirculate and lorries could be stacked floor to ceiling, thereby maximising the use of space in lorries. If a filter was added to the system then a water change may not even be needed.

4.4 Accutrend meter comparison:

The current study takes further a previous study in the literature which suggested that the Accutrend meter may be a suitable replacement for the traditional enzyme assay determination for L-lactate in *Nephrops norvegicus* and *Homarus gammarus* (Albalat et al., 2010; Bakke & Woll, 2014). The results in the present study suggest that the Accutrend handheld meter may be suitable for L-lactate determination, but it appears that this method for *Nephrops* was not suitable and gave a poor concordance correlation coefficient (CCC); this is in contrast to the findings in the literature (Bakke & Woll, 2014). The results from the *Homarus* trial were comparable to those of Bakke & Woll (2014), as both showed a moderate CCC and a significant correlation. Hence the meter appears to be an alternative tool to using a colorimetric diagnostic kit, at least in the case of *Homarus*. The poor CCC in the *Nephrops* trial may be as a result of diluting the haemolymph 50% after sampling but prior to all analyses, as this resulted in the L-lactate levels being measured as 'low' (below the limit of detection) by the meter in 45 of the samples, which resulted in only 13 usable samples. The 'low' error for this equipment presents the most substantial disadvantage of using this equipment for L-lactate analysis, as the relatively narrow working range (0.8 – 21.7mmol/L) meant that control readings (L-lactate levels during immersion in sufficiently oxygenated water) and many of the samples could not be quantitively measured. The proportion of samples that

could be quantifiably measured using this equipment would increase if the haemolymph was not diluted, but this is often a requirement to prevent coagulation during storage (Schmitt & Uglow, 1997; Lorenzon et al., 2007; Lorenzon et al., 2008; Bernasconi & Uglow, 2008, Bernasconi & Uglow, 2011; Smyth & Uglow, 2015). This means that the haemolymph would have to be used immediately to prevent coagulation from affecting the results. However, the benefits of using this equipment on undiluted haemolymph must not be overlooked, as it can offer a 60 second response time, which may be suitable for some studies carried out in the field, or studies that require an almost instantaneous L-lactate measurement. The portability of this meter is also a benefit which may lend to the perception that it may be suitable for field studies. However, field studies using this equipment may also be difficult in some conditions, as the narrow working temperature range (15-35°C) may provide numerous erroneous results while exposed to temperatures outside this range.

In conclusion, the niche that this meter is likely to fill in the sampling of L-lactate in decapod crustaceans is small, and therefore the lack of versatility may suggest that it is not suitable for most studies if the enzyme assay approach is an option. The speed and portability are the main benefits of this system, but the low CCC for the *Nephrops* trial suggests that the equipment may not be sufficiently accurate for reproducible peer-reviewed scientific studies.

5.5 General discussion:

The equipment required for producing the mist may be a large factor in whether its use is feasible in a commercial setting. The observations in this study suggests that particularly in a re-circulating system the mist system required 'servicing' after each trial (48 – 96 hours of operation). However, the servicing of the equipment did not require any specialised training or equipment. It was a procedure that took no more than 10 minutes, and consisted of removing the internal filters of the misting nozzles using open-ended spanners and an O-ring pick, then removing detritus from the filters using a wash bottle and tap water. This maintenance could also be carried out in-situ if required, and so blocked misting nozzles are not an insurmountable obstacle and can easily be managed in an emergency. During this study, the system was also completely cleaned with 5% acetic acid (household vinegar) and distilled water to prevent salt-crystal build-up, as the equipment used here was not salt-water rated. The cleaning regimes proved

to be successful in ensuring the longevity of the equipment, as over the course of 6-8 months of heavy usage, there was no noticeable depreciation in the performance of the pump. Despite the cleaning efforts made there was some evidence of minor corrosion on some of the external, non-essential metal parts of the pump, which may be resolved if the pump was placed in a permanent water-tight location.

