Effects of selected Post Harvest Processes on Brown Crab (*Cancer pagurus* L.) and European Lobster (*Homarus gammarus* L.) from Irish Crustacean Fisheries.

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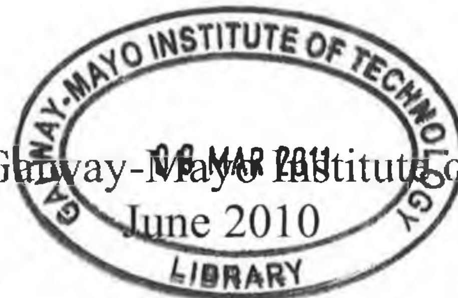

Submitted to Galway-Mayo Institute of Technology
June 2010

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Abstract

The crustacean fishery is important to the socio economics of rural and island communities around Ireland; with brown crab (*Cancer pagurus*) and European lobster (*Homarus gammarus*) being the most valuable shellfish species. Brown crab and lobster are marketed live with the majority being exported from Ireland to southern Europe. Post capture processes used in Ireland are very subjective but promote fresh, live products. Common practices used in the crustacean fishery include nicking of brown crab and long term storage of lobster. This study showed that nicking resulted in elevated mean lactate levels of 17.90% (StDev ± 1.74) and elevated mean glucose levels of 120.55 % (StDev ± 0.26) with mean circulating bacteria levels 9 times greater in nicked crab. Nicking resulted in 96.3% increase in tissue necrosis and a subsequent reduction in product quality. These factors possibly compromise the host's defense system, which may ultimately reduce the animal's ability to cope with additional stressors caused by post harvest processes. Long term storage allows lobster to be stored until the market is less saturated and prices are higher. This investigation found that some lobsters contracted bacterial biofilms as a result of long term storage. Bacteria isolated from biofims were identified as Arcobacter and Campylobacterales with identity and alignment scores of 80% andd 88% respectively.

Acknowledgments

I would like to thank Brendon Allen, Patsy Kelly and Ciara Ní Chualáin for their assistance whilst sampling. I would also like to thank Louise van der Heijden for her continual support. Finally, the project would have been possible without Pauline King, Eugene MacCarthy and all the examiners involved with the project.

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1.0 INTRODUCTION

Within Ireland the total seafood sector employs circa 15,720 people (EPA, 2000), of which a significant number are located in rural coastal communities. In addition to this, the number of persons reliant on the fishing industry within Ireland is considered to be much greater. These rural communities often have few other employment opportunities.

The Irish crustacean fishery targets a multitude of species for example brown crab (*Cancer pagurus*), European lobster (*Homarus gammarus*); prawn (*Palaemon serratus* Pennant); velvet crab (*Necora puber*); spider crab (*Maja squinado*) and crawfish (*Palinurus elephas*) (Paust & Rice, 1999; Browne *et al.,* 2001). The fishery is primarily inshore using small vessels and pots or creels. There are circa 350,000 pots or creels used in the inshore sector (www[1].). Soft eye creels are the most commonly used pot type, specifically for brown crab and lobster. However, most crustacean fishers carry more than one style of pot (www[1].) Pots typically have a soak time of two days, though this does vary widely depending on fisher and catch per unit effort (Bennett, 1974; Meredith and Fahy, 2005).

The west coast inshore fishing fleet has the most vessels in Ireland. In county Galway and Mayo many traditional vessels such as currachs (<5m in length) are still used due to the considerable number of sheltered bays (Lawler, 2001). However, traditional boats are now powered by outboard motors.

In recent years Bord Iascaigh Mhara (the Irish Sea Fisheries Board) commissioned research on Irish shellfish stocks and their management (Tully *et al.,* 2006a/b; Anon, 2009). Brown crab and lobster contribute the largest landings (circa 7,700 tonnes, www.[1]) to the Irish crustacean fishery, accounting for circa 90% of the total catch.

1.1.0 *Cancer pagurus*

Cancer pagurus (Infraorder, *Brachyura;* Superfamily, *Cancroidea;* Family, *Cancridae;* Genus, *Cancer)* are commonly known as Brown crab or edible crab. They are identified by their flat, ten lobed 'pie crust' carapace and their stout black dactyli and propodi on their chelae (Hayward *et al.,* 1996). Brown crab distribution ranges from the Mediterranean and West Africa to the northern waters of Scotland and Norway. Brown crab predominantly inhabit rocky beds but can be found on substrates from soft mud to shingle at mid-tidal to sub littoral (100m) depths (Hayward and Ryland 1995). Females (hens) grow to around 150 mm carapace width (CW), maturing at 115 mm CW. Males (cocks) can reach sizes of around 250 mm CW and mature at 110 mm CW with both sexes living for circa 20 years (Tully *et al,* 2006a). Moulting occurs once a year though this may decrease with an increase in size and age. Strong migratory components have been identified in hen crabs, with migration into shallow waters during summer (Fahy *et al.,* 2004; Meredith and Fahy, 2005).

1.1.1 Fishery

Brown crab are considered to be the most commercially valuable shellfish species within Ireland (Tully *et al.,* 2006a) and the UK (Barrento *et al.,* 2010). Since the 1960's the Irish fishery has expanded with increased landings. Production between 1990 and 1999 showed the greatest increase, with national landings rising from circa 3,500 tonnes to >7,000 tonnes respectively (Paust and Rice, 1999; Tully *et al.,* 2006a). In 2008, Irish brown crab landings reached circa 6284 tonnes, worth approx. €13,228,812 (Anon, 2009). The majority of live crab is exported, with around 3.6 thousand tonnes being exported in 2008 worth approximately €13 million (BIM/CSO unpublished trade data). Additional preserved and frozen crab were also exported. However, actual captured brown crab is estimated to be higher with rates of crab caught in the lobster fishery reaching 20-30% of targeted crab landings per unit effort.

The Irish brown crab fishery is categorised into offshore and onshore sectors (Lawler, 2001). The Irish offshore brown crab fishery was developed in the early 1990's (Tully *et al.,* 2006) and covers <60 miles offshore, in depths between upto 200 meters using large purpose built vivier boats (Lawler, 2001) and is reliant on catching the species all year round (Ní Chualáin *el al.,* 2009). The offshore fishery accounts for around 3,500 tons of brown crab annually (Lawler, 2001).

The inshore brown crab fishery is subdivided into two main regions, the northwest fishery which ranges from Donegal to Mayo and includes South West Scotland, and the South East Fishery, which spans along the Cork coast (Tully *et al.,* 2006a). Tully *et al.,* (2006a) proposed other divisions including a western fishery ranging from North Mayo to Shannon and a south west fishery spanning from the Shannon estuary to Cork.

The northwest fishery accounts for c.75% of national landings (Lawler, 2001; Tully *et al.,* 2006a). The Irish inshore crab fishery utilizes a greater range of vessel types and sizes compared to the offshore fishery.

There is little information available on precise brown crab fishing and storage practises. This may be primarily due to the lack of regulation and standardisation in methods. However, some decree does exist. Fishing methods are restricted to licenced vessels; a minimum landing size of 130mm from 48-56^{0}N to 140mm CW north of 56^{0}N; and limited fishing effort depending on region fished (EC regulation 1415/2004 & 1954/2003; Tully *et al.* 2006). In regards to holding practices there are no enforced regulations. Therefore, brown crab holding practices are not restricted by parameters such as stocking densities, water quality or length of storage. The most common practice observed in the Irish crab industry involved holding brown crab in high densities. The tanks in which the crab were held worked on a 'flow through' system whereby they were emptied and refilled on a daily basis.

1.1.2 Pathogens

It is hypothesised that any individuals with pre-existing infections would have a reduced survival rate when subjected to post harvest processes. Therefore, it is important to monitor diseases of brown crab in Irish waters and be aware of diseases on a global and multi species scale.

Several publications review marine invertebrate (Kinne, 1980a; Sparks, 1985; Couch, 1992) and crustacean (Kinne, 1980b; Provenzano, 1983; McVey, 1993) pathogens. However, these are relatively dated and predominantly concentrate on cultured species, such as penaeid shrimp, and not wild crustaceans. Fisheries and Oceans, Canada, has carried out extensive research and reviews on pathogens of valuable fish and shellfish found globally. This

information has been collated into 'quick reference' guides, which form a synopsis of infectious diseases and parasites of commercially exploited shellfish (Bower and McGladdery, 1994).

The current literature on marine pathogens shows two clear pathways to infection (Bower and McGladdery, 2003). Elston (1984), Bower and McGalddery (1994) and Stentiford *et al.*, (2009) identified poor husbandry and water quality as causative agents for opportunistic infections as it renders the animal more susceptible. Secondly, infectious agents may be introduced to indigenous populations through alien species (Sinderman, 1991; Stewart, 1993; Kern, 2001; Edgerton, 2002; Harper, 2002). Furthermore, individuals of the same species may act as vectors (Owens and McElnea 2000; Stentiford *et al.*, 2009), which poses particular implications in crustacean farming.

Opportunistic infections may arise from epibenthic fouling organisms utilizing shells as substrata (Cranford *et al.*, 2003). These may then traverse the shell and epidermis when damage occurs (Dyrynda, 1998; Vogan and Rowley, 2002). It is hypothesized that such infections progress slowly and, in the case of bacterial infections, are often only fatal in numeration. Such infections may become problematic in post harvest processes if the shell and epidermis are broken.

Many diseases and infections have been reported in brown crab. These infections range from ciliates (Bang *et al.*, 1972; Cawthorn, 1997), bacteria (Vogan *et al.*, 2002), viruses (Corbel *et al.*, 2003), to fungi and microsporidians (Stentiford, 2008). A comprehensive review of the current status of brown crab and their pathogens was published by Stentiford (2008). Stentiford (2008) concentrated on recently identified pathogens which have a potential economical impact. It was noted that viral, bacterial and fungal pathogens cause the greatest limitations on growth and survival of brown crab in captivity (Stentiford and Bateman, 2007; Stentiford *et al.*, 2007; Bateman and Stentiford, 2008; Stentiford, 2008).

Several commercially significant pathogens in brown crab have previously been described. Perhaps the best known is the parasitic dinoflagellate *Hematodinium* which causes bitter or pink crab disease (BCD or PCD respectively). BCD has been found to infect many crab species affecting global fisheries (Couch, 1983; Stentiford *et al.*, 2003; Stentiford and Shields 2005; Ní Chualáin *et al.*, 2009) and was first reported in the Irish brown crab fishery in 2004

(Tully *et al.,* 2006a). Ní Chualáin *et al.,* (2009) reported economic loss as a result of shell discoloration, tissue degradation, unpalatable taste and mortalities caused by *Hematodinium*. A similar infection, ciliate disease (*Mesanophrys spp.*), has also resulted in mortalities in crab species by degrading haemocytes and muscle tissue (Bang *et al.,* 1972; Armetta, 1990; Love *et al.,* 1993). *Mesanophrys spp.* has only been reported in stored brown crab which were held in high densities (Bang *et al.,* 1972).

Other pathogens which have negative effects when crabs are in captivity include white spot syndrome virus (Li-Li *et al.,* 2000) and *Paramoeba perniciosa* (Johnson, 1980b).Corbel *et al.,* (2003) showed that *Cancer pagurus* systemic bunya-like virus (CpSBV) may also be a significant contributor to brown crab mortalities held in captivity.

Shell disease is a commercially important disease of *Cancer pagurus* and has been associated with poor water quality (Young and Pearce, 1975; Delves-Broughton and Poupard, 1976; Vogan *et al.,* 2002; Barrento *et al.,* 2008). Chitinolytic bacteria, affiliated predominantly with the genus *Vibrio* are postulated to be the causative agent (Vogan *et al.,* 2002). The disease is not recognised as causing high mortality rates; however, it does cause shell discolouration resulting in a low quality or unmarketable product. Gill damage, hepatopancreatic haemocyte nodules and tubular degradation, and secondary infections have been associated with bacterial shell disease (Baross *et al.,* 1978; Comely and Ansell, 1989; Vogan *et al.,* 2001). Bacterial shell disease was first described in Ireland in 1982 (Ayres and Edward, 1982). An overview of other commercially important diseases of crab has been summarized in Appendix 1.

As a result of increasing risk of disease outbreaks, new legislation was introduced (EC directive 2006/88/EC) in 2008 and a designated European Community Reference Laboratory (CRL) for crustacean diseases was established. The objectives of the CRL are to synchronize information on diagnosis and prevalence of crustacean diseases in National reference laboratories (NRL) in EU member states. The Marine Institute, Galway was awarded the accredited status of NRL for crustacean diseases in Ireland in 2008 to coordinate the findings of crustacean disease from research and monitoring within Ireland with the CRL (Annex VI (Part II and III) of Directive 2006/88). For many EU countries, including Ireland, this will result in increased monitoring of crustacean farming, fishing and handling practices.

1.2.0 *Homarus gammarus*

Homarus gammarus (Order, *Decapoda*; Infraorder, *Astacidea*; Family: *Nephropidae*, Genera: *Homarus*,) is a member of the clawed lobster family and is also known as the European or blue lobster. European lobsters are identified by their blue colouration with coalescing spots, large chelae and lack of spines (Hayward and Ryland, 1995). The pincers differ in size; one is used to cut prey, the other for crushing (Fish and Fish, 1996). They are found on rocky substrates ≤60m (Hayward and Ryland, 1995) from the Northeast Atlantic, Mid Norway and the North Sea to the Mediterranean (Tully *et al.* 2006b); from the shoreline to the continental shelf. Lobsters have been known to be cannibalistic but mainly feed on live prey (fish, molluscs, polychaetes etc) with occasional scavenging (Hallbäck and Warén, 1972). European lobsters have revealed no long distance migrations so adult stocks generally remain local (Smith *et al.,* 2001); however, currents may displace pre-settlement larvae. Triantafyllidis *et al.,* (2005) recognised four distinct populations of *Homarus gammarus* based on genetic differentiation. The regions for these groups were identified as the Mediterranean, northern Norway, Netherlands, and the Atlantic.

1.2.1 Fishery

Lobsters have been reported to be hatchery reared and ranched since the 19th century (Addison and Bannister, 1994; Nicosia and Lavalli, 1999). In Ireland lobster hatcheries have been used for stock enhancement (Bannister and Addison, 1998; Linnane and Mercer, 1998) and research (Mercer and Browne, 1994; Beal *et al.,* 2002; Prodöhl *et al.,* 2006). In addition, methods of stock conservation have been introduced such as v-notching of female lobsters (Browne *et al.,* 2001; Tully, 2001; Tully, 2004). The method is reliant on fishers nicking a 'v' shape into the tail of females and returning them to the sea. Tully (2001) showed how v-notching methods physically mark mature females deeming them 'illegal catch' and therefore non-saleable. This allows female lobsters to produce eggs for a minimum of two moults before being caught and landed.

Lobsters are targeted by the Irish inshore fishing industry. They are caught using creels or pots in vessels ≤ 12m in length and are fished between May and October (Lawler, 2001). Reduced temperatures during the winter results in lobster having low metabolism and reduced activity, therefore, lobster landings are low. Female lobsters carry their eggs over winter for dispersal in late spring to early summer (Hepper and Gough, 1978). Tully *et al.,* (2006b) reported annual landings of circa 850 tonnes within Ireland in 2004, worth a value of around

€13m. However, the most recent published data for 2008 reported landings of only 497 tonnes, worth €7,533,99 (Anon, 2009). Live and processed lobster were exported (BIM/CSO unpublished trade data), primarily to France where the average value is €29.2/kg (Bord Bia 2010 statistics to date 26/04/2010).

1.2.2 Pathogens

There is a large amount of information available on diseases and parasites of lobsters, predominantly of the genus *Homarus*. This is due to the extent of the *Homarus americanus* (American lobster) fishery, their high commercial value, culture research, and the fact that the fishery is based on the sale of live animals resulting in closer observations over time during holding (Stewart, 1984).

Gaffkemia (*Aerococcus viridans*) is reported to be the most commercially important disease of *Homarus* species, occurring in European and American waters (Stewart and Marks, 1999; Lavallée, 2002). The pathogen has no invasive properties but has been isolated from individuals with handling damage (Stewart and Marks, 1999). Gaffkemia increases haemolymph clotting time and causes septicaemic disease (Stewart and Marks, 1999; Bower, 2007). It was hypothesized that *A. viridans* could have a sharp elevation in incidence rate when infected animals are placed in high densities within holding tanks like those commonly used in lobster fisheries (Stewart *et al.,* 1969).

Multiple fungal species have been associated with *Homarus* lobsters. *Legenidium sp.* has been indentified in *H. americanus* and *H. gammarus* (Fisher, 1988) when in captivity. It is reported to be lethal and is typically found in lobster larvae (Bower *et al.,* 1994). *Haliphthoros sp.* has been reported to cause high mortality rates in captive post larvae of *H. american* and *H. gammarus* (Fisher, 1988; Bower *et al.,* 1994). It is thought that *Haliphthoros sp* is ubiquitous. Other major fungal species known to infect lobsters include *Fungi imperfecti, Fusarium sp.* and *Ramularia homari.* These three types are reported to cause fungal shell disease (or burn spot disease) in *H. american* and *H. gammarus* (Alderman, 1987) rendering them unmarketable.

Vibriosis (*Vibrio anguillarum*) in captive juvenile lobsters is reported to be lethal (Rosemark and Fisher, 1988; Bower and McGladdery, 1994). Vibrios are ubiquitous with some species considered opportunistic and affecting most shellfish species (Sochard, 1979; Bower *et al.,*

1994). In addition to this, *Vibrio parahaemolyticus*, a marine vibrio associated with gastroenteritis and faecal matter has been isolated from lobsters (Brinkley *et al.,* 1976; Su and Liu, 2007).

Paramoeba perniciosa was associated with mass mortalities of *H. americanus* in 1999 (Mullen *et al.,* 2004). A combination of infection intensity and adverse environmental conditions resulted in the host's inability to survive the infection (Pearce and Balcom, 2005). Paramoebiasis prevents haemolymph clotting and causes tissue displacement and lysis, and reduction in key proteins (Sawyer, 1978). A reduction in key proteins and tissue displacement may be particularly harmful to lobster being starved when undergoing long term storage.

Further clawed lobster pathogens are summarised in Appendix 2.

1.3.0 Crustacean immunity

Innate immune systems have been identified in crustaceans (McKay and Jenkin, 1970; Paterson and Stewart, 1974; Smith and Ratcliffe, 1980; Sönderhäll and Thörnqvist, 1997; Sritunyalucksana and Sönderhäll, 2000; Flajnik and Pasquier, 2004; Little *et al.,* 2005; Fotedar *et al.,* 2006; Hauton and Smith, 2007).

Decapods have an open circulatory system (Fretter and Graham, 1976). The open circulatory system provides a rapid transportation system for infections. A quick responding clotting mechanism allows blood loss to be kept to a minimum when limb loss and other wounds occur during post harvest processes.

Advances in understanding invertebrate immunity have been made since the recognition of phagocytosis by Metchnikoff in 1901 (Metchnikoff, 1901; Bang, 1970; Tauber, 2003). More recently phagocytosis has been shown to be a part of a complex innate immune defence system (Johansson, *et al.,* 2000; Zong *et al.,* 2008). This defence mechanism can be found in all animals (Roch, 1999). The two primary innate immune response systems found in crustacea are the proPo-system (prophenoloxidase activating system) which initiates phagocytosis and the *Limulus* coagulation cascade (Cerenius and Söderhäll, 1995; Liu *et al.,* 2007). In decapods the cellular immunity response path works by encapsulating the foreign body (often shown as haemocyte aggregations) and phagocytosis with opsonisation, followed

by the release of lymphokines (Cerenius and Söderhäll, 1995) and nodule formation (Söderhäll and Ajaxon, 1982; Factor and Beekman, 1990; van der Braak *et al.,* 2002). These nodules can appear as elongated, circling haem cells when young and eventually becoming darker and melanised with the oldest nodules appearing as chambers. Such formations are used in histopathology to help establish the age and location of cellular damage.

In addition to cellular immunity other humoral responses in marine decapods can also occur (Stewart and Zwicker, 1972; Chisholm and Smith, 1992; Noga *et al.,* 1996; Khoo *et al.,* 1999; Haug *et al.,* 2001). Such responses include antimicrobial properties (Söderhäll and Ajaxon, 1982; Haug *et al.,* 2002; Brockton *et al.,* 2007) and multimeric coagulation (Sritunyalucksana and Söderhäll, 2000). Such properties may be beneficial when opportunistic bacteria breach the carapace (Pyle, 1950).

The micro-epifauna of crustaceans is a result of their benthic niche. Exoskeletons provide a substantial epibiotic and endobiotic habitat for biofouling communities (Austin and Austin, 1989). Cottrell and Kirchman (2000) recorded the typical marine microbial ecology as being dominated by gram-negative heterotrophic bacteria. Three governing bacterial groups in marine microbial ecology are the *Flavobacteria*, the *Pseudoalteromonids* and the *Vibrios*. These groups can be identified by their 16S, 18S or 23L rRNA sequences (Woese *et. al.,* 1990). Amplifying rRNA sequences through polymerase chain reaction (PCR) and identifying organisms through molecular sequencing can aid in identifying problematic bacteria from infections during post harvest processes.

1.4.0 Post harvest htressors

Examples of post capture stress factors are shown in Table 1 (adapted from Evans, 1999). From point of capture to consumption, shellfish are subjected to multiple handling techniques (Figure 2) and stressors (Paterson and Spanoghe, 1997; Lorenzon *et al.,* 2008). Stressors during the marketing chain include aerial exposure, emersion, handling, vibrations, and environmental (temperature and chemical) changes (Spicer *et al.,* 1990; Paterson and Spanoghe, 1997; Taylor *et al.,* 1997; Lorenzon *et al.,* 2008).

Factor	Examples
Handling Stress	Winching up in pot Removal from pot Transfer to factory Packaging operations
Hypoxia Stress	On boat handling Transport to factory and to markets Exposure to low oxygen levels in tanks
Temperature Stress	Exposure to variations in environmental temperature on boat and during transport Dip treatment prior to packaging
Behavioural Stress	Limb autonomy from a variety of stimuli Crowding and aggression
Toxicity Stress	Exposure to high environmental ammonia Exposure to other dissolved toxins (e.g. copper, excreta)
Salinity Stress	Exposure to high and low salinity environments

Table 1. Post capture stress factors (Evans, 1999).