The results from this study also raised concerns regarding the efficacy of behavioural responses as a measure of stress parameters. Behaviour was a concern for this study, as the subjective nature of the parameters may have meant that it did not accurately represent the health of the animals, which may have resulted from unsuitable behavioural endpoints or because there was no sub-lethal behavioural response before death (Olla et al., 1979b). This was clear for the whelk trial, as the behavioural responses would be expected to be the greatest (deviates most from normal; Kittredge, 1979) in the group which saw the highest rates of mortality, which was not the case. Although it did appear to be more suitable for determining the health of the decapods in this study (*N. norvegicus* & *H. gammarus*), which may be related to their increased size, and the increased number of appendages which aid in determining suitable behavioural categories for comparisons. This allowed for improved behavioural metrics for the crustaceans used in this study, and may have contributed to improved overall stress measurements. Hence, this study questions the efficacy of using behavioural parameters for measuring stress, particularly in marine gastropods. The use of D-glucose was also scrutinised during this study, as there was no measurable difference between or within any of the transport treatmment types of this study. It would be expected that D-glucose levels would increase within the animals as a response to stress (in this case, emersion) as the animals are known to mobilise glucose stores to increase haemolymph glucose levels (Spicer et al., 1990; Speed et al., 2001; Lorenzon et al., 2007). This result may suggest that D-glucose should be used in a more supplementary role in stress measurements in marine decapods, and may not be as useful as the other indicators in this study. Finally, the changes in total protein within this study were observed to be minimal, though there were some sporadic changes at times during the study which did not suggest any specific trends. This parameter was not valuable in many of the analyses of the animals, as the changes in total protein concentrations were expected to increase during times of emersion reflecting an increase in hemocyanin to improve gaseous

exchange. In addition, protein metabolism involved in acid-base regulation of the haemolymph and body tissues (Hosie, 1993; Ridgeway et al., 2006; Lorenzon et al., 2008), which was not the case in this study.

The efficacy of behavioural parameters for determining the stress of the animals are often overlooked when physiological/biochemical biomarkers are used. This is likely because the measurement of behaviour is a subjective parameter, and the observed effects can largely depend on the metrics chosen for the given study (Olla et al., 1979a). It may be that unsuitable metrics were chosen for the *B. undatum* trial of this study, as it is expected that using aberrant (behavioural responses to a stressor that do not increase fitness for survival) behaviours any further deviation from the normal behaviours should correlate with a decrease in health of the animals (Kittredge, 1979). However, behavioural parameters may offer valuable data on the overall health of the animals, because many physiological and biochemical biomarkers can have high levels of variability, meaning a low signal:noise ratio (Thurberg, 1979; Bayne, 1979; Lee et al., 1979). Physiological and biochemical biomarkers may offer a much quicker measurable response to the stressors, as they act at a lower level of biological organisation compared to behaviour, and so the behaviour responses measured in this study may be a delayed response to the changing biochemical and physiological aspects within the animals (Adams et al., 1989). The use of behavioural parameters for the stress determination of animals is often best used in conjunction with biochemical and physiological biomarkers in order to negate the negatives of each method, and to gain a more holistic view regarding the health of the animals (Olla et al., 1979b).

In general, it was clear that the mist systems offered some benefits over the dry systems typically used for *B. undatum* and *H. gammarus* through some gaseous exchange at the gills, and better general condition in some cases. The improved stress parameters in some areas of the two species raises the question of whether the benefits are sufficient to warrant a change to this system from the current transport practices. As previously discussed, this system does require more maintenance than a dry system, but the low initial cost of the equipment, and little maintenance cost associated with this system is likely to make this a quite cost-effective transport method for many fishers. This method also allows for a wider range of storage systems to be implemented, as polystyrene

boxes are not necessary (which are usually single use, and contribute to a growing waste problem), therefore typical industry standard mesh boxes or other non-insulating crates/boxes could be (re)used, or in some cases a more efficient storage method could be designed for this method.

This proof-of-concept study made significant progress in achieving the aims set out for the project, as evidence was provided to show that a chilled mist may be a suitable transport method for at least *B. undatum* and *H. gammarus*. The objectives of the current study were mostly achieved except for the testing of *B. undatum* haemolymph for glucose and L-lactate, as the concentrations of these chemicals were below the detection range of the equipment and testing kits used. This study also adds to the knowledge base of information on the stress responses of decapods, and has provided the first experimental insights into the stress responses of *B. undatum* to emersion which had previously not been studied. The data provided by this study could be used by fishers to improve current transport techniques for commercially important marine decapods and gastropods.

5.6 Critique of the Study

The current study was successful in achieving the aims and objectives set out; despite this, there were aspects of the project that were not successful and require modification if applied to future projects. The analysis of whelk haemolymph for both L-lactate and glucose was a shortfall of this study, as the concentrations measured of these parameters were below the detection range of the equipment and assay kits used. Upon reflection, glycogen and D-lactate may have been better parameters to measure for *B. undatum* as it appears that they are likely to be detected in the animals and therefore may offer further insights into the metabolism during emersion. Furthermore, a more reliable method of haemolymph withdrawal from whelks would have to be developed, as the mixing of water with the haemolymph may have influenced the measurements of biochemical biomarkers in this study. The behavioural parameters used for the whelks also appeared to be insufficient in determining the overall health/stress of the animals, and so if a repeat of this study was carried out, more robust behavioural parameters would have to be determined. The high mortality of *Nephrops* was also a shortcoming of the present study, as 2 and a half months was spent attempting to find a suitable way of ensuring the survival of the animals whilst

emersed; despite the efforts applied to this issue, little was found in the way of improving survival during emersion. Overall, the methods applied in this study were scientifically sound, but the issues raised above illustrate how improvements could be made to similar projects, as to allow for the formulation of a methodology with fewer shortcomings.

5.7 Directions for Future Study:

Despite fulfilling the aims and objectives and testing the hypotheses set for the current study, there are suggestions for further study and for the further implementation of the results from the study. Further work on the stress of *Buccinum undatum* should aim to improve on the behavioural parameters used in the present study, as to better reflect the overall stress of the animal. Work should be carried out to determine the exact ammonia excretory mechanisms used by *B. undatum* which would help future studies on emersion stress of this species. Moving forward from the findings of the present study, efforts should be made to determine the effect mist droplet size has on the efficacy of stress parameter reduction. Throughout this study, the continuous misting environment has also been shown to give poorer results regarding some stress parameters when compared to the intermittent mist. At the present, it is unclear as to the primary method of L-lactate removal from crustaceans, and so more work should be focussed on this area so it can give a better insight of how animals recover during immersion. As for the *H. gammarus* trials, it was unclear why there was an increase in ammonia from the intermittent mist treatment after immersion, any further tests should aim to discover why this was the case. Work should be done to determine the causal mechanistic effect of the difference between the continuous and intermittent mist transport groups. Finally, there should be some investigation as to why the *Nephrops* performed so poorly in the misted transport, and did not perform equal to the emersed conditions in the literature. All future work should be focussed towards what changeable aspects of the mist system contribute to improved efficiency of the system, and allows for the lowest stress responses in the animals being transported. The results presented in this study can be used by commercial fishers to improve the current practices of live shellfish transport, to lower the volume of water transported, whilst ensuring a suitable quality of product.

6 Conclusions:

This study is the first to provide evidence that an intermittent mist system reduces stress when compared to the dry transport treatments of both *B. undatum* and *H. gammarus*, through reduction in haemolymph ammonia concentrations, and an increased pH compared to dry treatment groups. The intermittent mist system was also shown to reduce the mortality level compared to the dry transport method in *B. undatum*, and a similar rate of mortality to the dry treatment in *H. gammarus*. So, for both above species it is likely that the hypothesis "Crustaceans (and Molluscs) kept under the mist conditions will have a lower overall stress level and lower mortality, when compared to the traditional means of transport" can be accepted, but for the *Nephrops* trial the hypothesis must be rejected, as in its present state the technology is unsuitable for maintaining the animals in a state that they can survive.

The Accutrend handheld meter was shown to be a suitable measurement tool for L-lactate levels in *H. gammarus*, although this was not the case for *Nephrops* where the meter was shown to have a poor concordance correlation coefficient. The meter was also shown to have quite a narrow range of detection which meant that 62.66% of lobster samples could not be measured, and 78% of *Nephrops* samples could not be measured using this method. This result suggests that we can accept the hypothesis 'The Accutrend handheld lactate meter will not produce statistically different lactate measurements compared to the traditional method of L-lactate determination' for the *H. gammarus*, but the hypothesis would have to be rejected for the *N. norvegicus*.

7 Ethical Statement:

This study was carried out in accordance to the Guidelines for Ethical Research set out by the University of Hull. Measures were taken in order to reduce the stress impacts on individuals during this study. Effects of this study on natural populations were not a concern, as the animals were obtained from a commercial fishery, and were destined for human consumption. Any animal that was deemed too weak to proceed with testing was removed to ensure survivability.

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