Idiopathic muscle necrosis (IMN) and multi-species bacterial septicaemia is often caused by manhandling damage, allowing opportunistic bacteria to invade (Stentiford & Neil, 2000; Ridgeway *et al.,* 2007). La Via and Hill (1975) suggested that mortality was primarily a result of physical injury or an impairment of the individual's host defence response resulting in bacterial infections. Johnson (1976) and Evans (1999) also noted bacterial infections as a result of post capture stress and damage, which was postulated to increase during transport. External surfaces of crabs yield a film of high bacterial diversity (Johnson, 1976). Whilst this film is likely to be a result of the animal's benthic niche it is possible that the film may support potential pathogens which could become problematic once the carapace has been breached. Colwell *et al.,* (1975) showed that 82 % of blue crab contained haemolymph infected with bacteria after undergoing post capture processes with higher bacterial counts being reorded in the haemolymph of crabs with missing appendages (Tubiash *et al.,* 1975). Environmental stressors such as those caused during post capture procedures (Table 1) have been shown to have severe effects on marine crustaceans (Evans, 1999).

It is imperative that correct procedures are used whilst handling the animals from the time pots are lifted to when they reach their final destination. Rough handling and general inadvertent abuse results in limb damage or autotomy which reduces their market value (Dunlin, 1996; Uglow *et al.,* 2005). SeaFish recommend that animals caught for human

consumption with broken limbs have the limb removed at the casting joint and that any bleeding animals are removed from the batch. Evans (1999) identified four possible post capture stressors resulting in morbidity or mortality as:

- Cell injury and organ failure due to physiological disturbances-air exposure, rough handling and other stressors.
- Opportunistic bacterial infections resulting from impaired immunity induced by the above stressors.
- Wounding increasing the likelihood of bacterial infection.
- Pre-existing disease conditions, which reduces the individuals' ability to resist stress.

The above factors can result in morbidity and mortality occurring through a multitude of pathways (Table 1; Figure 1). Environmental stressors such as those caused during post capture procedures (temperature, salinity changes etc.) have been shown to have adverse effects on marine crustaceans (Table 1). However, little information is available on *Cancer pagurus* and *Homarus gammarus* in the Irish fishery.

Pathogens such as epizootic viruses (Arcier *et al.,* 1999), bacteria (Cheng and Chen, 1998) and ciliates (Bang *et al.,* 1972; Armstrong *et al.,* 1981) have all reported to cause microfaunal changes during post capture. Other unidentified agents or idiopathic phenomena have caused mass mortalities of crustaceans (Lindqvist and Mikkola, 1978; Stentiford and Neil, 2000). The literature suggests that secondary infections also play a key role in the individuals health during holding, therefore, morbidity is likely to be due to a multitude of infections and stressors.

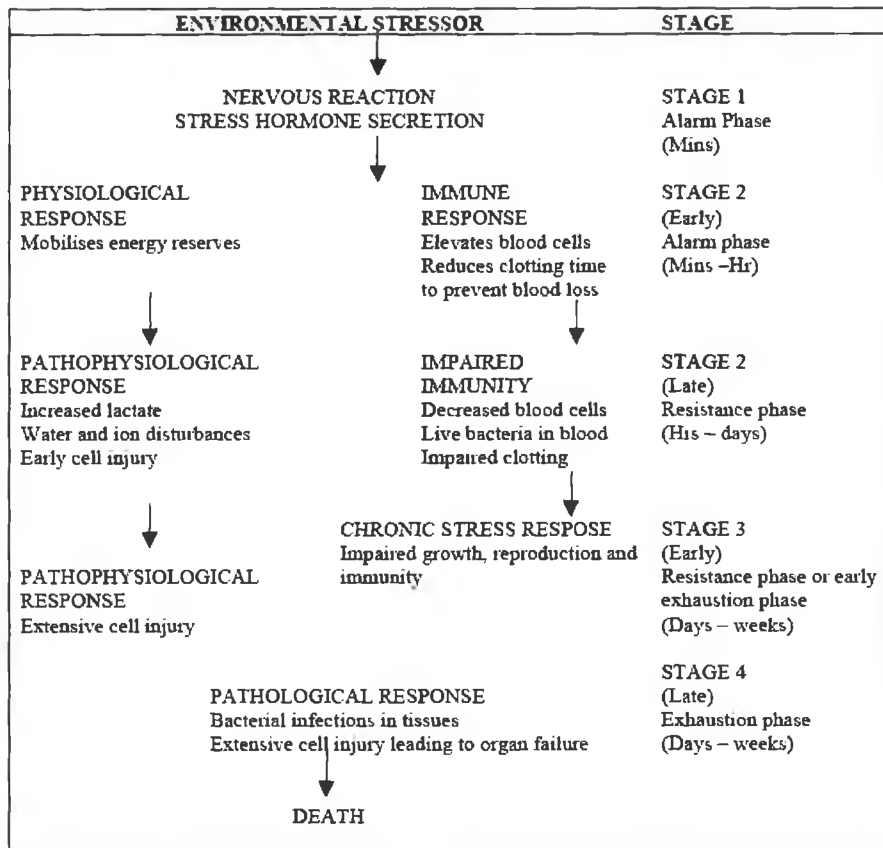

Figure 1. Stress response pathways in decapods (Evans, 1999).

Greater awareness of animal health and human food sources has lead to increased regulation. SeaFish developed recommendations for holding conditions to be applied to decapods captured by the UK fishery (Jacklin, 2007). These guidelines suggest cool, lowlight conditions within the holding tank building with water temperatures of between 5 and 10^0C. Brown crab should be kept at densities of up to 250kg/m^2 (in comparison to other decapod species this is relatively high), reaching depths no greater than 500 mm. Holding tank filters should remove debris of sizes over 100 microns and water quality should be monitored regularly.

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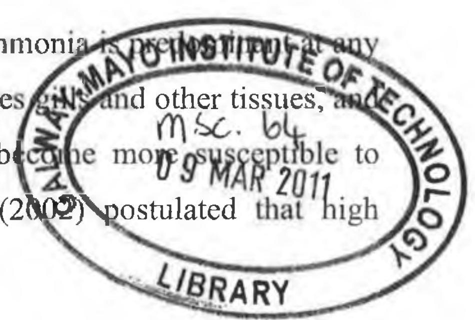
After oxygen, ammonia is the most important substance to monitor, particularly in intensive systems. Two forms of ammonia occur in water, i.e., NH_4+ and NH_3 collectively termed total ammonia nitrogen. Ionized ammonia (NH_4+) is toxic to marine crustacea at certain concentrations. Temperature and pH can affect which form of ammonia is predominant at any given time in an aquatic system. Ammonia causes stress, damages gills and other tissues, and low levels of exposure over time may cause the animal to become more susceptible to bacterial infections (Danford *et al.,* 2002). Danford *et al.,* (2002) postulated that high



ammonia levels may reduce an animals' tolerance to routine handling and is the reagent causing unexplained mortalities. However, brown crab produce substantial amounts of ammonia whilst in captivity (Regnault, 2005).

Stress caused by handling and storage can either be reversible or irreversible (Taylor *et al.,* 1997; Barrento *et al.,* 2009). Stress within the homeostasic capabilities of the animal may be reversed through reducing aerial exposure (Brown and Caputi, 1983) or by placing them in well oxgenated storage tanks (Whiteley and Taylor, 1992; Taylor *et al.,* 1997; Barrento *et al.,* 2009). Irreversible stress may result in permanent damage or mortalities. Handling damage is dependent on the size, age, diet and reproductive state of the individuals (Kruse *et al.,* 1994). Aerial exposure and handling longevity are two of the main causes of deterioration in marine decapod health. For a summary of literature on capture and handling effects on crab and lobster see Murphy and Kruse (1995).

In recent years physiological variables have been measured to assess stress levels in shellfish during the live marketing chain (Webster, 1996; Patterson and Spanoghe, 1997; Taylor *et al.*, 1997; Fotedar *et al.*, 2006; Lorenzon *et al.,* 2007, 2008; Barrento *et al.,* 2009). Typical, moderately invasive physiological measurements include: blood glucose, lactate, pH and total protein concentration of haemolymph and total haemocyte counts. Lorenzon *et al.,* (2008) used such physiological profiles in brown crab to determine suitable transportation methods. The results showed an initial increase in glucose, lactate and total protein concentrations in haemolymph, when animals were transported chilled in tanks of seawater. Animals transported in boxes with high humidity only showed high levels of lactate. In both scenarios, lactate levels returned to normal after 24 hours. Barrento *et al.,* (2009) also showed that lactate levels returned to control levels after 24 hours with $_{D}$-glucose and $_{L}$-lactate increasing during transportation. Fotedar *et al.*, (2006) identified alterations in clotting times of western rock lobster (*Panulirus cygnus*) during holding, with significantly prolonged clotting times and alterations in haemolymph pH and haem cell ratios. Such studies are imperative in identifying stressors and areas of improvement in the live marketing chain (Fotedar *et al.,* 2006)

1.5.0 Aims

The quality of live crustaceans for human consumption have frequently been observed as unsatisfactory with animals having reduced survivability; and are therefore often discarded in the later stages of the marketing chain. To help understand further how post harvest processes affect brown crab and European lobster in the Irish crustacean fishery this study aims to:-

- Describe the effects of nicking on brown crab tissues,
- Describe the effects of nicking on brown crab physiology,
- Describe the effects of long term storage on the European lobster

The research shall assess these aims through the following hypotheses:-

H_0 1. There is no difference in granulocyte counts from nicked crab and non-nicked crab.

2. There is no difference in viable bacteria counts from nicked crab and non-nicked crab

3. There is be no difference in tissue degradation from nicked crab and non-nicked crab

H_0 1. There is no difference in lobster granulocytes counts with increase in time spent in captivity.

2. Lobsters will not show pathologies throughout the trial.

The data generated from this project shall provide base line information which can be used as a source of comparison for future research.

2.0. CHAPTER ONE

Histopathological and bacterial progression in Irish brown crab (*Cancer pagurus*) subjected to industrial fishing practise of claw immobilisation by 'nicking'.

2.1 Introduction

2.1.1 Marketing chain

Methods used for handling, storage and transportation in the Irish inshore fishery are very subjective. Rural coastal fishermen have been observed to rely upon a cooperative system where crab are held in onshore concrete flow-through ponds until it is feasible to transport. Densities of brown crab in such ponds can be high with animals being held for >7 days. Transportation often occurs over 28-72hours in vivier lorries (refrigerated lorries with tanks). In addition, the live marketing chain may be long with multiple handling stages depending on fisher and retailer (Lorenzon *et al.,* 2008; Barrento *et al.,* 2009). A generalised chain is summarised in Figure 2. The periods between capture, distribution and consumption can be circa 20 days with storage periods of around 14 days (Uglow *et al.,* 2005) and < 3days in transportation (Uglow *et al.,* 2005) and often involves the crab being exported to central and southern Europe (Barrento *et al.,* 2009, Tully *et al.,* 2006).

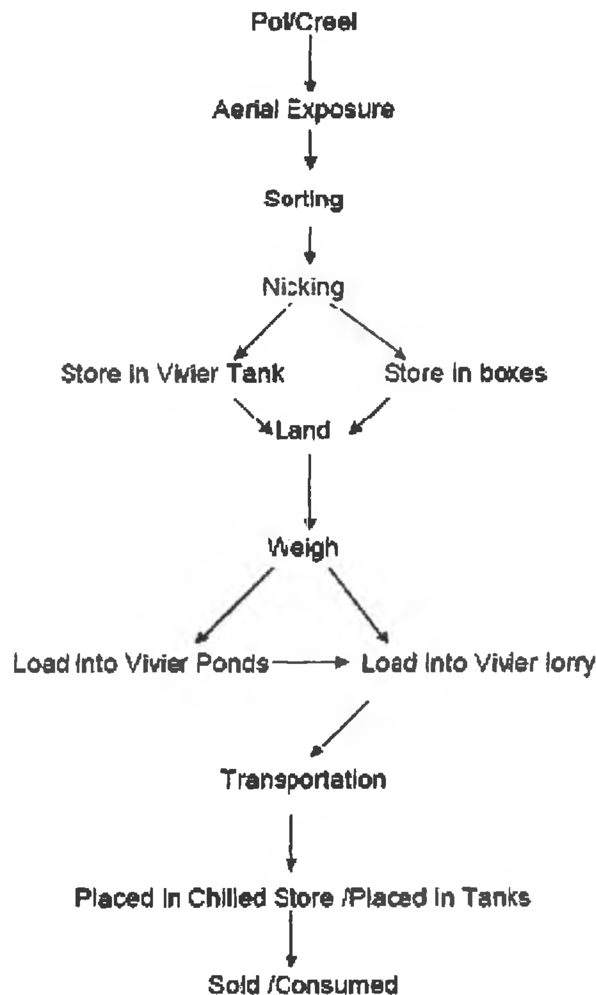


Figure 2. Generalised marketing chain for live brown crab in Ireland .

Previous studies have reported mortalities during transportation (Stentiford *et al.,* 2002; Stentiford and Shields, 2005) and during holding of brown crab and European lobster (Barrento *et al.,* 2008). Mortality rates of 15 -70% in brown crab have been recorded (Uglow *et al.,* 2005) with rates of up to 50% occurring in soft shelled-crab (Barry, 1981). Higher levels of mortalities (60-70%) have been recorded during the acclimatisation period when crab are held in tanks after transportation (Uglow *et al.,* 2005; Barrento *et al.,* 2009). Mortalities have been reported to be much greater in animals transported from northern UK waters to central and southern Europe during the summer (Uglow *et al.,* 2005).

One common mistake occasionally made within the crab fishery is for fishers to walk on the crabs whilst the crabs are in high densities in drained ponds. This was highlighted as a major contributing factor to increased mortalities (Jacklin, 2007). Tanks should be drained regularly and moribund individuals should be removed. Furthermore, it is recommended that stocks

from different fishers should be kept separate and crabs should be kept in holding tanks for a minimum of 24 hours before transit. During transport, recommended conditions equated to maximum densities of 1 kg crab to 1 kg of water, a salinity of 34 at 8^0C and an O_2 concentration of at least 5mg/l (Jacklin, 2007). These guidelines are aimed at improving the survivability, and therefore profit to fishers, and other industry groups, an increased standard of product offered to the public and importantly, a move towards improving the welfare of fished crabs.

2.1.2 Chelea retention methods

Claw nicking is the typical method of immobilising chelae of brown crab for fishery purposes. Nicking renders the claw non-functional by cuttg a tendon (Figure 3).

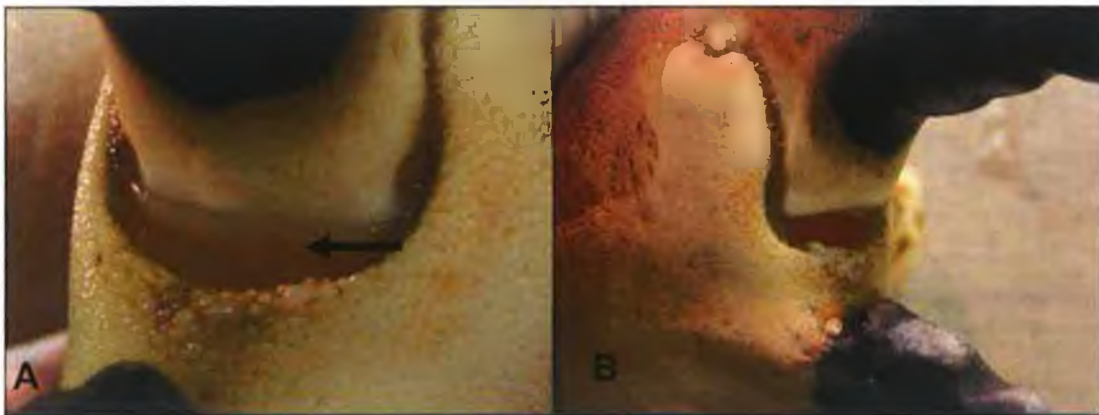


Figure 3. A) An un-nicked brown crab chelae showing conective tissue (Arrow) and B) Nicked chelea using the French nicking method with obvious lack of connective tissue between palm and pincer.

The tendon may be fractured by a) French nicking, where the tendon is cut in the claw hinge joint between the upper and lower dactyl (Haefner, 1971; Figure 4), or b) English nicking, where the tendon is cut between the palm and pincer (Jackilin, 2005; Figure 4).

morphology of the upper chelae. Other methods have been considered, such as the use of individual sacks and boxes. Although these prevent fighting, they are time consuming, expensive and require more space. They are therefore not viable due to the value of the crab as a product. No publications have considered the effects of nicking as a potential point of entry for opportunistic bacteria and potential pathogens. Furthermore, there is a demand for knowledge regarding brown crab health. Stentiford (2008) highlighted that studies on brown crab are inadequate and made a request for further baseline data.

The aim of study is to provide histopathological information on the effects of post harvest processes on brown crab from the Irish fishery.

2.2.0 Materials and Methods

A multi-disciplinary investigation of brown crab was conducted using histology, polymerase chain reaction (PCR) and physiological variables to assess affects of selected post harvest processes. The techniques employed were chosen for their reliability, the amount of information they produce and feasibility. Histology was selected as a visual guide to the progression of the tissue deterioration, haemocyte response and identifying the location of potential pathogens within tissues. PCR was chosen to validate information gathered from bacterial culturing. Microbiological methods were utilised to gain pure isolates to assess colony morphology and bacterial characterisation using chemical tests. Bacterial isolates were archived pending further investigation.

A total of 270 female brown crab where caught off Malin Head, County Donegal during February 2009. From the sample, 135 crabs were nicked using the commercial French method and 135 were banded using 5-10cm inner tube lengths. Upon landing 10 nicked and 10 non-nicked crabs were dissected (Table 2). The crab were transported to commercial ponds (not owned by GMIT) in Clarinbridge, County Galway (approximately 331km) in fish boxes and placed in two tanks with re-circulating natural seawater. The nicked crab were placed into an identical tank adjacent to the non-nicked crab. Both tanks were rigged to the same re-circulation system and filter, and therefore used the same water. On each sampling day, for 20 days, 10 nicked and 10 non-nicked crabs were dissected and the following samples were taken (Figure 5).

Day	Dissected		Samples		
	Nicked	Non-nicked	Histology	Microbiology	Physiological Variables
0	10	10	X	X	X
1	10	10	X		
2	10	10	X	X	X
3	10	10	X		
4	10	10	X	X	X
5	10	10	X		
7	10	10	X	X	X
10	10	10	X	X	X
14	10	10	X	X	X
17	10	10	X	X	X
20	10	10	X	X	

Table 2. Techniques used on nicked and non-nicked brown crab on each sample day (X) throughout the trial.

Figure 5. Natural seawater recirculation tanks used for holding live brown crab during the trial. Clarinbridge, Co. Galway.

2.2.1 Observations

Prior to dissection, the crab were subjected to a visual check and observations were recorded including sex, carapace length, limb loss, shell damage and fouling.

Water quality was monitored throughout the trials. Variables measured included temperature, salinity, pH, Nitrate, Nitrite and Ammonia using Hach Lang DR 800 colorimeter, unless otherwise stated.

2.2.2 Histology

Approximately 7mm^2 samples of heart, hepatopancreas, gill, gut, gonad, claw muscle and epidermis were placed into Davidson's seawater fixative (Hopwood, 1996; Howard *et al.*, 2007) for 24 hours, after which they were stored in 70% ethanol pending further investigation. The samples were then run through a process of graded ethyl alcohols, clearing and infiltration of polymer wax. The samples were placed in paraffin wax, embedded (Leica EG 1150H) (Sheehan and Hrapchak, 1973; Luna, 1992) and sectioned using a microtome (Leica RM2125) (Howard *et al.* 2007). Sections (3-5µm) were mounted on slides and stained using the Hematoxylin and Eosin Y (H&E) stain method (Johnson, 1980a). H&E stain is widely used and was chosen because it does not fade with time, it is compatible with fixatives and is not affected by the long term storage of the tissues (Johnson, 1980a). Four slides per tissue sample containing a total of eight sections were used to assess pathologies within the tissues of each animal. The tissues were then examined using a standard light microscope (Olympus BX41) and camera system (Olympus E330 and Olympus eye programme). Each pathology observed within a tissue was counted as one. Total pathologies were calculated by adding all the pathologies found in all tissues and subsequently all animal within the sample group. In addition, the severity of the pathology was noted. The severity was established by the size of the area of tissue it affected.

2.2.3 Physiological Variables

All reactions were carried out in 96 microlitre well plates.

Haemolymph Glucose

Haemolymph was extracted (0.2ml) using a 19 gauge syringe, placed directly into a 1.5ml micro-centrifuge tube containing 0.6M Perchloric acid and stored in a freezer. Later that week, samples were removed, defrosted and centrifuged for 3 minutes at 8000g. 2M Potassium bicarbonate (7µl) was added and gently shaken to form a supernatant, followed by chilling at 4^0C for 5 minutes to allow for neutralisation. Samples were then centrifuged for 1 minute at 8000g and the supernatant removed. The Supernatant (50µl) was then diluted with 100µl of deionised water.

Supernatant dilute (50µl) was added to 100µl glucose oxidase reagent (Sigma-Aldrich, Ireland), vortexed and incubated for 30 minutes at 37^0C. Once removed from the heating block, 100µl 12N sulphuric acid was added. Samples were vortexed for 15 seconds. 250µl of sample was then placed into a corresponding well on a 96 well plate.

To obtain a standard curve (range from 0-80µg/ml) and blank, the following series of dilutions was made using glucose and deionised water:

Standard	Glucose Standard	Deionised Water
Blank	0 µl	100 µl
0 µg	0 µl	100 µl
20 µg	2 µl	98 µl
40 µg	4 µl	96 µl
60 µg	6 µl	94 µl
80 µg	8 µl	92 µl

Table 3. Glucose dilutions used to obtain a standard curve and blank when testing for .

Samples and blanks were read using a photometric microplate reader (Labsytems Multiskan RC) at 540nm.

Haemocyte Lactate

Haemolymph extraction and initial processing was carried out as for haemolymph glucose analysis, described above.

Supernatant dilute (2.5µl) was added to 250µl lactate reagent (Sigma-Aldrich, Ireland) and then placed into a corresponding well on a 96 well plate. Samples were read using a photometric microplate reader (Labsytems Multiskan RC) at 540nm.

Haemolymph Refractive Index

Haemolymph was extracted (0.2ml) using a 19 gauge syringe and placed directly onto refractometer (E-Line ATC refractometer, Bellingham & Stanley Ltd, UK).

Granulocyte Counts

Haemolymph was extracted (0.2ml) using a 19 gauge syringe and placed directly into 4% neutral buffered formalin (NBF) for fixation. Fixed haemolymph was smeared onto slides and analysed for granulocyte counts using a standard light microscope (Olympus BX41) and camera system (Olympus E330 and Olympus eye programme). Granulocyte counts were obtained by counting the number of granulocytes in 100 haemocytes.

Granulocytes (Figure 6) are characterised by a distinctive eosinophilic granular cytoplasm and dense basophilic nucleus. The nuclear-cytoplasm size ratio of granulocytes (cell Ø = cc.18-35µ; nucleus Ø = cc.7-9µ) is lower than that of other haemolymph cells. Hyaline cells (Figure 6) contained a lightly staining cytoplasm with a high nuclear-cytoplasm size ratio (cell Ø = 7-10µ; nucleus Ø = 5-7µ) (Wood and Visentin, 1967). For the purpose of this study, semi-granulocytes were counted as granulocytes.

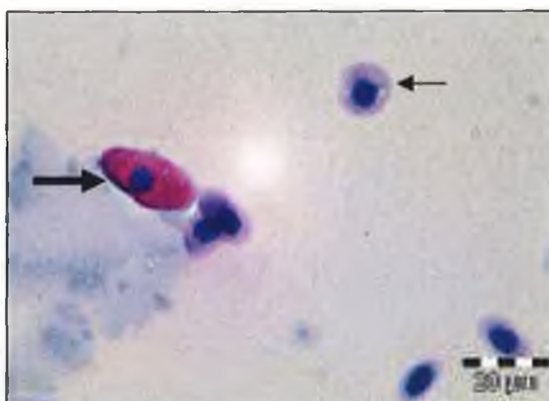


Figure 6. Granulocyte (thick arrow) and hyaline cell (thin arrow).

2.2.4 Microbiology

The methods used for bacterial identification were adapted from Cowan and Steel's Manual for the Identification of Medical Bacteria (Barrow and Feltham, 1999). Haemolymph (0.5ml), claw shell and claw muscle was extracted using sterile 19 gauge syringe and placed into a 2ml centrifuge tube and placed on ice. The samples were weighed before adding sterile artificial seawater until a volume of 2ml was reached. The samples were then homogenised and 200µl was pipetted onto seawater agar plates and then spread using sterile hockey sticks. The plates were incubated at 10^{0}C for seven days before colony counts and sub culturing occurred. Primary tests were carried out on fresh, pure isolates, which were obtained through multiple sub culturing. Measurements taken to describe bacteria were colony morphology, shape, motility, Gram, aerobic/anearobic growth, catalase, oxidase, glucose and carbohydrate. Other variables included testing for vibrios using marine Thiosulphate Citrate Bile-salt Sucrose (marine TCBS) medium and chitinlytic activity.

2.2.5 Molecular Analysis

Approximately $5mm^2$ samples of heart, hepatopancreas, gill, gut, gonad, claw muscle, epidermis and observed bio films were placed into 1.8ml cryovials containing 1.5ml of 100% ethanol. DNA extraction was performed using DNeasy® Blood & Tissue extraction kit (Qiagen, UK). Universal forward and reverse primers designed to target the conserved 16S rRNA region of bacteria were used for PCR amplification. The forward, (S-D-Bact-008-A-s-20 5'-AGA GTT TGA TCC TGG CTC AG-3') and reverse (S-*-Univ-0536a-A18 5'-GWATTA CCG CGG CKG CTG-3') primers were designed to generate approximate bands of 529 bp corresponding to nucleotides 8-536 of the 16s rRNA *Eschenichia coli* genome

(Pond *et al,* 2006) and were selected from Suau *et al* (1999). DNA was amplified in 25µl reaction mixture containing 12.5 µl GoTaq Green master mix (Promega, USA); 6.5µl DNA/RNA free water (Invitrogen, UK); 0.5 µl of each primer (10pmol/ul each) and 5 µl of template DNA. Template DNA consisted of DNA extracted from a selection of tissue samples and purified cultured bacterial colonies. Concentration and purification of genomic DNA was checked using BioPhotometer (Eppendorf, Germany) after extraction and after PCR.

PCR reaction was performed as follows: initial denaturation at 94^0C for 10 mintues. Subsequently, 35 cycles of the following were carried out: denaturing at 94^0C for 1 minute, annealing at 55^0C for 1 minute and elongation at 72^0C for 1 minute with a final elongation step at 72^0C for 10 minutes (Pond *et al.,* 2006). PCR was carried out on PxE 0.5 Thermo cycler (Thermo Scientific). Amplified PCR products (5µl) were electrophoresed in a 1% agarose/TBE buffer gel containing 2.4µl SYBR® Safe (Invitrogen, Uk) and visualised using DNR Gel capture imaging software. A 1kb ladder (Invitrogen, Ireland) molecular marker was used for comparison.

2.2.6 Statistics.

Descriptive statistics was carried out on all the data using Minitab 15. Bartlett's test for normality was followed by Levene's test for equal variance. Homogeneous data (parametric data) was subjected to an analysis of variance (ANOVA). If the Lavene's test showed the data to be non-parametric, a square root transformation was used (unless otherwise stated) to obtain parametric data. Transformation of non-parametric data was chosen because parametric tests are considered stronger than non-parametric tests (Underwood, 1996). Tukey's post hoc test was used to show where variance occurred between groups of data.

2.3.0. Results

2.3.1. Observations

Water quality was monitored throughout the trial period at Clarinbridge, County Galway. Temperature stayed relatively constant at 12.7^{0}C from day 0 to 5 and then peaked on day 7 at 14.9^{0}C. A subesquent drop in temperature to circa 6.60^{0}C from day 10 to 17 and a increase to 12.7^{0}C on day 20 occured (Table 4). Salinity varied between 27.00 and 32.50. The ammonia levels were below the toxic level of 6mg/L, ranging from 0.27-0.73mg/L. The water was changed on day 17 due to the Nitrite reaching the maximum reading for that monitor. Nitrate ranged from 5.50mg/L to 24.30mg/L, the maximum limit for the monitor. The greatest increase in Nitrate was between day one and two, with an increase of 15.1mg/L. Nitrite ranged from 9.0mg/L to 165 mg/L with the greatest range occurring between day 10 and 14 (Table 4).

Day	Temp (^{0}C)	Salinity	pH	Nitrate (mg/L)	Nitrite (mg/L)	Ammonia (mg/L)
0	12.8	32.5	7.1	5.5	29.0	0.6
1	12.8	32.5	7.1	5.5	29.0	0.6
2	12.8	32.0	7.1	20.6	67.0	0.7
3	12.7	31.3	7.1	24.3	23.0	0.7
4	12.7	31.4	7.1	23.0	47.0	0.7
5	12.7	31.0	7.0	24.3	9.0	0.7
7	14.9	31.1	6.2	24.3	9.0	0.7
10	6.6	29.5	6.3	24.3	9.0	0.7(L)
14	6.5	29.0	6.6	24.3(L)	165(L)	0.6
17	6.8	27.0	6.8	24.3	18.0	0.3
20	12.7	27.3	6.1	24.3(L)	77.0	0.6

Table 4. Water quality results. showing monitors limits (L) and water change (grey shading) .

Recorded mortalities were >50% higher in the non-nicked crab, with the greatest loss on day four. A total of 6 nicked (of which half died on day four) and 14 non-nicked crabs died during the trial period. However some mortalities were not recorded on the day due to their removal prior to site visit by fishers working at the Clarinbridge facilities.

Claw loss in non-nicked crab was considerably lower than that of nicked crab. The nicked crab array lost a total of 18 claws from 16 animals whilst the non-nicked crab total claw loss was 8 from 8 animals. The greatest loss of claws was on day 0 and 17, both with 3 claws lost from 3 nicked crab.

Total limb (2nd- 4th pereiopods) loss in non-nicked crab was considerably higher than that of nicked crab. The non-nicked crab array lost a total of 287 (from a possible 904) limbs from 78 out of the 117 animals whilst the nicked crab total limb loss was 37 from 24 animals. The greatest loss of limbs was on day 17 with a total loss of 48 limbs lost from 9 non-nicked crabs.

Nicked crab showed blackened tissue around the nicking wound (Figure 7, Figure 8). The blackening of tissue was observed in crab dissected from day 7 but not in those dissected on day 5. Note the extent to which internal tissue is exposed to external environment.

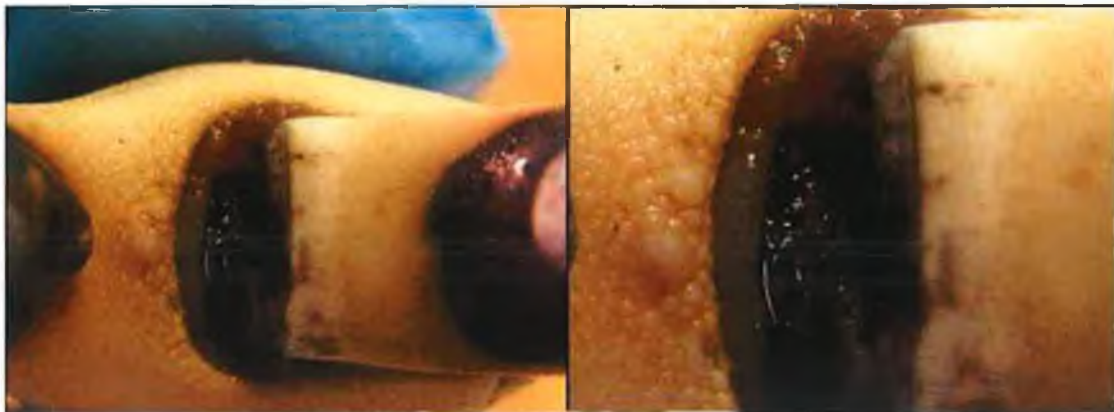


Figure 7. The blackening of claw tissue at the point where the tendon is fractured during French nicking. Photograph from an animal dissected on day seven.

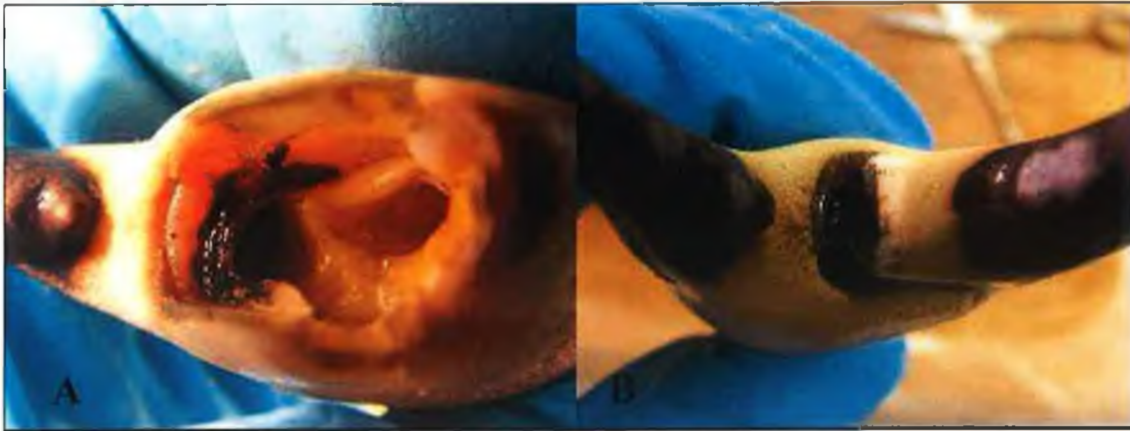


Figure 8. An internal (A) and external (B) view of the blackening of claw tissue at the point where the tendon is fractured during French nicking.

2.3.2. Histology

Both nicked and non-nicked crab showed pathologies throughout the trial (Table 5). Pathologies observed during the trial included granulomas, haemocyte aggregates, necrosis and gill biofilms. More pathologies were observed in nicked crab than in non-nicked crab.

Day	Nicked	Non-Nicked
0	4.50 ± 9.56	1.50 ± 1.65
1	3.90 ± 3.70	1.90 ± 1.91
2	7.30 ± 5.77	3.90 ± 2.38
3	6.50 ± 3.27	4.80 ± 2.15
4	17.30 ± 28.43	5.00 ± 1.15
5	8.70 ± 28.24	6.50 ± 1.25
7	17.90 ± 1.77	5.40 ± 1.18
10	11.20 ± 4.42	9.00 ± 2.00
14	17.00 ± 2.26	15.00 ± 1.76
17	19.00 ± 0.47	17.20 ± 0.42
20	22.30 ± 1.64	20.00 ± 0.00

Table 5. Mean number of pathologies in all tissues of nicked and non-nicked brown ± standard deviation.

Figure 9 and Figure 10 show a selection of pathologies found through out the trial. Figure 9A shows necrosis and initial stages of melanistaition in the myocardium of a nicked animal dissected on day 0. Figure 9C shows multiple granulomas and melanised nodules at varying stages in the connective tissue of a nicked crab on day one. By day three both nicked and non nicked crab were showing dense biofilms on the secondary gill lamella (Figure 9 E/F). Figure 10 shows melanised nodules in connective tissue (A), gill (B-E) and ovary (F).

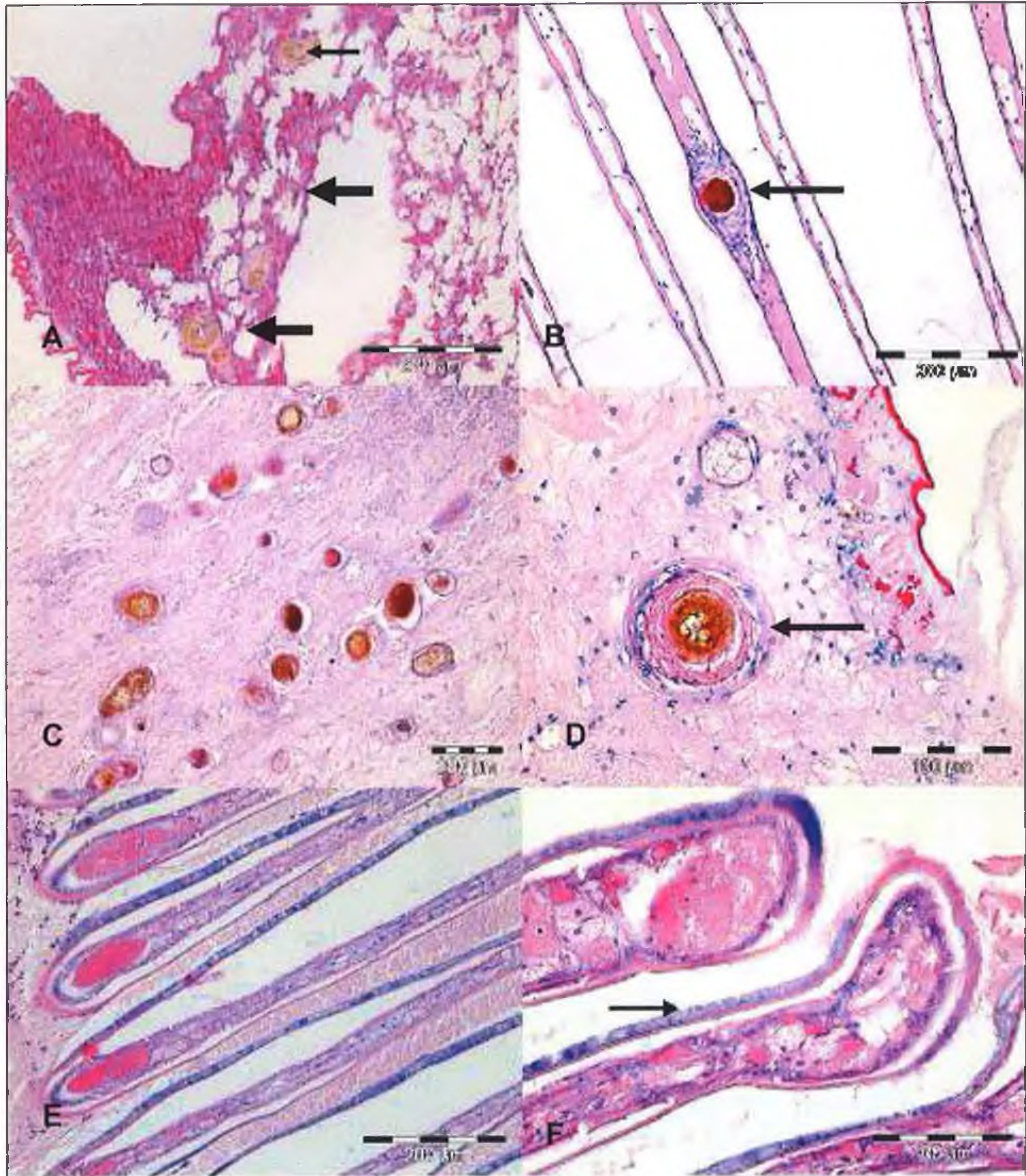


Figure 9. A) Necrosis (small arrow) and advanced melanised nodules (large arrow). B) Swelling, haemocyte aggregate and melanization within a secondary gill lamella from a nicked crab on day one. C/D) Melanised nodules (arrow) in connective tissue from a nicked crab on day four. E/F) Dense bacterial biofilm found on the secondary gill lamella of a nicked crab on day three.

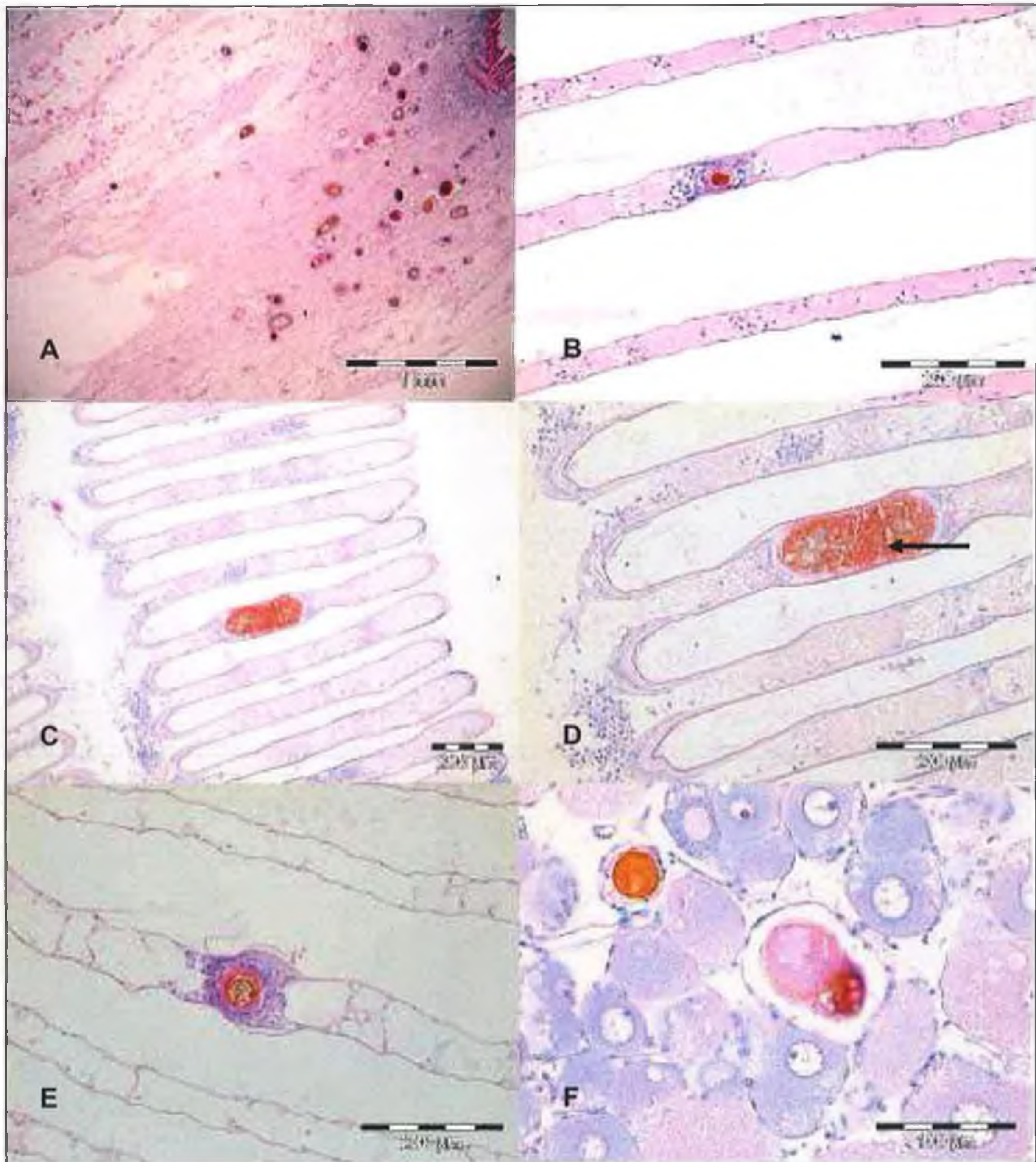


Figure 10. A) Varying stages of phagocytosis in nicked crab from day four. B) Swelling, haemocyte aggregate and melanization within a secondary gill lamella from a nicked crab on day one. C/D) Prolific swelling and melanisation in secondary gill lamella of a nicked crab on day four. E) Prolific swelling and melanisation in secondary gill lamella of a nicked crab on day four. F) Melanised nodule in ovary of a nicked crab on day two (arrow).

The number of pathologies observed over time in nicked and un-nicked crab is shown in Figure 11. Overall, there was a significant difference ($P<0.05$) in pathologies observed between nicked and non-nicked crab, with nicked crabs showing more pathologies. Pathologies in the muscle and hepatopancreas of nicked crab were significantly higher ($P<0.05$) than those in non-nicked crab.

The results showed a significant difference ($P = 0.02$) in total number of pathologies found in nicked (Mean = 11.94; STDEV = 10.90) and non-nicked crab (Mean = 9.10; STDEV = 6.60) with more pathologies observed in nicked animals.

In nicked crab there was a highly significant difference between day 0 (Mean = 4.50; STDEV = 9.56) and day 20 (Mean = 22.30; STDEV = 1.63) with a significant ($P = 0.00$) increase on day four (Mean =17.30; STDEV = 28.43). However, one animal on day four was classed as an outlier with 97 pathologies, when excluding this animal the mean was 8.44 (STDEV = 5.22). Non-nicked crab also showed a highly significant difference between day 0 (Mean = 1.50; STDEV =1.65) and day 20 (Mean = 20.00; STDEV = 0.00). Non-nicked crab proved a significant ($P = 0.00$) increase between day seven (Mean = 9.00; STDEV = 2.00) and ten (Mean =15.30; STDEV = 4.08) (Figure 11; Table 5).

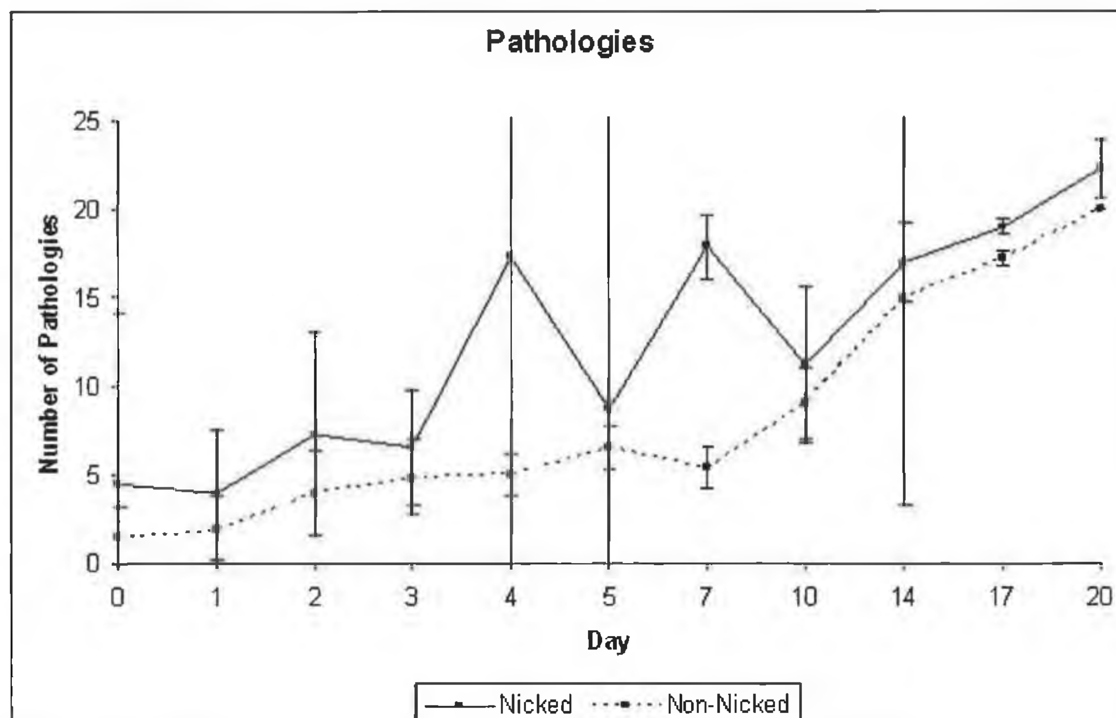


Figure 11. Mean Pathologies found in all tissues in nicked and non-nicked crab. Values shown ± Standard deviation.

Necrosis was consistently higher in the muscle of nicked crab compared to non-nicked crab mean= 1.18, STDEV = ± 1.30 and 0.22, STDEV = ± 0.41 respectively. Over twice the amount of melanisation occurred in the hepatopancreas of nicked crab than in non-nicked crab (mean = 0.31, STDEV = ± 1.73 and 0.90, STDEV = ± 1.16) respectively).

Other observations included a change in hepatopancreatic tubule lumen size. In non-nicked crab lumen size increased after day 5. However in nicked crab this increase occurred after day 14. Most samples showed normal levels of reserve inclusion cells and mature oocytes.

2.3.3 Physiological variables

Glucose values in nicked and non-nicked crab on day 0 were 0.57µg/mL (STDEV ± 0.21) and 0.33µg/mL (STDEV ± 0.08) respectively, decreasing to 0.29µg/mL (STDEV ± 0.05) and 0.13µg/mL (STDEV ± 0.02) on day two. The values then increased to 0.77g/mL (STDEV ± 0.18) in nicked crab and 0.38 µg/mL (STDEV ± 0.11) in non-nicked crab on day four, after which they decreased to 0.17µg/mL (STDEV ± 0.08) and 0.12µg/mL (STDEV ± 0.04) respectively on day 17 (Table 6). There was a highly significant difference (P= <0.000) between nicked and non-nicked crab, with nicked crab showing elevated levels of haemolymph glucose. Tukey's family error also showed a significant difference (P= <0.005) between days for both nicked and non-nicked crab.

Initial lactate values in nicked and non-nicked crab on day 0 were 0.20µg/mL (STDEV ± 0.6) and 0.15µg/mL (STDEV ± 0.05) respectively, decreasing to 0.05µg/mL (STDEV ± 0.01) and 0.04µg/mL (STDEV ± 0.00) on day two. The values then increased to 0.11µg/mL (STDEV ± 0.04) in nicked crab and 0.05µg/mL (STDEV ± 0.01) in non-nicked crab on day four. After a decrease on day seven the average lactate values increased to 0.08µg/mL (STDEV ± 0.03) in nicked and 0.05µg/mL (STDEV ± 0.00) non-nicked crab on day fourteen followed by a decrease on day seventeen to 0.04µg/mL (STDEV ± 0.02) and 0.03µg/mL (STDEV ± 0.02) respectively (Table 6). There was a significant difference (P= <0.005) between nicked and non-nicked crab, with nicked crab showing elevated levels of haemolymph lactate. Tukey's family error rate also showed a significant difference (P=<0.005) between days in nicked but not in non-nicked crab.

Day	Glucose (µg/mL)		Average Lactate (µg/mL)		Refractive Index (n)	
	Nicked	**Non-Nicked**	**Nicked**	**Non-Nicked**	**Nicked**	**Non-Nicked**
0	0.57 ± 0.21	0.33 ± 0.08	0.20 ± 0.06	0.15 ± 0.05	11 ± 1.99	12 ± 1.25
2	0.29 ± 0.05	0.13 ± 0.02	0.05 ± 0.01	0.04 ± 0.01	11 ± 2.23	11 ± 2.75
4	0.77 ± 0.18	0.38 ± 0.11	0.11 ± 0.04	0.05 ± 0.01	12 ± 1.32	11 ± 0.99
7	0.37 ± 0.07	0.21 ± 0.06	0.05 ± 0.01	0.04 ± 0.01	10 ± 0.96	11 ± 0.57
10	0.35 ± 0.07	0.25 ± 0.02	0.06 ± 0.01	0.05 ± 0.01	9 ± 1.01	11 ± 2.13
14	0.23 ± 0.10	0.13 ± 0.02	0.08 ± 0.03	0.05 ± 0.01	11 ± 1.29	10 ± 1.63
17	0.17 ± 0.08	0.12 ± 0.04	0.04 ± 0.02	0.03 ± 0.02	7 ± 3.99	7 ± 4.19

Table 6. Mean glucose and lactate in the heamolymph of crab undergoing two different chelea retention methods showing ± standard error. Mean refractive index of haemolymph of crab undergoing two chelea retention methods ± standard error).

Glucose and lactate values were higher in nicked crab than non-nicked crab throughout the trial. The initial time course for glucose and lactate in nicked and non-nicked crab follow a similar pattern (Figure 12). The three variables decreased from day 0 to day 2, peaked on day 4 and then decrease to day 7. However, lactate and RI measurements increased on day 14 and thereafter decreased whilst glucose levels continued to decline. Glucose was on average >120.55% (STDER = ± 0.58) higher in nicked crab and lactate was 17.90% (STDER = ± 0.87) higher in nicked crab compared to non-nicked crab

Refractive index values on day 0 in nicked and non-nicked crab were very close (11.46, STDER = ± 0.99 and 11.62, STDER = ± 1.25 respectively). These values decreased on day two to 10.96 (STDER = ± 2.23) in nicked crab and 10.56 (STDER = ± 2.75) in non-nicked crab. The values then increased to 12.04 (STDER = ± 1.32) and 11.44 (STDER = ± 0.99) respectively on day four, after which they decreased to 8.96 (STDER = ± 1.01) and 10.64 (STDER = ± 2.13) on day ten. Refractive index values then increased in nicked crab to 11(STDER = ± 1.29) whilst non-nicked crab values continued to decrease. This was followed by a decline in RI values in both nicked and non-nicked crab (7.08, STDER = ± 3.99 and 7.48, STDER = ± 4.19 respectively) (Table 6; Figure 12).

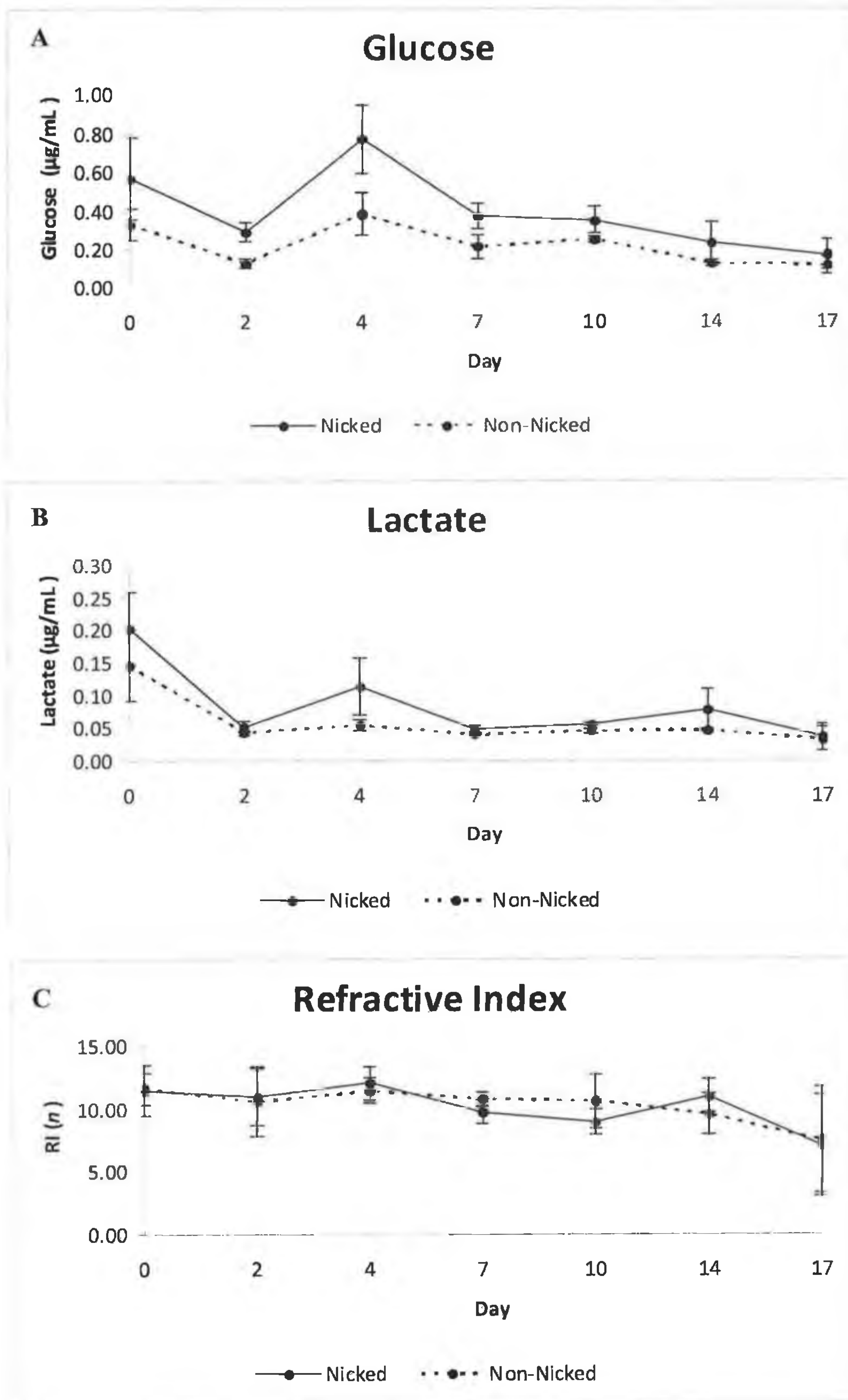


Figure 12. Time course of Glucose (A) and Lactate (B) in the heamolymph of nicked and non-nicked crab. Time course of refractive index (C) of haemolymph in of crab which are either nicked or not nicked. Values shown as means ± standard error.

Granulocyte Counts

Table 7 and Figure 13 show the increase in granulocyte means and standard deviation over the trial period. There was a significant difference (P <0.05) in granulocyte numbers between groups. Tukey's post hoc test also showed a positive regression (highly significant Coefficient (P <0.00)) for granulocyte counts from nicked crab.

On day 0, both nicked and non-nicked crab had a mean granulocyte count of circa 32 % (STDEV = ± 12.97, ± 5.65 respectively), whilst the granulocyte cell count on day 20 for nicked crab was >39% (STDEV = ± 6.70) and non-nicked crab was 37% (STDEV = ± 4.24). However, there was only a significant difference (P = 0.01) in granulocytes over time in nicked crab after data had been transformed. Granulocyte counts in nicked crab peaked on day 17 at 45.70 (StDev ± 5.38) and on day 10 in non-nicked (mean = 41.80; StDev ± 5.38) animals.

Day	Granulocytes	
	Nicked	Un-Nicked
0	32.90 ± 12.97	32.10 ± 5.65
1	40.00 ± 10.22	37.00 ± 10.92
2	36.30 ± 5.41	31.90 ± 8.21
3	37.60 ± 7.90	34.50 ± 6.96
4	33.50 ± 6.38	36.30 ± 7.72
5	39.40 ± 10.05	37.80 ± 6.56
7	43.80 ± 7.15	39.60 ± 7.44
10	42.60 ± 6.08	41.80 ± 7.87
14	42.60 ± 9.62	38.30 ± 7.24
17	45.70 ± 5.38	39.30 ± 4.95
20	39.20 ± 6.70	37.00 ± 4.24

Table 7. Mean granulocyte counts (%) of nicked and non-nicked brown crab over time in holding including Values shown as mean ± standard deviation.

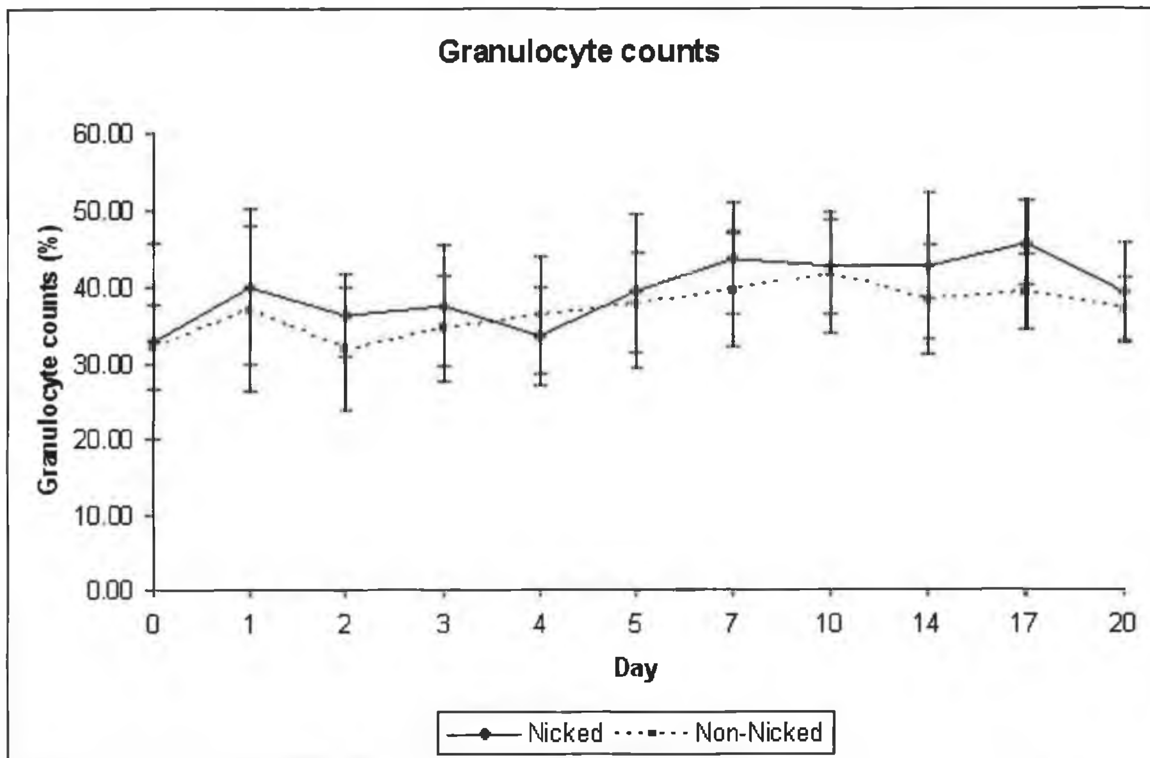


Figure 13. Change in mean granulocyte counts (%) in nicked and non-nicked brown crab over holding period. Values shown as means ± standard deviation.

2.3.4. Microbiology

Colony Counts

Colony forming units (CFU), which measure viable bacteria isolates, were counted after three days incubation and results are presented in Figure 14.

Total CFUs were not significantly higher (P = 0.15) in nicked crab than non-nicked crab with the greatest difference occurring on day four when mean total CFUs in nicked crab was 710 (STDEV = ± 413.95) and 234 (STDEV = ± 114.98) in non-nicked crab. Overall mean total CFUs increased in nicked crab (154.33, STDEV = ± 47.73 to 292.67, STDEV = ± 120.00) and in non-nicked crab (147.67, STDEV = ± 82.77 to 390.67, STDEV = ± 279.75) (Table 8; Figure 14).

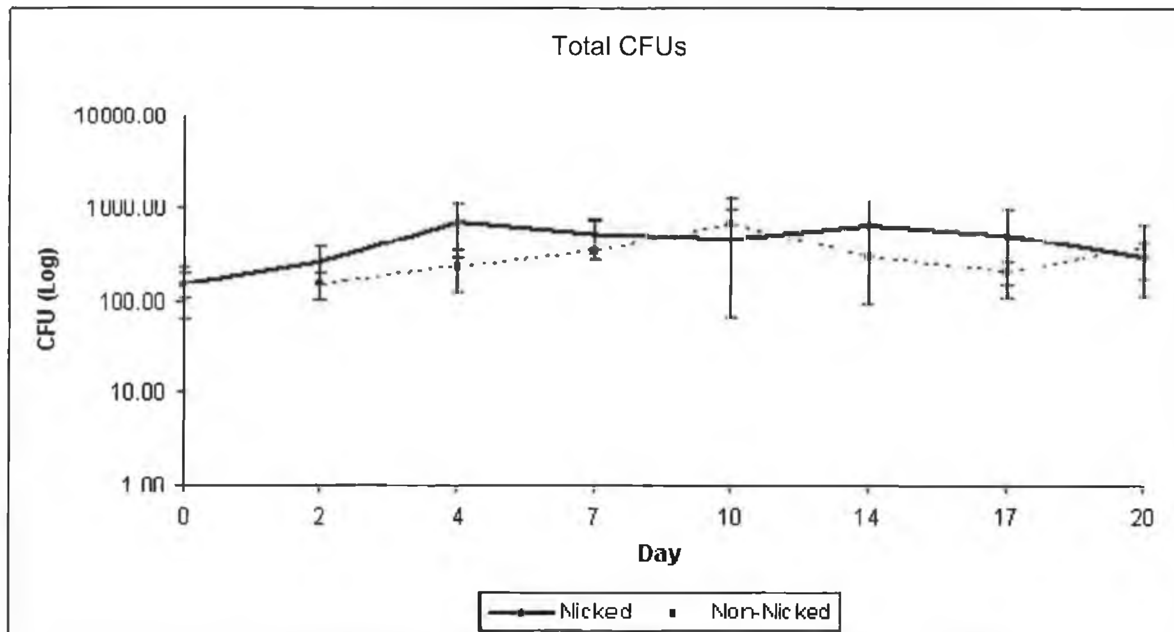


Figure 14. Mean CFU in nicked and non-nicked crab over time in holding ponds. Values shown as means ± standard deviation

Colony forming units from haemolymph were significantly higher (P = 0.009) in nicked crab (Mean =557.2, STDEV= ± 313.0) than in non-nicked crab (Mean = 210.3; STDEV = ± 74.2) (Figure 15; Table 8). Haemolymph colony forming units in nicked crab increased until day four, decreased on day seven then reached a plateau thereafter, finally declining on day 20. Overall, haemolymph CFUs in nicked crab increased from 111.00 (STDEV = ± 47.16) to 174.00 (STDEV = ± 118.42) and from 102.67 (STDEV = ± 41.19) to 159.32 (STDEV = ± 163.66) in non-nicked.

Data on CFUs from claw muscle was non parametric and was transformed using Log_{10}. Colony forming units from claw muscle were not significantly higher (P = 0.27) in nicked crab. However there was a difference in nicked (Mean = 2.63, STDEV = ± 0.29) than in non-nicked crab (Mean = 2.482; STDEV = ± 0.236) (Figure 15; Table 8). On days 0 and 14 CFUs were higher in the claw muscle of non-nicked crab then nicked crab. CFUs from claw muscle of nicked crab increased from day 0 to day two, reached a plateau until day seven, increased until day ten, then increased again between day 17 and 20. In non-nicked crab CFUs declined between day 0 and two, increased from day two to 14, followed by a decrease until day 17 and a final increase on day 20. Overall, haemolymph CFUs in nicked crab increased from 130.33 (STDEV = 40.65) to 1083.00 (STDEV = 722.27) and from 238.67 (STDEV = 44.79) to 266.33 (STDEV = 238.54) in non-nicked specimens.

Group	Day	Total	Haemolymph	Claw Muscle	Claw Shell
Nicked	0	154.33 ± 47.73	111.00 ± 47.16	130.33 ± 40.65	157.00 ± 60.31
	2	264.33 ± 117.11	321.67 ± 190.69	283.67 ± 38.55	244.00 ± 111.73
	4	710.00 ± 413.95	930.33 ± 507.61	296.67 ± 35.57	468.67 ± 323.04
	7	524.00 ± 243.77	664.67 ± 269.52	362.67 ± 62.07	578.33 ± 297.67
	10	476.33 ± 488.54	854.33 ± 569.03	744.33 ± 154.94	744.33 ± 662.89
	14	649.00 ± 554.07	656.00 ± 735.08	629.00 ± 754.71	306.33 ± 67.04
	17	519.00 ± 412.90	745.67 ± 660.81	562.00 ± 242.13	510.33 ± 375.16
	20	292.67 ± 120.00	174.00 ± 118.42	1083.00 ± 722.27	615.33 ± 781.86
Non-Nicked	0	147.67 ± 82.77	102.67 ± 41.19	238.67 ± 44.79	744.33 ± 50.46
	2	151.67 ± 51.20	150.67 ± 58.05	169.33 ± 67.87	174.67 ± 45.08
	4	234.67 ± 114.98	219.00 ± 57.24	233.67 ± 30.09	228.00 ± 30.05
	7	339.33 ± 399.85	311.00 ± 43.71	303.33 ± 97.53	716.33 ± 678.70
	10	687.33 ± 618.38	280.67 ± 64.07	670.00 ± 720.21	1081.33 ± 725.15
	14	304.00 ± 405.04	279.00 ± 81.50	728.67 ± 668.21	279.33 ± 32.13
	17	210.00 ± 59.09	180.00 ± 15.72	193.33 ± 58.32	167.00 ± 98.97
	20	390.67 ± 279.75	159.33 ± 163.66	266.33 ± 238.54	451.67 ± 404.63

Table 8. Mean colony forming units from haemolymph, claw muscle and claw shell ± standard. Total colony forming units for all tissues combined, ± standard deviation in crab undergoing two chelea retention methods.

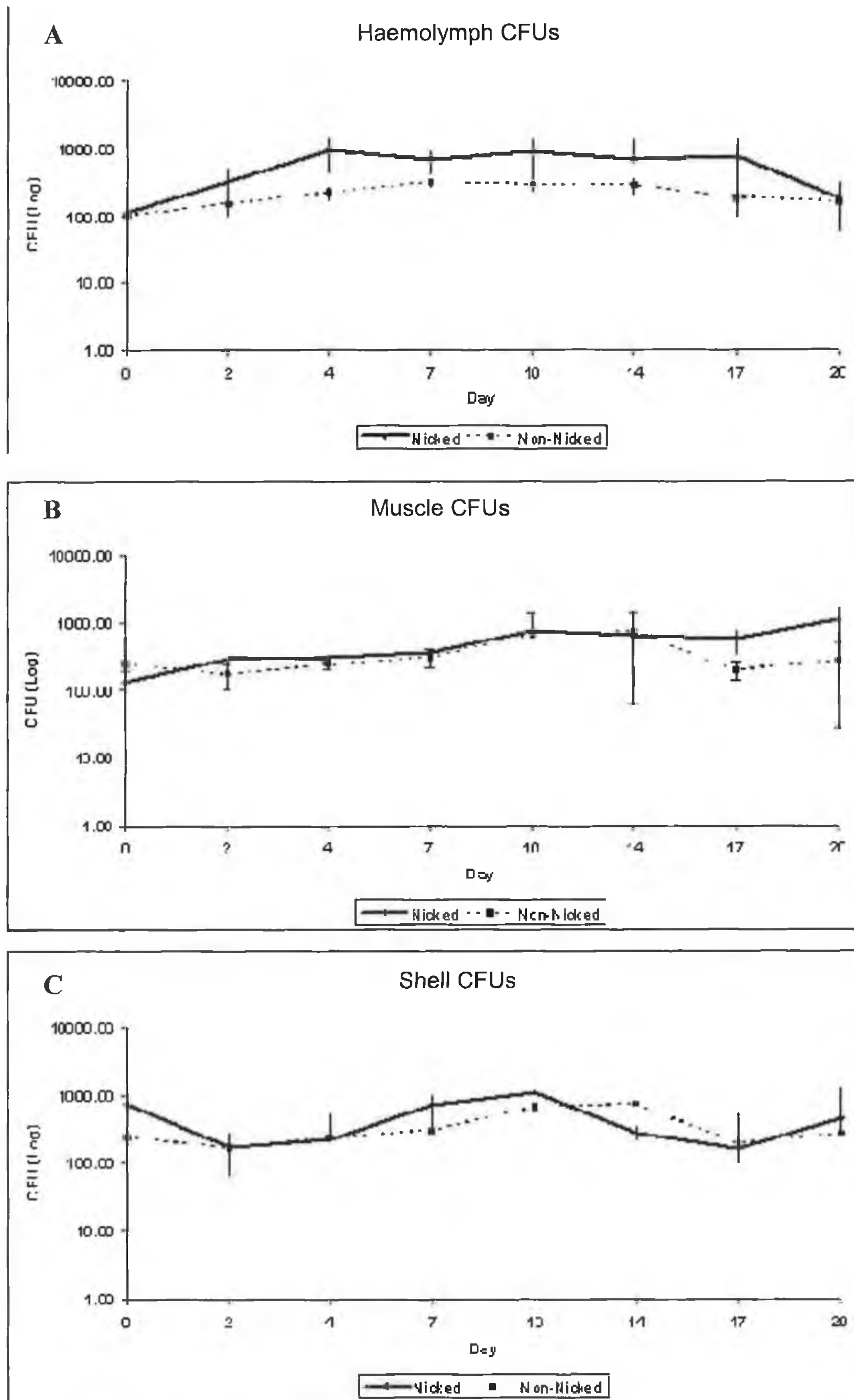


Figure 15 Changes in total colony counts from haemolymph (A), muscle (B) and claw (C) shall samples from nicked and non-nicked crab over time. Values shown as means ± standard deviation.

Colony forming units from claw shell were not significantly higher (P = 0.846) in nicked crab. However, there was an overall difference in means between nicked (Mean = 453.0, STDEV = ± 201.2) and non-nicked crab (Mean = 480.3; STDEV = ± 334.5) (Figure 15; Table 8). On day 14 CFUs were higher in the claw shell of non-nicked crab then nicked crab. CFUs from claw muscle of nicked crab decreased from day 0 to day two, increased until day ten, then decreased again between day 10 and 17 and finally increased between day 17 and 20. In non-nicked crab CFUs between day 0 and seven were at a plateau, increased from day seven to 14, followed by a decrease until day 17 and a slight final increase on day 20. Overall, haemolymph CFUs in nicked crab increased from 157.00 (STDEV = 60.31) to 615.33 (STDEV = ± 781.86) and from 744.33 (STDEV = ± 50.46) to 451.67 (STDEV = ± 404.63) in non-nicked crab.

In nicked crab, total CFU samples showed an exponential phase between day 0 and four, followed by a stationary phase between days four and 17 and a death phase after day 17. Non-nicked crab samples showed initial exponential phase between day 0 and ten followed by a death phase from day 14 to 20.

Overall the claw muscle of nicked crab showed the highest average colony counts with a total of 4091.67CFUs. Also nicked crab showed higher levels of viable bacteria, with a total of 15763.33 CFUs than non-nicked (10793.67 CFUs).

Classical Identification.

The methods used for bacterial identification were adapted from Cowan and Steel's Manual for the Identification of Medical Bacteria (Barrow and Feltham, 1999). Physiological observation and chemical test were used and results were compared to identification information in Barrow and Feltham (1999).

Vibrio spp. were found throughout the trial, primarily in haemolymph samples. Other bacteria found in the haemolymph were *Colweilliaceae* and *Psuedoalteromonadacea.* Bacteria found on the claw shell throughout the trial were *Flavobacteriaceae* and *Psuedoalteromonadacea. Vibrionaceae* were isolated from claw muscle samples along with *Psuedoalteromonadacea* and *Flavobacteriaceae.*

Whilst these groups of bacteria were identified by the above characteristics, further molecular investigation would confirm the identification and group up to strain level.

2.3.5 Molecular Analysis

Bacteria from muscle samples were extracted and amplified using universal primers (Suau, 1999; see 2.1.3) and visualized on 1% agarose gel using SYBR® Safe (Invitrogen, Uk).

Figure 16 and Figure 17 show whether bacterial DNA was present in the samples. Bacterial DNA after PCR amplification of the 16srRNA region is signified by a band in the region of the 506bp mark on the 1kb molecular marker (L). Lanes 1-13 show 16s rRNA PCR amplification of genomic DNA from the claw mucscle of non-nicked crab. Lane 13 shows the results from 16s rRNA amplification of genomic DNA extracted from the hepatopancrease of a non-nicked crab. Lanes 14-17 in Figure 16 show results from template DNA extracted from the claw muscle of nicked crab. Each pathology observed within a tissue was counted as one. Total pathologies were calculated by adding all the pathologies found in all tissues and subsequently all animal within the sample group. In addition, the severity of the pathology was noted. The severity was established by the size of the area of tissue it affected.

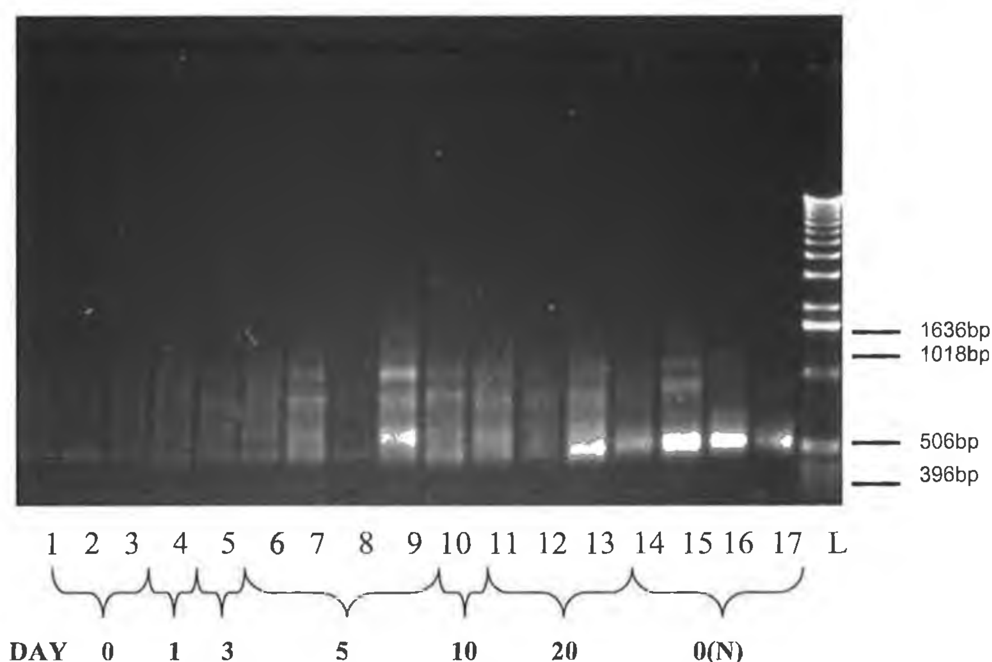


Figure 16. 1% Agarose gel with samples obtained from muscle of non-nicked crab on days 0, 1, 3, 5, 10, 20 and day 0 in nicked crab (0(N)) Visualised against a 1KB ladder (L).

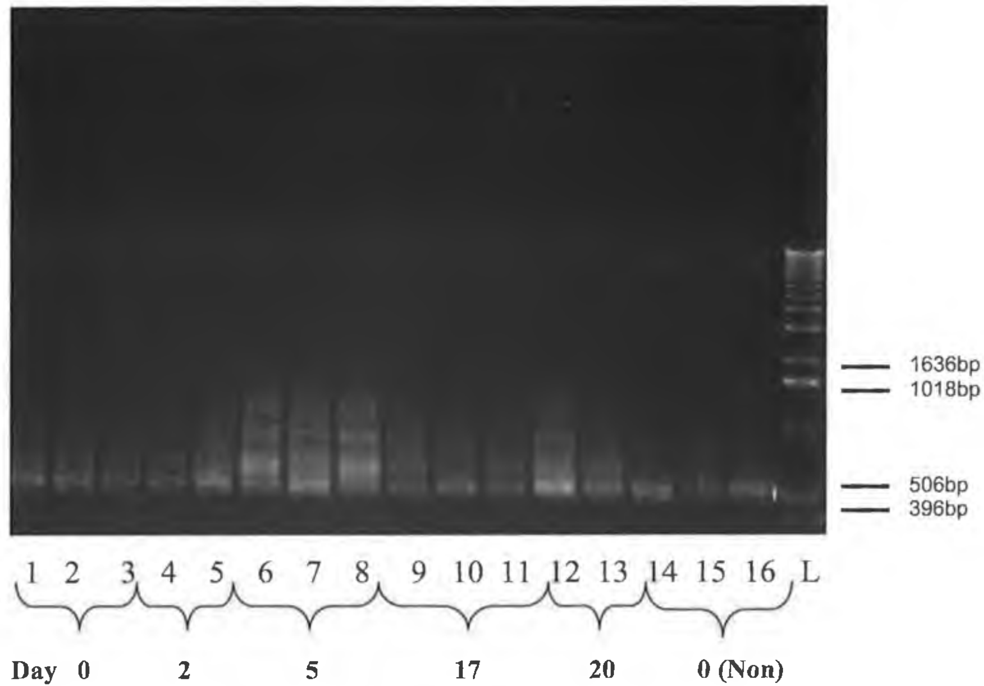


Figure 17. 1% Agarose gel with samples obtained from muscle of nicked crab on days 0, 2, 5, 17, 20 and day 0 in non-nicked crab (0(Non)) Visualised against a 1KB ladder (L).

Lanes 1-14 in Figure 17 show results from template DNA extracted from the claw muscle of nicked crab. Lanes 14-16 show the results from 16s rRNA amplification of genomic DNA extracted from the hepatopancrease of a non-nicked crab. The results showed a similar strength 16s rRNA signal in both nicked (lanes 1-13) and non nicked (lanes 14-16) with the exception on one sample (lane 15). This sample showed a very low signal.

All template samples showed some genomic DNA around the expected 526bp marker. Several of the samples showed multiple bands and slight smearing in the direction of migration. Nicked crab showed higher relative levels of genomic DNA yield than non-nicked crab.

2.4.0 Discussion

2.4.1 Observations

Non-nicked crab endured the greatest limb loss (31.75 %) and the highest mortality rates. This may be due to the method of chelae retention chosen for this study as many of the crab removed the restraint during the trial. The most appropriate method of chelae retention available at the time of the study was tubing covering the knuckle to the end of the chelae and imposed the least amount of implications to the design of the experiment. The selected method enabled the two sample groups to be subjected to the same conditions. However several of the brown crab removed the tubing, freeing their chelae and enabling them to cause damage to other crab in the pond. Using tubing for chelae would not be recommended as it is time consuming and not effective enough in reducing damage.

The highest levels of mortalities were recorded on day four; which also showed the highest mean glucose levels in both nicked and non-nicked crab (Nicked = 0.77µg/mL, STDEV = ± 0.18; Non-nicked = 0.38µg/mL, STDEV = ± 0.11); mean RI levels in nicked crab (12.04, STDEV = ± 1.32); mean levels of total CFUs (710.00, STDEV = ± 413.95) and the highest level of mean CFUs (930.33, STDEV = ± 507.61) in the haemolymph throughout the trial period. The levels of mortality on day four may be a result of these combined factors. Jacklin (2007) hypothesised that bacterial infections are the causative agent of mortality in stored nicked brown crab. Due to the open circulatory system in brown crab (Fretter and Graham, 1976) the colony forming units obtained from haemolymph samples would be considered 'circulating CFUs whereby the bacteria rapidly spreads to all areas of the animal where haemolymph is found. However, there appeared to be no correlation between an increase in CFUs in one tissue and changes in CFU's of another tissue.

2.4.2 Histology

Pathologies have previously been observed in brown crab with experimental and natural infections (Stentiford *et al.,* 2002; Vogan *et al.,* 2002; Stentiford *et al.,* 2003; Costa-Ramos *et al.,* 2004; Stentiford and Bateman, 2007) but few scientific studies have compared the histology of brown crab under commercial conditions.

The results showed a significant difference (P = 0.020) in total number of pathologies found in nicked (Mean = 11.96; STDEV = ±1 0.90) and non-nicked crab (Mean = 9.10; STDEV = ± 6.60) with more pathologies observed in nicked animals.

The significant increase in mean total pathologies on day four (Mean =17.30; STDEV = ± 28.43) was likely due to outliers, with one animal having a total of 97 pathologies. If this animal is excluded the mean is 8.44 (STDEV = ± 5.22). This anomaly showed numerous areas of phagocytises at varying stages of melanisation from granuloma to melanised nodules within the connective tissue surrounding the gut.

Non-nicked crab showed a highly significant increase (P= <0.01) in total pathologies observed between day 0 (Mean = 1.50; STDEV = ±1.65) and day 20 (Mean = 20.00; STDEV = ± 0.00) with non-nicked crab showing a highly significant increase (P = 0.00) in total pathologies between day seven (Mean = 9.00; STDEV = ± 2.00) and ten (Mean =15.30; STDEV = ± 4.08). The increase in pathologies did not appear to be linked with water quality or physiological parameters which decresed with time in storage. However, there was an increase in circulating CFUs with time.

Gill bio-films were not observed on all crab and their occurrence throughout the trial was sporadic. Gill bio-films were observed on days two and seven in nicked crab and on day 14 in non-nicked crab. However, it is hypothesised that gill bio-films would increase with time in storage or with an increase in claw shell bacteria concentrations.

Observations showed that both nicked and non-nicked crab had an increase in hepatopancreatic lumen size. Increased lumen size is considered a result of reduction in reserve inclusion (RI) cell size. RI cells are found in the hepatopancreas tubule wall and are used for storage. Reduction in size often occurs during starvation (Johnson, 1980a). However most samples showed normal levels of reserve inclusion cells and mature oocytes, which also often decline in numbers during periods of starvation.

Perhaps the most interesting observation was the difference in number of melanised hepatopacreatic tubules. The total number of nicked animals showing hepatopancreas degradation through melanisation was 14 compared to 3 in non-nicked crab. Whilst the

hepatopancreas is not the most valuable product from crab it is still sold for human consumption.

In nicked crab there was a highly significant difference in pathologies over time in holding. Most of the pathologies occurred in the claw muscle of the nicked crab. Observed muscle necrosis severity varied with the worst cases occurring in nicked animals. Jacklin (2007) noted that the claw meat from nicked crab started to blacken after ten days in storage. Observations from this experiment showed that the blackening and scaring occurred within the first seven days with pathologies including necrosis-like degradation occurring from day 0.

The implications of damage to the claw muscle are two fold. Firstly, the damage results in a reduction in the animal's health and possibly reduced survivability. Such damage triggers an innate immune system response; this response uses energy resources (Smith and Metchnikoff, 1901; Bang, 1970; McKay and Jenkin, 1970; Paterson and Stewart, 1974; Tauber, 2003). Therefore, when an animal is subjected to additional stressors, such as storage and handling, it is possible that the innate immune system may be unable to respond so efficiently to infections occurring elsewhere in the anatomy. This has been shown in mammals (Maslanika *et al.,* 2009), invertebrates (Mydlarz *et al.,* 2008) and crustaceans (Stewart and Zwicker, 1972; Le Moullac and Haffner, 2000; del la Vega *et al.,* 2007).

Secondly, the claw muscle is the most valuable product of brown crab. Discolouration and a reduction in quality of claw muscle results in a lower value or unmarketable product. This, with the addition of mortalities, has an effect on the economics of the Irish shellfish fishery. It is often the upstream primary producers (the fisher) which incur the loss.

2.4.3 Physiological variables.

As suggested by the literature (Wheatly and Taylor, 1992; Lorenzon *et al.,* 2008), this study showed that glucose and lactate levels receded between 0 and 48 hours. Lorenzon *et al.,* (2007), Ridgeway *et al,* (2006) and Spicer *et al.,* (1990) all noted that glucose levels in the haemolymph are related to the availability of energy during stressful circumstances and should return to normal once the stressor has been reduced. The 48 hour period of decline was followed by a peak on day four (96 hours) in glucose, lactate and RI levels. Glucose is

used for anaerobic glycolysis which results in accumulation of lactate in the muscle (Paterson *et al.,* 2005). Ridgeway *et al.,* (2006) suggested that an increase in haemolymph lactate may be a result of an animal's inability to supply tissues with sufficient amounts of oxygen. At the time of the trial it was not possible to measure the dissolved oxygen of the water, therefore, reduced oxygen levels can not be eliminated as a possible cause of the increased glucose and lactate levels. Water quality tests for day four showed an elevated nitrite level (47.00mg/mL), which was above the safe levels for brown crab as recommended by Estrella (2002). This decrease in water quality may have compromised microbiological and physiological data obtained from the experiment but may also be indicative to the increased mortality rates that occurred on this day.

Furthermore, daily checks for mortalities and moribund individuals necessitated that the crab be handled. This additional stressor, which would also be encountered in the fishery, may have caused the levels of glucose and lactate to be continuously higher than that of crab in the wild. Diurnal glucose changes in the haemolymph of crustaceans (Kallen *et al.,* 1988; Aguzzi and Sardà, 2007) and crab (Rosas *et al.,* 1992; Tilden *et al.,* 2001) have also been reported. However such patterns would not have been encountered during this study due to the measurements being taken once a day at approximately the same time.

There was an increase in granulocyte means (%) in both nicked and non-nicked crab during the trial period. However, only nicked crab showed a highly significant increase ($P = 0.011$). There was a significant difference in mean granulocytes (%) of nicked and non-nicked crab, with nicked crab showing higher levels of granulocytes on all days via day four when granulocyte counts where higher in non-nicked crab. Day 4 showed the lowest and day 17 the highest granulocyte mean in nicked crab. On these days glucose and lactate showed elevated and reduced levels (respectively) in nicked crab. Results from Paterson *et al.,* (2005) also showed a negative correlation between granulocytes and lactate. Patterson *et al.,* (2005) also identified a correlation between mortality, lactate accumulation and haemocyte-related effects in western rock lobster (*Panulirus cygnus*) undergoing handling and storage. It could be presumed that an increase in total lactate would result in an increased demand for oxygen and as hyaline cells are the primary carriers of oxygen to tissues there would naturally be a decline in granulocytes. However this theory did not apply to non-nicked crab.

2.4.4. Microbiology

Total muscle and shell colony forming units were higher in nicked crab than in non-nicked crab. Colony forming units are an indicator of bacterial levels in the tissue. The higher levels of CFUs from muscle in nicked crab are likely to be due to the fracturing of the chelae tendon. The fracturing caused by nicking resulted in the claw muscle coming in direct contact with the re-circulated sea water. Microbe species inhabiting biological filters used for marine aquaria typically include autotrophic ammonia-oxidizing bacteria such as strains of *Proteobacteria.* Other strains of bacteria which have been identified from marine biofilters include strains archetypal to the marine environment such as *Pseudomonas*, *Oceanospirillum*, *Marinobacter*, *Paracoccus*, *Erythrobacter*, *Vibrio* and *Aeromonas* (Leonard *et al.,* 2000). Hovanec and DeLong (1996) identified 20% of bacteria from marine biofilters as *Nitrosomonas europaea* and related species. It would be expected that such bacteria would be found in the tanks in small numbers, particularly as a biofilm, as biofilter bacteria require a solid growth media. Therefore it is possible that similar bacteria would colonise the claw muscle of nicked crab.

Colony forming units from claw muscle in non-nicked crab were higher on days 0 and 10 than in nicked crab. On the same days non-nicked crab also had higher RI readings than nicked crab. In addition, non-nicked crab showed a peak in granulocyte counts (%) and pathologies on day ten. There were no significant changes in water chemistry or obvious additional stressors on day ten compared to the rest of the trial period but there was a reduction in water temperature between day seven (14.9^0C) and ten (6.60^0C). This may indicate an increase in a bacterial strain which grows at an optimum temperature of circa 6^0C or the decline of one dominant strain allowing others to multiply.

Total colony forming units increased over time in nicked and non-nicked crab. The re-circulation system meant that the seawater was not changed until day 17. The decline in haemolymph CFUs in nicked and non-nicked crab and total CFUs in nicked crab between day 17 and 20 suggests that bacteria present on day one had enough available nutrients to multiply and CFUs may have continued to increase had the water not been changed.

It must be noted that colony forming units do not necessarily represent the full bacterial diversity. Media is selective and it is not always feasible to produce media tailored to the nutrient requirements of all bacterial strains. In addition, it is common knowledge that only a small percentage of bacteria can be grown under laboratory conditions (Fry, 2000). Also, competition (due to growth time and nutrient uptake) between strains in cultures can result in reduced diversity and the mucoid nature of some strains makes it impossible to define individual colonies.

Vibrio spp., Flavobacterium spp. and *Psuedoalteromonas spp.* were identified throughout the trial using methods adapted from Barrow and Feltham (1999). *Vibrio* species were the most dominant from CFUs and were primarily isolated from haemolymph samples. Other suspected bacteria found in the haemolymph were *Colweilliaceae* and *Psuedoalteromonadacea*. Bacteria indentified from the claw shell throughout the trial were *Flavobacteriaceae* and *Psuedoalteromonadacea. Vibrionaceae* were identified in claw muscle samples along with *Psuedoalteromonadacea* and *Flavobacteriaceae*.

The bacteria identified through classical methods were species typical of the benthic niche of brown crab (Austin, 1982; Sapp *et al.* 2007). *Vibrio* species are ubiquitous to the marine environment with some strains being associated with tissue degradation and human pathogens (Ayres and Barrow, 1978; Yano *et al.*, 2006, 2004).

Flavobacterium spp. have been isolated from marine benthic sediments (Fu *et al.*, 2010) and have been isolated from crustaceans (Jussila, 1997; Ceccaldi, 1998). *Flavobacterium spp.* have been associated with mortalities in wild and farmed fish with the strain *Flavobacterium psychrophilum* being the causative agent of coldwater disease in trout and salmon (Rahman *et al.*, 2002). However no direct association of *Flavobacterium spp.* and brown crab mortalities has been made.

Psuedoalteromonas spp. are also ubiquitous to the marine environment (Akagawa-Matsushita *et al.*, 1992; Pernthaler *et al.*, 2001) and can also be found in estuarine habitats (Costa-Ramos *et al.*, 2002). *Psuedoalteromonas spp.* are gram-negative and have been isolated from shellfish (Costa-Ramos and Rowely, 2004; Pernice *et al.*, 2007). There are 12 species within the *Psuedoalteromonas* group (Gauthier *et al.*, 1995). The *Psuedoalteromonas* strain *P. atlanticus* has been isolated from brown crab (Vogan *et al.*,

2002) and has been associated with shell disease and crab mortalities (Vogan *et al.,* 2002; Costa-Ramos and Rowely, 2004). Costa-Ramos and Rowely (2004) described the strain as lethal due to the production of extracellular products (ECP). Death resulted within 90 minutes of brown crab being injected with 1% total haemolymph volume of ECP.

Bacteria cultured from the trial could not be identified to strain level and the lack of mass mortalities of crab suggests that no pathonogenic bacteria were present. However, it may be postulated that low levels of mortalities may have been caused by an excess of non pathogenic bacteria. Methods such as quantitative PCR would enable enumeration of the strain specific bacteria in the tissues and would allow for a more accurate account of changes over time.

Identification methods from Barrow and Feltham (1999) are based primarily on medical practices and were originally designed for the identification of bacteria which use humans as hosts. Despite this, chemical tests for bacteria used in Barrow and Feltham (1999) have previously been used in aquatic research (Verner-Jeffreys, 2003; Pond *et al,* 2006). PCR using universal 16S rRNA primers and gel electrophoresis confirmed that bacteria were present in all tissues.

2.4.5 Molecular Analysis

The univerisal 16s rRNA forward and reverse primers for bacteria (Suau *et al.,* 1999), successfully amplified nucleotides corresponding to those at 8-536 of the 16s rRNA *Escherichia coli* genome (Pond *et al,* 2006). The results visualised on electrophoresis gels showed approximate 529 bp nucleotides for most samples including claw muscle and haemolymph, confirming the presence of bacteria and the results from microbiological testing.

The gel electrophoresis analysis using universal 16s rRNA bacterial primers (Suau *et al.,* 1999) showed multiple bands and some smearing for all of the samples. The smearing suggests that there was incomplete separation of genomic DNA. The smearing could be reduced by increasing the agorose concentration of the gel or by applying a lower voltage, allowing the separation of smaller DNA molecules. Moreover, additional purification and cloning prior to visualization would result in clearer, more defined bands. The use of a

smaller ladder incorportating the 526bp nucleotide is recommended for future work. This would allow for a more accurate analysis of the samples.

The multidisciplinary approach used in the current study has resulted in the null hypotheses being rejected. The results showed that there was an increase in granulocytes over time in nicked crab and non-nicked crab with higher levels of granulocytes recorded in nicked crab. Furthermore, more viable bacteria were isolated from the claw muscle of nicked crab in comparison to non-nicked crab and histology showed higher levels of degradation of the commercially important tissues in nicked crab compared to non-nicked crab.

This study has shown that chelae restraints are important in the survival and quality of live crab in the Irish marketing chain. To reduce autonomy and physical damage to brown crab undergoing post harvest processes, it is suggested that further investigations are carried out to find an alternative method to nicking. Enforcing the use of non invasive methods, such as boxing or bagging the crab individually, would possibly increase the quality of the white meat and reduce stressors such as increased haemolymph glucose and lactate levels. Furthermore, non invasive methods would be more ethically correct. However, the amended retention method would have to consider the fisher, vessel space and the economics of the fishery. This investigation and previous studies (Haefner, 1971; Newman and Ward, 1973) have shown that banding does not work as efficiently on brown crab as it does on other crustaceans. Therefore, it is suggested that either an adhesive or individual boxes or bags are used to prevent shell damage and claw loss in brown crab claws during post harvest processes.

3.0. CHAPTER TWO

Tissue deterioration and potential infections in European lobster (*Homarus gammarus*) undergoing long term storage.

3.1.0 Introduction

3.1.1 Live Marketing Chain.

The marketing chain for European lobster is often lengthy with longer periods of storage than with other species (Hearn, 2002). In addition, lobster are considered 'hardier', surviving > 5days in humid conditions (Estrella, 2002). Lobsters from the Irish fishery are often exported to central and southern Europe (BIM/CSO trade data) which requires multiple points of handling and changes in environmental parameters (Figure 18). Banding is essential to prevent cannibalism and interspecies damage during these processes (Beard and McGregor, 2004) adding further handling stressors. Transportation may occur over single or multiple trips with intermittent storage. Animals are usually kept at low temperatures to reduce metabolism; at high humidity to prevent gills from drying out (Estrella, 2002) and the animal becoming hypoxic (Taylor and Wheatley, 1980; Lorenzon *et al.*, 2007).

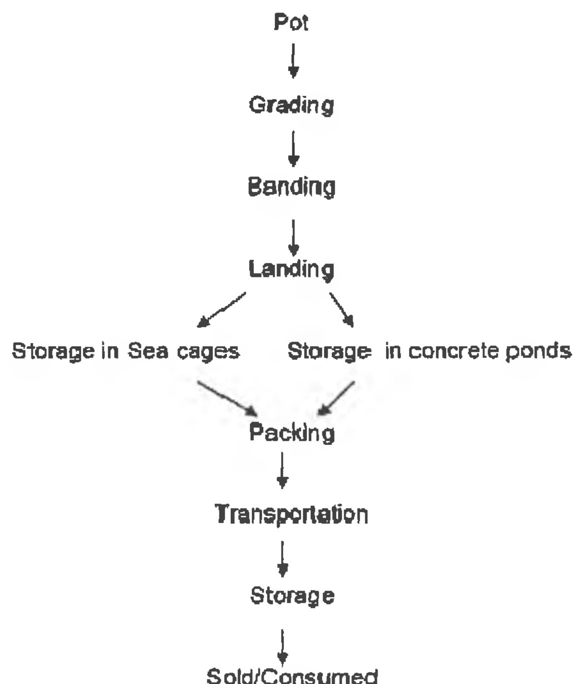


Figure 18. Generalised marketing chain for live lobster from the Irish fishery .

Initial post harvest processes (Table 9) impact on lobster health, possibly affecting survival rates and susceptibility to infections in further procedures such as transportation and storage.

Process	Stressor	Time (Hours)
Pot/Creel	Confined space	48-36
Deck	Aerial Exposure/ Environmental Variations	0.4 - 2
Visual Check/Grading	Handling	0.25
Banding	Handling	0.1
Packing	Handling/Hypoxia/Temperature/Crowding	0.25
Land	Environmental Variations	1-2
Store	Hypoxia	24+
Transport (including export)	Environmental Variations/Hypoxia/Crowding	2+
Stored	Environmental Variations/Hypoxia/Crowding	5+
Sold to Consumer	Handling	

Table 9. Marketing Chain processes, stressors and estimated length of time animals are subjected to each stressor.

3.1.2 Storing

Storing lobster has become common practice in recent decades (Pringle and Burke, 1993; Scarrett, 2001). Storage can occur over different lengths of time, from a few days to several months. Depending on storage time there are a variety of facilities available. Floating wooden crates ('keep boxes') kept inshore and onshore concrete flow through ponds are typically used for short periods (Figure 19). Self contained temperature controlled re-circulation systems are used for long term storage (Scarrett, 2001; Figure 20; Figure 21). Lobster keep boxes and flow through ponds are characteristically and traditionally used in Ireland. However, in recent years, long term re-circulation systems used for storing live lobster have been trialed in Ireland. Generally these systems are designed to the requirements of the user with storage capacity only being limited by available space, power and water supply. The systems necessitate minimal staff and use electronic monitoring systems. Such systems are designed for cooperative style circumstances.

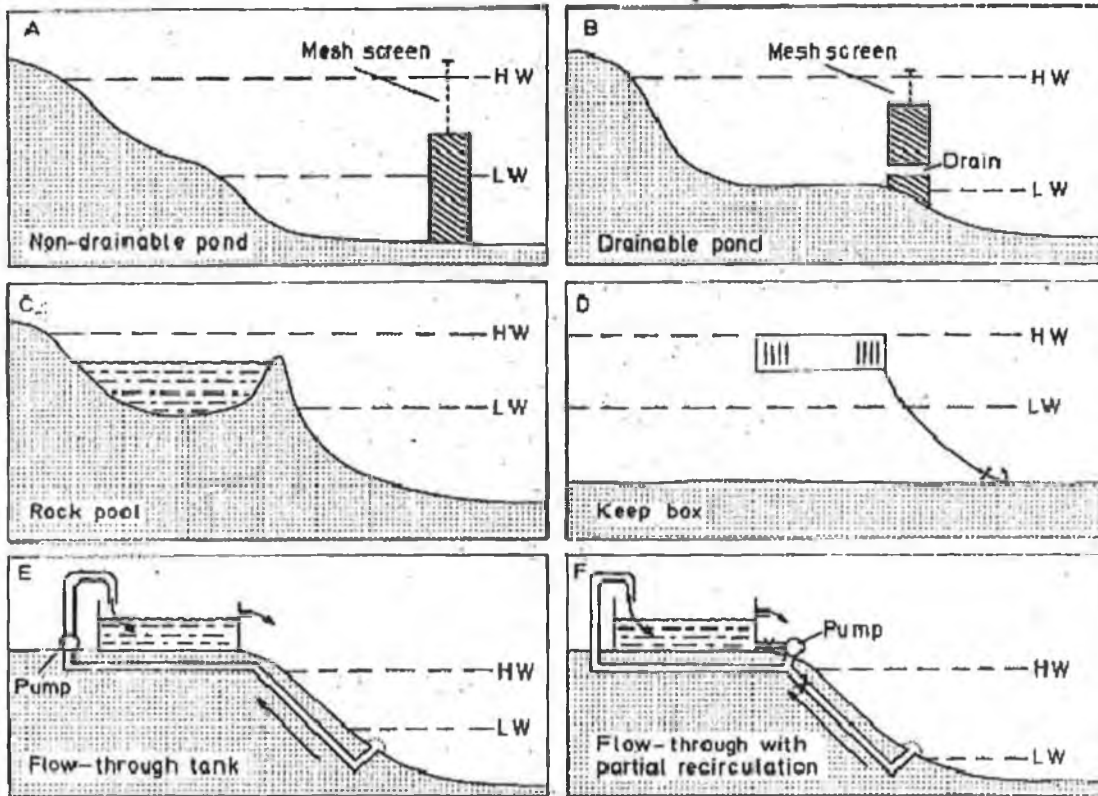


Figure 19. Types of inshore live lobster storage methods (Ayres and Wood, 1977). HW = High water; LW= Low water. A) Non drainable pond using retention of high water and adapted method with drain (B); C) Natural or artificial rock pool; D) Purpose built anchored floating crates. E/F) land based concrete ponds using natural sea water.

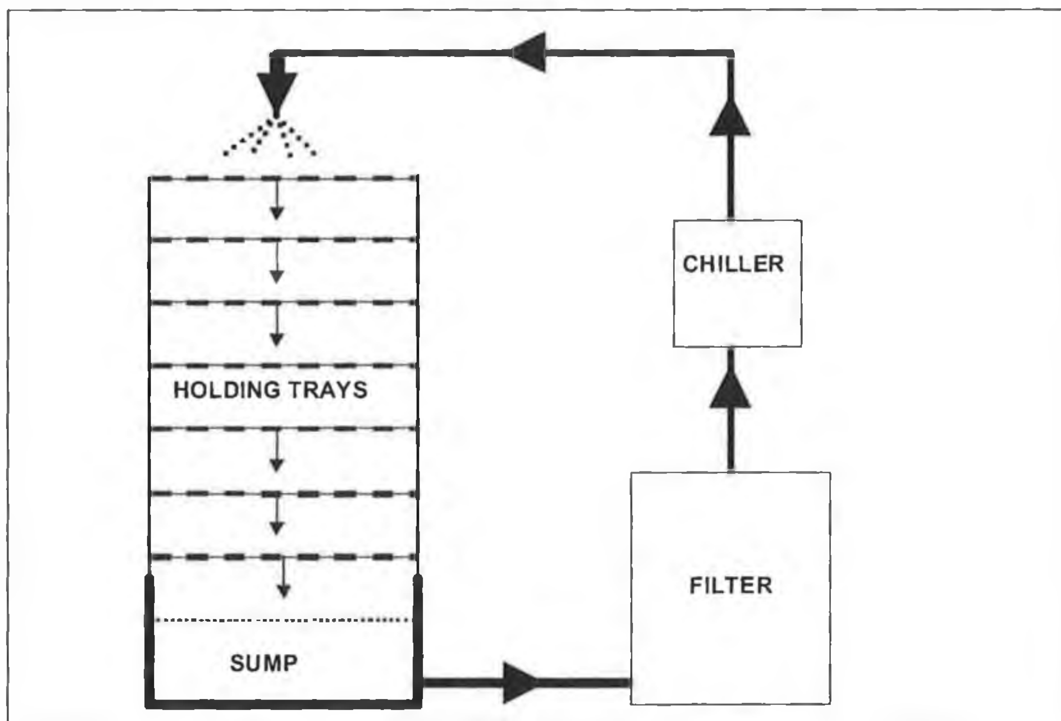


Figure 20. Land based re-circulation system for long term storage of lobster.

Figure 21. Re-circulatory long term shellfish storage facilities (AquaBiota™ Habitat) at ShellTec, GMIT and Inish Oirr, Co. Galway

Storage of lobsters typically occurs for economic reasons but requires a balance between market value and running costs. The Irish lobster fishery has a history of fluctuations in price/kg and landings (Browne *et al.,* 2001). These fluctuations are habitually due to moult cycle and environmental temperature (Tully *et al.,* 2006), imports of American lobster, fuel prices, national and international economy status and catch per unit effort. The Irish Lobster fishery is seasonal and occurs from May to September (Tully *et al.,* 2006b). Lobster value is at its highest in late December, when catches are at their minimum and demand is at its highest. This demand is generally driven by the Christmas market and the consumers want for luxury products. Long term storage facilities enable fishers to catch lobsters during the peak season (May-September, when lobster values are at their lowest) and store them live until the market is less saturated and when higher prices can be obtained (Cawthorn, 1997).

Irish lobster fishers are often geographically isolated or live on coastal islands. This has implications on transportation longevity and distance and may result in an extended period of time between capture and consumption of product. To overcome this, fishers often use a co-operative based system. Co-operatives are groups of fishers who catch shellfish along a relatively small area of coastline, landing catch to one quay and storing them communally. Storage of lobsters in such rural areas enables sufficient numbers to be stocked prior to collection by large vivier lorries (Estrella, 2002). Estrella (2002) highlighted that storage may also help maintain lobsters in prime condition ensuring a better quality product.

3.1.3. Implications of Storing Live Lobster

Particular environments, including those related to poor husbandry, are hypothesised to be the cause of multiple infections including shell disease, fungal disease and ciliate infections. Stewart (1984) reported the haemolymph of healthy lobsters to be sterile but noted transient infections of haemolymph bacteria as a result of handling. Up to 20% of fresh caught lobsters had non pathogenic strains of bacteria present in the haemolymph which were not reported in the same animals a few days after capture (Cornick and Stewart, 1966) when animals had been allowed to recover (Patterson and Spanoghe, 1997). Gaffkemia, (Stewart et al., 1969; Estrella, 2002; Battison *et al.,* 2004) and *Anophryoides haemophila* or Bumper car disease (Ragan *et al.,* 1996; Greenwood *et al.,* 2005) has been associated with handling and post capture damage (Stewart *et al.*, 1969; Estrella, 2002; Battison *et al.*, 2004). In addition, Battison *et al.,* (2008) characterized bacteria found on healthy (*Vibrio spp., Lutibacter litoralis* like *spp.*) and unhealthy (*vibrio spp.*) lobsters undergoing live storage.

It has been hypothesized that Gaffkemia could have a sharp elevation in incidence rate when infected animals are placed in high densities within holding tanks like those commonly used in lobster fisheries (Stewart *et al.* 1966). Suggested control and prevention methods used in lobster holding facilities include improving husbandry, reducing water temperatures to $<5^0$C and regular disinfection of facilities (Stewart and Marks, 1999). Other prevention methods concentrate on the predisposing factors resulting in Gaffkemia. These include prevention of interspecies damage, rough handling and over crowding. In addition, a vaccine has become available (Keith *et al.* 1992); however, this results in the necessary removal of animals from the marketing chain for a minimum of 30 days.

Bumper car disease was first reported in 1973 (Greenwood *et al.,* 2005 from unpublished data by Aiken *et al.* 1973) but was not characterized molecularly until 2005 (Greenwood *et al,* 2005). The need for molecular characterization followed an outbreak in a commercial holding facility in Nova Scotia, Canada, 2004. Greenwood *et al.,* (2005) reported daily mortality rates of 0.5–10% over a period of approximately 1 week prior to lobsters being distributed. The lobsters were reported to have undergone storage for 2–3 months. Furthermore, a histophagous ciliate infection has been reported in other stored crustacean

species from Scotland Small *et al.,* (2005). The first infection was found after a period of two days on storage, then later after 14 days.

The literature has shown that the impacts of specific pathogens are emphasized when animals are subjected to stressors and storage. Combined with the high densities at which the animals are kept and the re-circulatory nature of the systems utilized a disease outbreak could have significant economical implications. Furthermore, Fotedar *et al.,* (2006) noted that extended storage times may increase processing costs, additional stress, starvation, aggression and extended periods of confinement. There have been many documents recommending optimum holding conditions (Ayres and Wood, 1977; Evans and Jones, 1999; Estrella, 2002; Beard and McGregor, 2004; Jacklin, 2007); however, fishers are not legally required to adhere to such recomendations.

To stabilise price fluctuations in live lobster markets it is necessary to store the animals for lengthy periods of time. Previous research has concentrated on the effects of specific pathogens in lobster recirculation systesems. In addition, the majority of previous studies were carried out in the UK or Canada. This study looks at the physiopathology of European Lobster from the Irish fishery over time in storage and provides baseline data for future research.

3.2.0 Materials and Methods

Forty European lobster were caught off the coast of Galway, Ireland, transported to ARE ShellTec research centre, GMIT, Galway (41.5 km) and stored between November and December 2008. After transportation in covered fish boxes, the lobster were placed into a Shellfish AquaBiota™ Habitat ¼ tonne recirculation holding facility (Aquabiotech, Canada; Figure 21). Premixed artificial seawater (Peacock Salt Sea Mix) at a salinity of 32.00 was used in the system and kept at an average temperature of 7.12 ^{0}C throughout the trial. Water quality, temperature, dissolved oxgen (Enviro-Monitron monitor); salinity (Oakton Salt 6 Acorn salinity meter); Nitrate, Nitrite and Ammonia (Hach DR/890) were monitored. The system water was drawn from a sump below the trays and circulated through a sand filter (Sta-Rite S7S50) and an Aquabiotec biofilter. In addition, a protein skimmer was used. The system was topped up on day 17 due to a slight loss of water as a result of evaporation. During the holding period, handling and disturbance of the animals

was kept to a minimum. All techniques were kept as close to commercial methods as possible. The temperature controlled room recieved artificial light between the hours of 7.30am and 6pm.

Ten lobsters were first dissected on return to ShellTec (day 0). Thereafter, ten lobsters were dissected once a week over a period of 2 hours. Environmental and behavioural changes (such as animal activity levels) were observed and recorded. Physiological abnormalities were recorded upon dissection.

3.2.1 Observations

Prior to dissection, the lobster were subjected to a visual check and measurements were recorded including sex, carapace length, limb loss, shell damage and fouling.

Water quality was monitored throughout the trials. Parameters measured included temperature, salinity, pH, Nitrate, Nitrite and Ammonia using Hach Lang DR 800 colorimeter, unless otherwise stated.

3.2.2 Histology

Histology techniques employed were as outlined in 2.2.2 for brown crabs.

3.2.3 Physiological Variables

Granulocyte counts employed were as outlined in 2.2.3 for brown crabs.
To eliminate the effects of granulocyte aggregation in the limbs, the haemolymph was removed from the tail and not the peripheral sinuses (such as those in the limbs).

3.2.4 Molecular Analysis

Molecular techniques employed were as outlined in 2.2.5 for brown crabs.

3.2.5 Statistics.

Statistical tests employed were as outlined in 2.2.6 for brown crabs.

3.3.0 Results

3.3.1. Observations

Water quality was monitored daily for the trial period. There were no major fluctuations in temperature with a maximum of 1^{0}C difference between the highest and lowest temperature (Table 10; Figure 22). Temperature was kept low to reduce metabolism, demand for food and oxygen. Salinity varied between 34.60 and 36.00. Oxygen saturation was kept above the recommended (80%) saturation level (Beard and McGregor, 2004) throughout the holding period. The lowest saturation level (86%) was at the end of day 0 which is typical due to the high demand for O_2 by the lobsters when they were first placed into the system. The ammonia levels were significantly below the toxic level of 6mg/L (Figure 22).

Day	Temp. (^{0}C)	Salinity	O_2 (% Saturation)	pH	Nitrate (mg/L)	Nitrite (mg/L)	Ammonia (mg/L)
0	7.00	35.00	86.00	7.70	1.50	5.00	0.14
6	7.20	35.00	98.00	7.50	4.00	8.00	0.20
13	7.40	34.60	97.00	7.86	7.70	11.00	0.40
19	7.50	36.10	94.00	7.90	5.20	26.00	0.05

Table 10. Water quality parameters from the re-circulation system measured through out the trial on European lobster undergoing long term storage.

pH levels remained relatively constant at circa 7.70 until day 26 when pH reached 8.46. Nitrate levels increased from 1.50mg/L on day 0 and peaked on day 13 at 7.70mg/L and then decreased to 3.70mg/L on day 26. Nitrite increased from 5.00mg/L on day 0 to 26.00mg/L on day 19 and then decreased to 6.00mg/L on day 26.

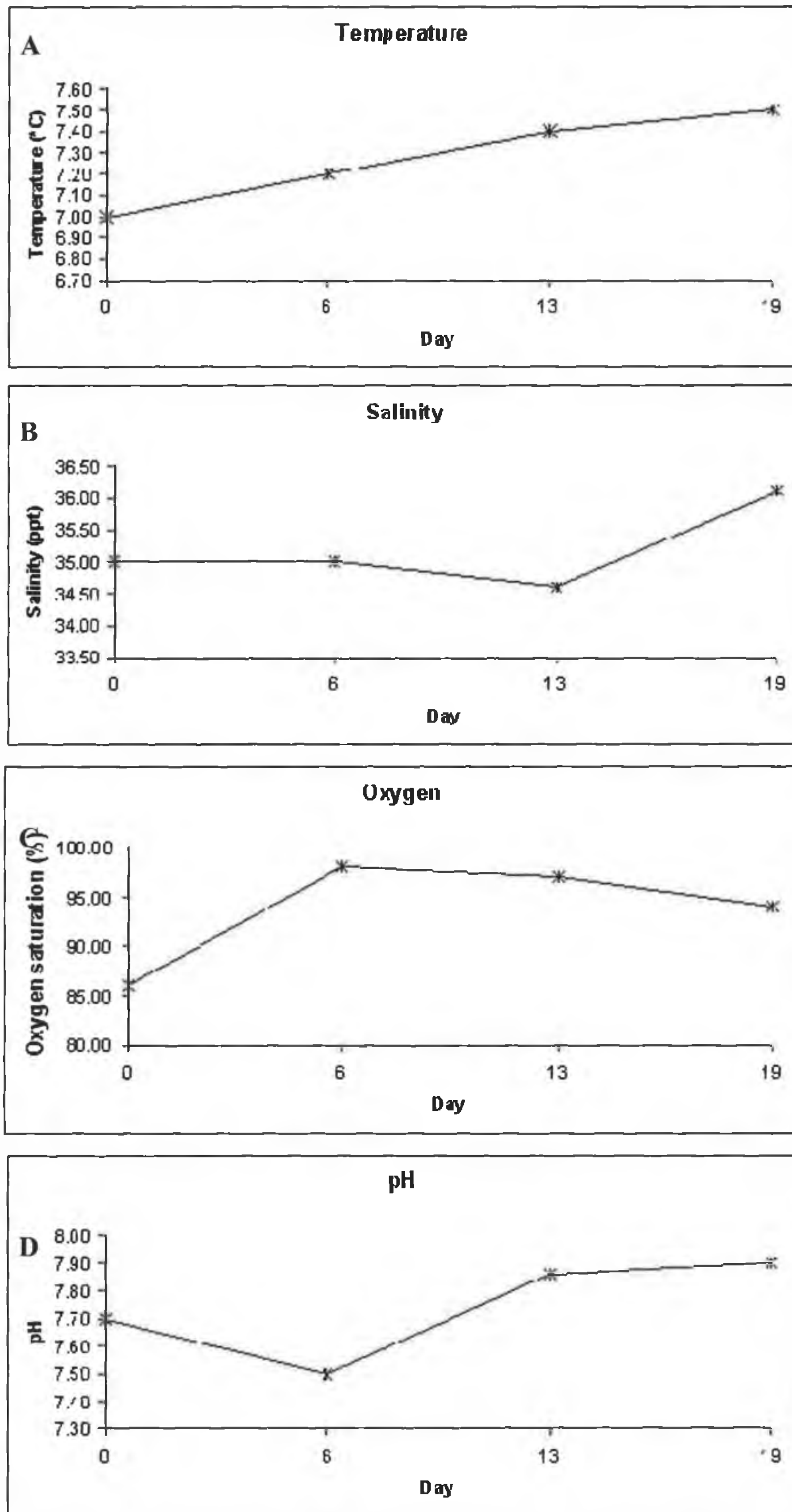


Figure 22. Variables measured from water in the the re-circulatory holding system through out the trial on European lobster undergoing long term storage.

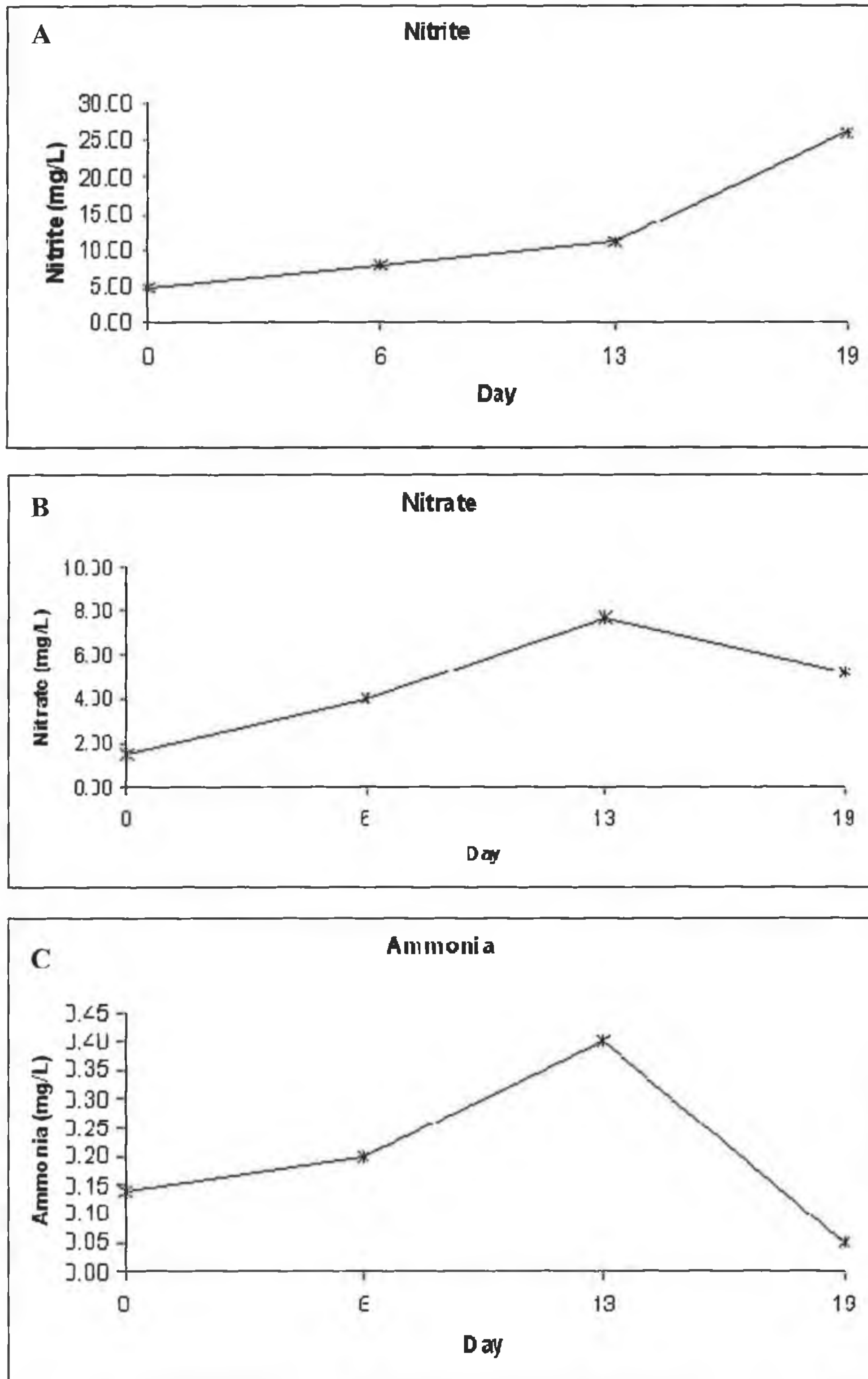


Figure 23. Levels of organic nitrogen compounds measured from the water in the re-circulatory holding system through out the trial on European lobster undergoing long term storage.

A preliminary study into multi species storage at ARE ShellTec, GMIT Ireland showed other microfauna including nematodes, digenea, ciliate and copeopods were present (Figure

24). As the study used artificial seawater it is assumed that the microfauna entered the system using crab and lobster as host species.

During the investigation a biofilm was observed on the lobsters (Figure 24 E). The film was stromatolite like in appearance with some aggregated detachment into the water column. The film also appeared to be periphyton in nature, adhering to the system walls below the water level.

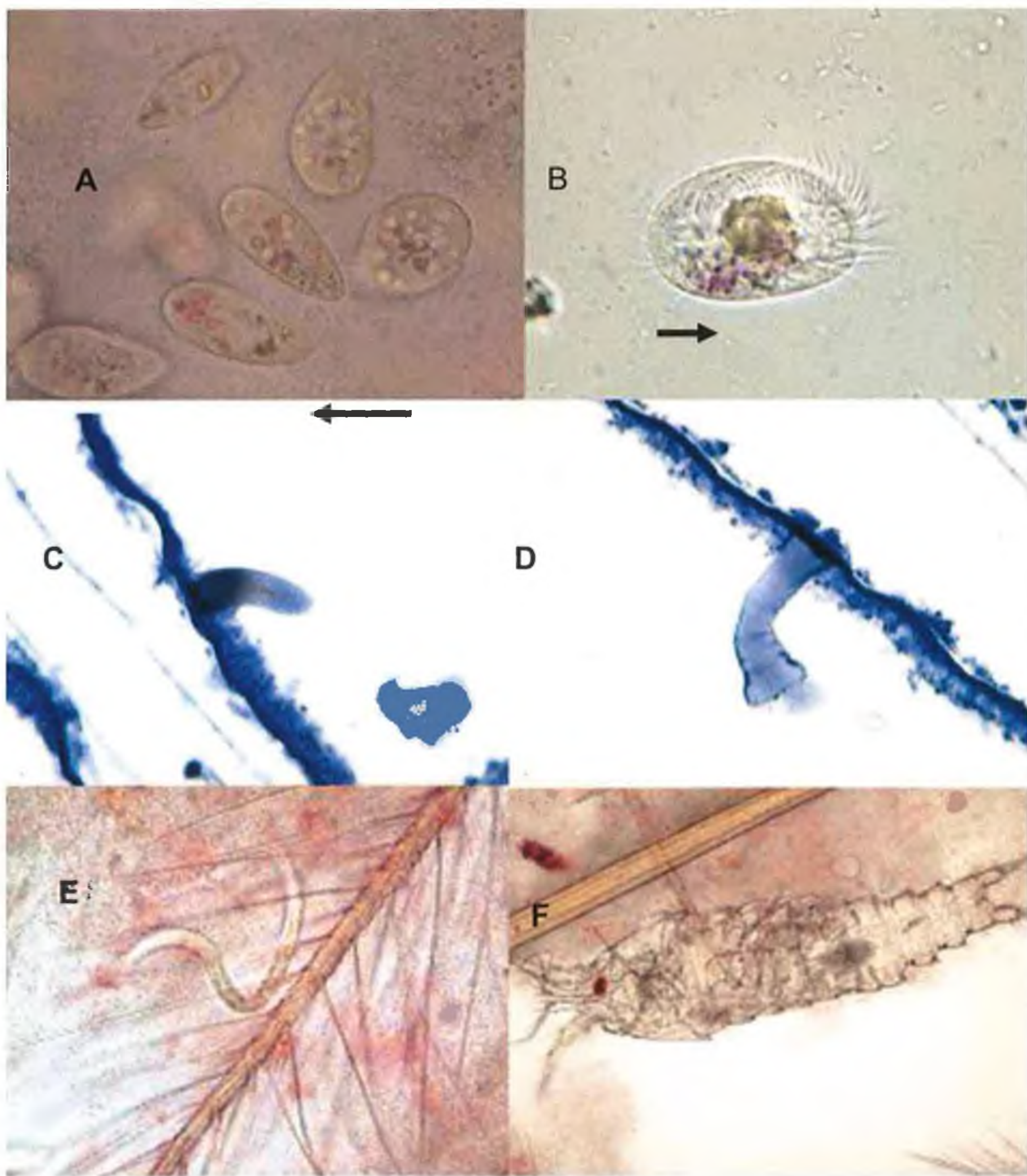


Figure 24 Parasites and simbionts found on crustacean exoskeleton and gills after four weeks in commercial holding facilities. A) Ciliates containing pink bacteria; B) Unidentified Ciliate; C/D) Unidentified hydrozoa spp.(large arrow) and bacterial film on secondary lamellae (small arrow) E) Nematode surrounded by biofilm. F) Copepod (*Harpacticus littoralis).*

3.3.2. Histology

Histopathology provided a holistic assessment of the health status of lobster throughout the trial. Pathologies found throughout the sample period included haemocyte aggregates, melanised nodules, necrosis and bio-films on the gills (Figure 26). The number of haemocyte aggregates and melanised nodules were found predominantly in the hepatopancreas and heart. Animals dissected on day 13 had the most pathologies with a total of 25 melanised regions. There was a fluctuation in pathologies throughout the investigation. However, the increase was not statistically significant, animals dissected on day 0, 13 and19 showed pathologies whilst those on days 6 and 26 did not (Table 11; Figure 25). Animal 22 from day 13 showed an infection that either emerged from or led to the collapse of a hepatopancrease tubule. Due to the low levels of pathologies found throughout the experiment it was not possible to accurately test for significant differences between tissues.

Day	Melanised nodules	Granuloma	Necrosis
0	0	8	0
6	0	0	1
13	25	0	2
19	1	0	2
26	0	0	0

Table 11. Total number of identified areas of tissue degeneration.

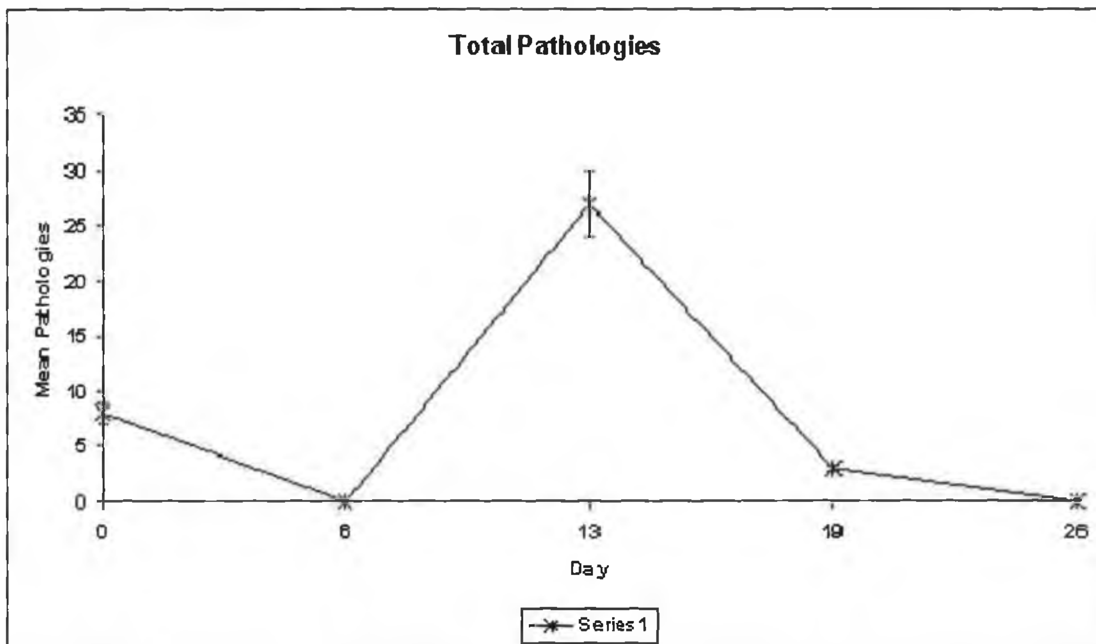


Figure 25. Total pathologies found in all examined tissues of lobster throughout the trial period. Values shown as means ± standard deviation.

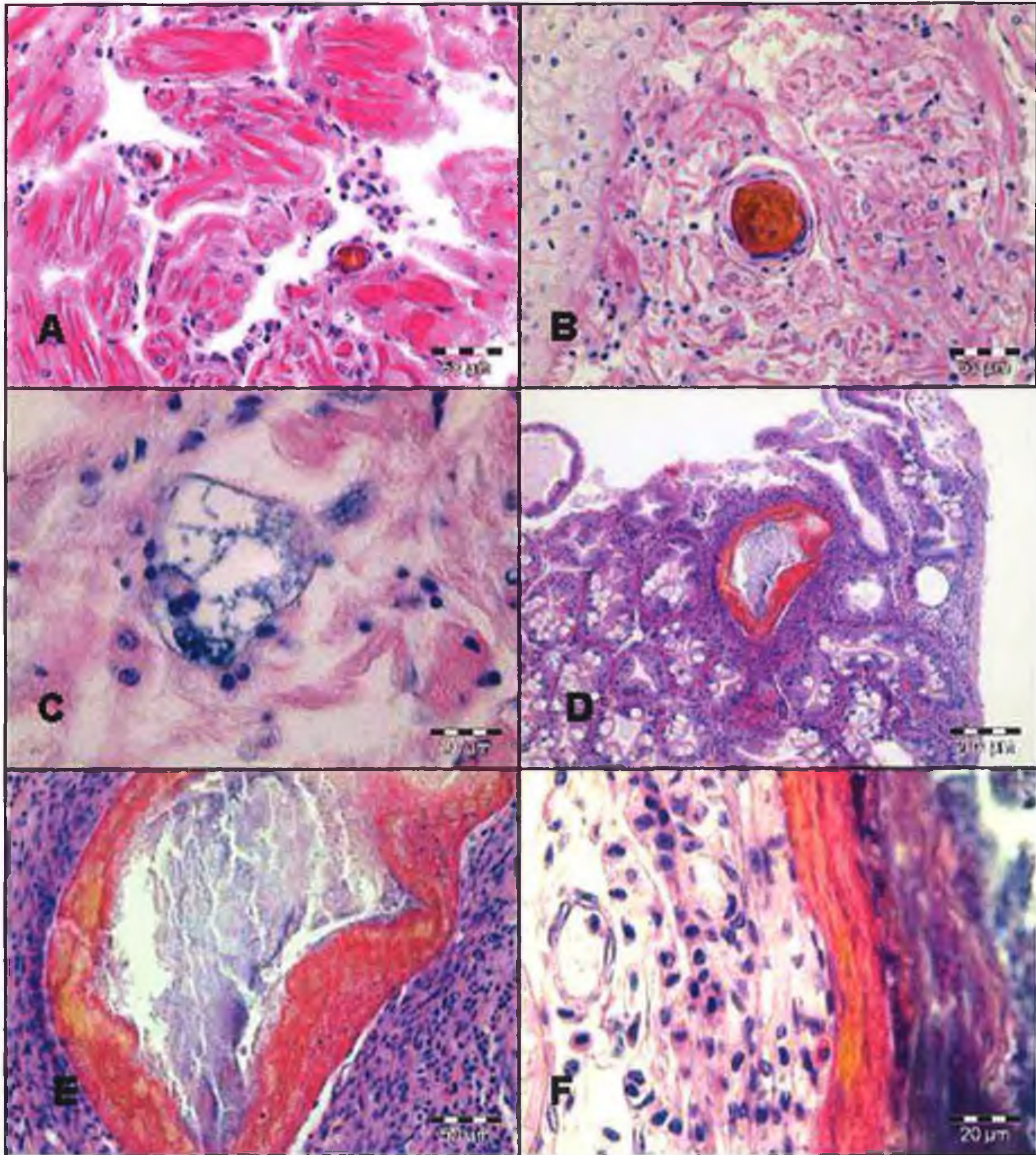


Figure 26. A/B) Melanised nodules and necrosis in myocardium; C) Putative fungal infection in connective tissue; D/E) Enveloped melanised hepatoprancreas tubule; F) Elongationj of phagocytes surrounding melanised hepatoprancreas tubule.

3.3.3. Physiological Variables

Granulocyte Counts

Differential haemolymph counts were carried out to obtain granulocyte counts (Table 12). For the purpose of this study, semi-granulocytes were also counted as granulocytes.

Day	Mean Granulocyte
0	16.00 ± 5.58
6	14.90 ± 0.01
13	22.70 ± 6.29
19	37.50 ± 4.67
26	49.78 ± 5.59

Table 12 Increase of granulocyte means (± standard deviation) over the holding period.

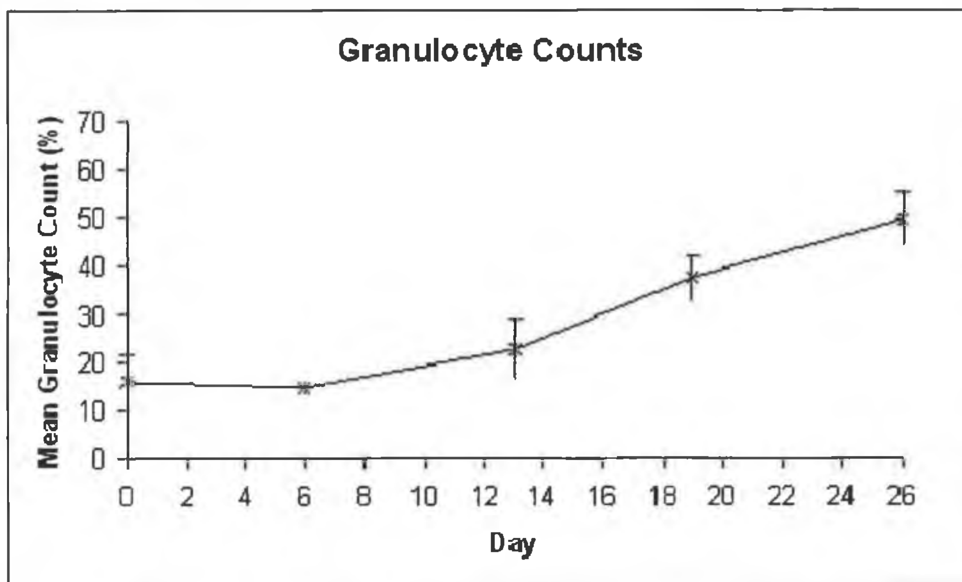


Figure 27. Granulocyte counts (%) over holding period. Values shown as means ± standard deviation.

Data was transformed from non-parametric to parametric data using $\log_{10}$. There was a highly significant difference in granulocytes over time (P = 0.000). The results showed an increase in mean granulocytes from 16.00 (STDEV = 5.58) on day 0 to 49.78 (STDEV = 5.59) on day 26 with the greatest increase in mean granulocyte counts occurring between day 13 (mean = 22.70; STDEV = 6.29) and day 19 (mean = 37.50; STDEV = 6.67). There was a small decline in granulocytes between day 0 (mean = 16.00; STDEV = 5.58) and day 6 (mean = 14.90; STDEV = 5.59) (Table 12; Figure 27).

3.3.4 Molecular Analysis.

Total genomic DNA was lysed from muscle samples and bacterial DNA was amplified using universal 16S rRNA primers (Suau *et al.*, 1999; Figure 28; also see 2.2.5). All samples showed products at the predicted 500bp marker (Figure 28). Total extracted genomic DNA was measured on a BioPhotometer (Eppendorf, Germany) and ranged from 40.8 μg/ml to 122.6μg/ml.

Figure 28 shows the template DNA after PCR amplification of the 16srRNA encoding region compared to a 1kb molecular marker (L). The template DNA was extracted from muscle samples. Lanes 1-4 and 17 in Figure 28 show results from template DNA extracted from lobster dissected on day 0. Lanes 5-6 show results from template DNA extracted from lobster dissected on day 6; lanes 9-10 show results from template DNA extracted from lobster dissected on day 13; lanes 11-13 show results from template DNA extracted from lobster dissected on day 19 and lanes 14-16 show results from template DNA extracted from lobster dissected on day26.

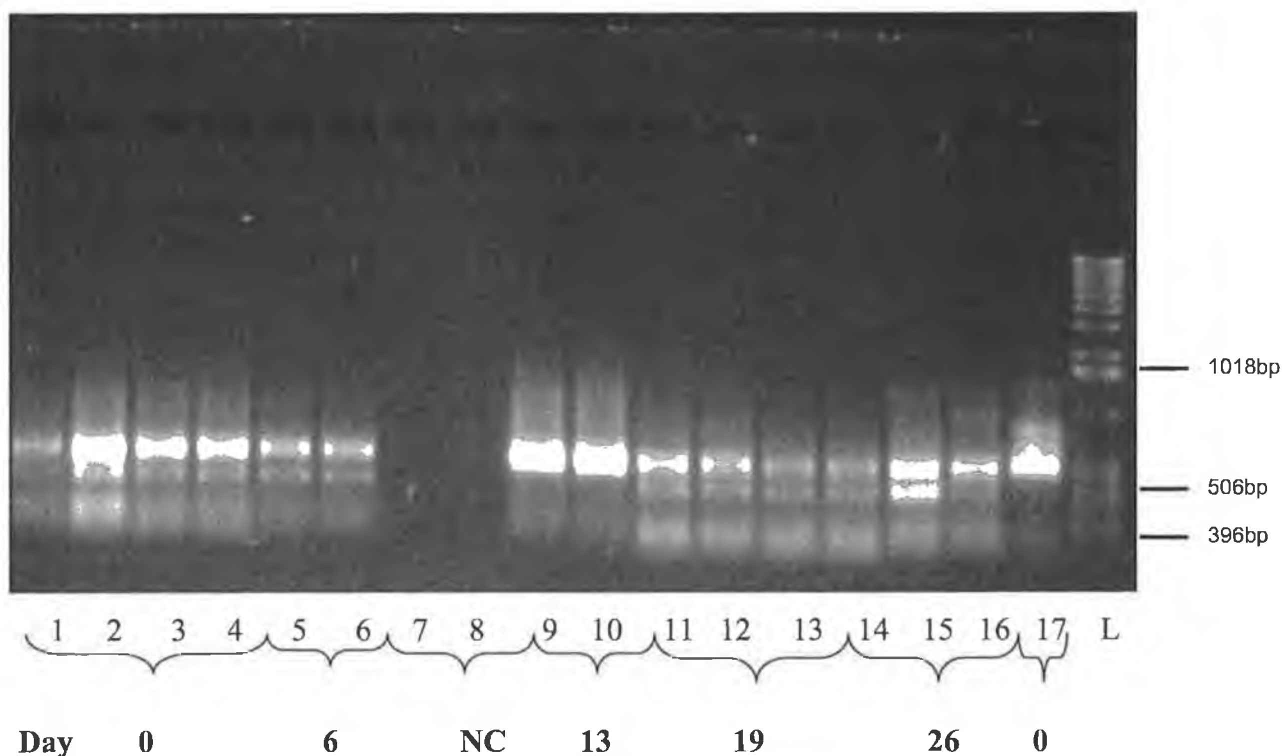


Figure 28. 1% Agarose gel with amplified genomic DNA obtained from the days 0, 6, 13, 19 and 26 of lobster trial. Visualised against a 1KB ladder (L) and showing negative control replicates (NC).

Lobster dissected on days 13 showed higher relative levels of genomic DNA yield (illustrated as more vibrant bands, Figure 28). For several of the template DNA samples more than one length of genomic DNA was visualised (Figure 28, 11-15 with particular emphasis on lane 15). All samples showed genomic DNA around the 526bp nucleotide which corresponds with the universal 16s rRNA bacterial primer. Lanes 2-4 and 9-10 showed very high relative levels of genomic DNA yield. Most samples also showed more than one band indicating the presence of different DNA structures or as a result of contamination during preparation.

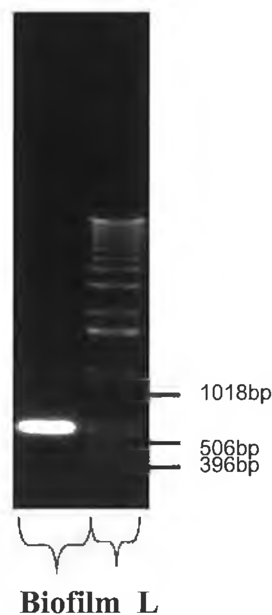


Figure 29. 1% Agarose gel showing PCR amplified DNA from the bio-film. Visualised against a 1KB ladder (L).

Figure 29 shows the template DNA after PCR amplification of the 16srRNA encoding region compared to a 1kb molecular marker (L). The template DNA was extracted from a bio-film found on the lobster and holding facility during the trial period. The gel shows the presence of genomic DNA at the approximate 529 bp nucleotide confirming that bacteria were present in the biofilm.

Sequencing

Purified PCR product from a biofilm sample were sequenced by MWG operon, Germany using 2pmol/ul universal bacterial forward and reverse primers (see 2.2.5). BLAST searches on the Ribosomal Database Project (www.[2]) and European Molecular Biology Laboratory (EMBL; www.[3]) returned sequences with phylum identity levels of >95% (Table 13). The table includes identity scores >80% resulting in identification to genus level. However, results with identity scores <95% should be considered with caution.

Sample	No. of bases sequenced	Primer	Identification	Identity (%)
Biofilm	901	1	Proteobacterium	98
			Epsilonbacteria	90
			Arcobacter	80

Table 13. BLAST results for bacteria extracted from biofilm samples found on lobster during holding. Primer 1 = forward primer S-D-Bact-008-A-s-20 5'-AGA GTT TGA TCC TGG CTC AG-3'. Primer 2.=reverse primer S-*-Univ-0536a-A18 5'-GWATTA CCG CGG CKG CTG-3' (Suau, *et. al.*, 1999).

The primer selected sequence of the identified *Arcobacter* (Domain, *Bacteria*; Phylum, *Proteobacteria*; Class, *Epsilonproteobacteria*; Order, *Campylobacterales*; Family, *Campylobacteraceae*) is as follows:

```
taccatgcaagtcgacgagaacggatattagcttgctaatattgtcagctaagtggcgcacgggtgagtaatatatagttaatctg
ccccaaagaagagaataattgttggaaacgacttgtaatgctctatatgcctttattacaaaagtaagcaagggaaacatttatgg
ctttgggatgagactgtacagtatcagttagttggtgaggtaatggctcaccaagacaatgacacttaactggtttgagaggatga
tcagtcacactggaactgagacacggtccagactcctacgggaggcagcagtggggaatattgcacaatggagggaactctg
atgcagcaacgccgcgtggaggatgacacatttcggtgcgtaaactccttttatatgagaagataatgacggtatcatatgaata
agcaccggctaactccgtgccagccgccggg
```

The sequence structural analysis and homology between the identified *Arcobacter* and the universal *Arcobacter* sequence (Miller et al., 2007) is shown in Figure 30. The *Arcobacter butzleri* strain RM4018 is the reference genome for *Arcobacter* and *Campylobacter* from the order Campylobacterales (Miller et al., 2007). An alignment score of 87.7% was achieved between between the identified *Arcobacter* and the universal *Arcobacter* sequence.

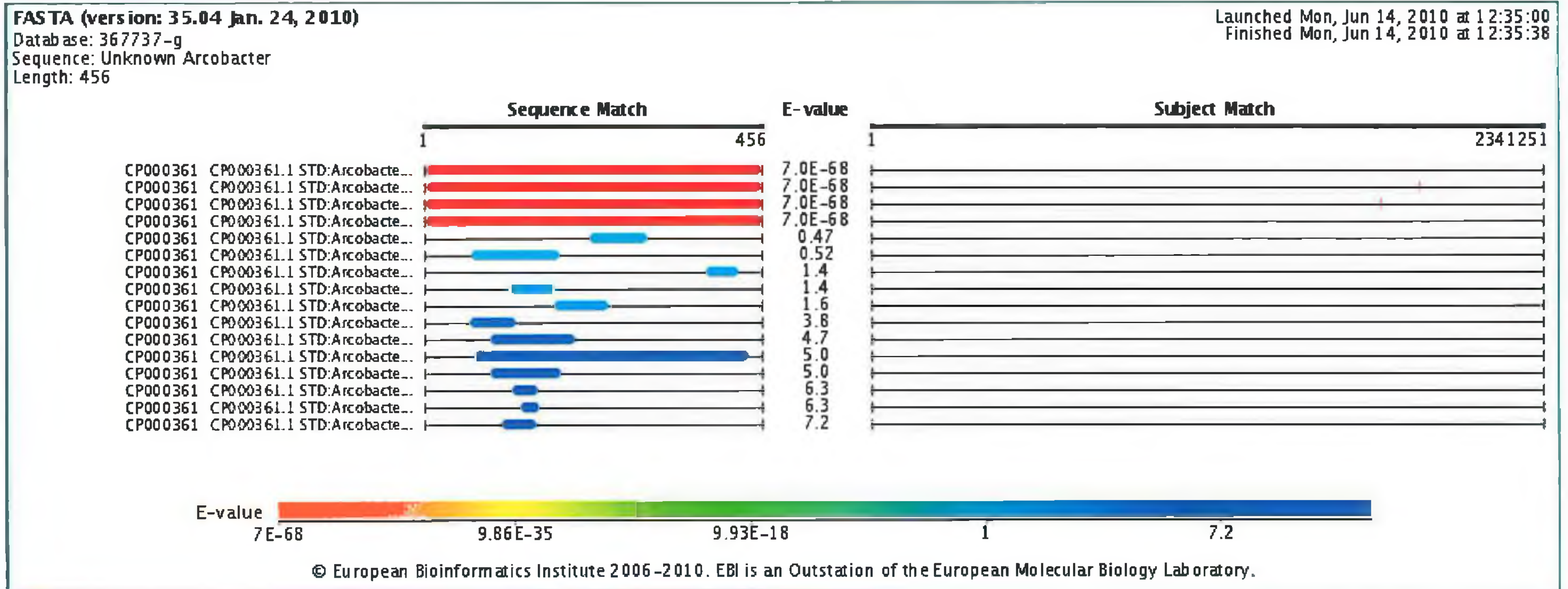


Figure 30. Homologies identified by FASTA software (version 35.04, Jan. 24, 2010; Pearson and Lipman, 1988) between the unidentified *Arcobacter* isolated from *Homarus gammarus* and the holding system compared to the full *Arcobacter butzleri* genome (EMBL strain RM4018; Miller *et al.*, 2007). E= the standardized score for random variables that result in HSPs (high-scoring segment pairs).

Full alignment, sequence structural analysis and homology details are shown in Appendix II.

3.5.0 Discussion.

3.5.1 Observations

This study showed that organic nitrogen compound levels in the water of a lobster holding facility altered the most between day 13 and 19. An increase in nitrite levels from 11.00 to 26.00mg/L respectively, a decrease in ammonia from 0.40-0.05 mg/L and nitrate from 7.70-5.20mg/L occurred.

Toxicity of ammonia is dependant on water temperature, salinity and pH. Beard and McGregor (2004) noted that at 10^0C, pH 8.0 and salinity of 30, levels of total ammonia above 6mg/L is considered toxic. Levels of around 0 should be anticipated, though readings under 2mg/l are acceptable (Beard and McGregor, 2004). The ammonia readings throughout the trial were circa 0 with a highest reading of 0.40mg/L on day 13.

An increase in salinity also occurred between day 13 (34.6‰) and 26 (36.10‰). Jacklin (2007) recommended salinity levels of 30 to 35 for storage of live lobster. The results showed that the salinity levels exceeded these recommendations between days 19 and 26. The increase in salinity was primarily due to water loss through evaporation and splashing, resulting in a concentration of water minerals. Recommendations of dissolved oxygen levels of 98% (8mg/L) were also made by Jacklin (2007). The levels of dissolved oxygen fluctuated throughout the trial with an initial increase between day 0 (86.00%) and 6 (98.00%). Lobster are known to have a higher respiration rate after handling and transportation (Clear and Forteath, 1997) due to increased anaerobic glycolysis which results in accumulation of lactate in tissues and haemolymph (Taylor *et al.*, 1987; Paterson *et al,* 2005; Ridgeway *et al.,* 2006). However, Lorenzon *et al.,* (2007), Ridgeway *et al.,* (2006), Spanoghe (1997) Spicer *et al,* (1990) and Wheatly and Taylor (1992, 1981) observed that most physiological variables, including oxygen uptake, return to normal once the stressors have been restored. These factors are likely to account for the initial low readings in dissolved oxygen on day 0, followed by a rapid increase to day 6 once the animals had recovered.

Estrella (2002) recommended pH levels of between 5 and 9, nitrite levels of circa 5ppm (approximately 5mg/L) and nitrate levels of <100ppm (100mg/L). Biological and carbon filters help maintain pH levels above 7.5 and ensure that there are sufficient levels of bacteria present to convert ammonia to nitrite then nitrate (Estrella, 2002). The levels of toxic organic ammonia compounds in the water are controlled by *Nitrosomonas* bacteria consuming ammonia and converting it to nitrite, followed by *Nitrobacter* converting nitrite to nitrate, the least toxic form of ammonia to lobsters. Results from Wickins (1976) indicated that the toxicity of nitrate is one-twentieth of that of nitrite. In addition, Jenson (1996), Stormer *et al.,* (1996) and Cheng *et al.,* (2002) reported that nitrite and nitrate diffuse into the blood of fish and crustaceans from their environment, which, has been reported to cause issues in penaeid shrimp inhabiting environments of elevated ambient nitrite and nitrate levels (Cheng and Chen, 2002). Nitrate and pH levels in this study were maintained below the recommendations of Estrella (2002) but nitrite levels from days 13 and 19 were higher than that recommended. It is hypothesised that the sudden increase in nitrite may be due to an increase in salinity. However the biofilter had been primed prior to the addition of the lobster and lobster densities had decreased with time, therefore, such a large fluctuation is inexplicable from the data available.

3.5.2 Histology

A range of pathologies were observed in lobsters throughout the investigation period. Pathologies included haemocyte aggregates, melanised nodules, necrosis and gill bio-films. Whilst there was no significant increase in pathologies, animals dissected on day 0, 13 and 19 showed pathologies but animals dissected on day 6 and 26 did not. Furthermore there was no significant difference in pathologies observed in different tissues. Whilst there was no significant difference in the number of pathologies observed in different tissue, there were more haemocyte aggregates and melanised nodules in the hepatopancreas and heart than any other tissue samples.

Animals dissected on day 19 had the most pathologies with a total of 25 melanised nodules. Animal 22 from day 13 showed an infection that either emerged from or led to the collapse of a hepatopancrease tubule. The hepatopancreas absorbs nutrients, stores lipids and produces digestive enzymes (Vonk, 1960, Barker and Gibson 1978, Johnson, 1980a).

Hepatopancreatic tissue consists of haemal sinuses, connective tissue and fixed phagocytes, through which run blind tubules connecting to a main hepatopancreatic duct (Johnson, 1980a). The results showed an extensive inflammatory response to these tubules (involving granulocytes and to an extent hyaline cells). This response is likely to have lead to a reduction in the circulating level of cells (particularly granulocytes) which in turn, correspond with the low level of granulocytes in the haemolymph ratio counts for this particular animal (Figure 27). It is postulated that the destruction of hepatopancreas tubules may have a possible impact on the hosts' ability to absorb nutrients. Such damage would result in the animal being unable to store lipids and produce digestive enzymes (Barka and Gibson, 1977; Johnson, 1980a). In holding, the animals are not typically fed and the hepatopancreas is of little commercial value; however due to the severity of the tubule melanisation it is likely that this would affect other aspects of the animal's health. The muscle, which is commercially valuable, showed no deterioration during the holding period. However, it is postulated that the muscle would deteriorate if held for longer periods of time due to the design of the holding systems restricting the lobsters' movement, resulting in reduced use of the animals' muscles.

3.5.3 Physiological Variables

Previous studies have shown post harvest stressors to affect haemocyte counts (Jussila *et al.*, 1999; Lorenzon *et al.*, 1999; Gomez-Jimenez *et al.*, 2000; Perazzolo *et al.*, 2002). Jussila *et al*, (1997) showed that haemocyte counts may be a useful tool in assessing lobster health. The results showed a significant increase in granulocytes from day 0 to day 26. However, initially there was a decline in granulocytes between day 0 and 6. This decline in granulocytes concurred with a small decrease in observed pathologies, salinity and oxygen readings.

There was a highly significant increase in granulocytes between day 13 and 19. This increase coincided with an increase in nitrite, salinity and pathologies and a respective decrease in ammonia and nitrate. The data showed that the granulocyte counts continued to increase after day 13 whilst the number of pathologies decreased. Therefore it is assumed that there is no link between the number of pathologies and granulocytes. Only nitrite and salinity levels continued to increase.

Haemocytes play an active roll in defence against injuries and infections. A typical response in a healthy animal to a wound would result in haemocytes aggregating at the site, depositing of plasma and causing a clot to close the wound. It has been suggested that in some decapod species granulocytes deposit collagen in reaction to parasites (Hubert *et al.* 1976) and are attracted to surgically damaged nerves by the development of junctional complexes (Shivers, 1977). Unlike other trials, the haemolymph in this study was not removed from peripheral sinuses where granulocytes are thought to collect. The significant increase in semi-granulocytes and granulocytes with an increase in time in holding, suggests that the animals may have had phagocytic reaction to underlying stressors. These stressors may have been a result of undetected infections such as an increase in bacteria within the tissues.

3.5.4 Molecular Analysis

Sieburth (1975) described the chitin of crustacean carapaces as a good source of nutrients which encourages the attachment and colonization of bacteria. This study showed the presence of a bacterial biofilm on the lobster carapace and within the re-circulatory sytem. The biofilm identified during this study was stomatolite and perphyton in appearance (Hall-Stoodley *et al.*, 2004). Gel electrophoresis of PCR products showed positive for DNA of the targeted base pair lengths. In addition, electrophoresis gels showing the presence of bacterial genomic DNA from lobsters tissues also showed other bands which are postulated to contain bacterial genomic DNA fragments of different lengths. However, incomplete separation of genomic DNA and contamination during preparation can also result in multiple bands or 'smearing'. This may be resolved by increasing agarose concentration or by encourage the separation of smaller DNA molecules by applying a lower voltage. As mentioned in 2.4.5, further purification and cloning may also result in clearer bands. In addition, the application of a smaller ladder incorportating the 526bp nucleotide would result in a more accurate analysis of the samples.

BLAST searches carried out on sequenced bacteria, which was isolated from a biofilm observed during lobster holding returned >96% identity scores with proteobacteria. The Phylum proteobacteria are a major group of predominantly gram-negative bacteria. The Phylum is divided into five classes, which include many important nitrogen fixing and

pathogenic strains. Proteobacteria have been isolated from the marine environment (Cifuentes *et al.*, 2000) and have been associated with intensive shellfish aquaculture (Asami *et al.*, 2005).

BLAST searches on sequenced bacteria from the biofilm returned >80% identity scores with *Arcobacter*. The genus *Arcobacter* (family *Campylobactereaceae*) was formed after research by Vandamme *et al.*, (1991) and Vandamme and De Ley, (1991). Subsequently, the *Arcobacter* genus has been associated as a food (Vandenberg *et al.*, 2004; Prouzet-Maule´on *et al.*, 2006) and waterbourne pathogen (Ho *et al.*, 2006; Collado *et al.*, 2008). Ho *et al.*, (2006) reviewed associations of *Arcobacter* spp. with diarrhoea and human gastroenteric illness.

Gene alignment between the unidentified *Arcobacter* isolated from the biofilm and the full reference genome for *Arcobacter butzeri* (EMBL strain RM4018) showed an 87.8% exact match with multiple conserved regions. The high alignment score is a result of their phylogenetic proximity, however, despite the multiple conserved regions occurring a alignment score of 87.8% suggests that the unidentified *Arcobacter* is *Arcobacte buster* but does belong to the order *Campylobacter*.

Arcobacter contain several species associated with marine sources (Romero *et al.*, 2002; Irrera *et al.*, 2003; Fera *et al.*, 2004; Maugeri *et al.*, 2005;). Specifically *A. marinus* (Kim *et al.*, 2010), *A. mytili* (Collado *et al.*, 2009), *A. butzleri*, *A. cryaerophilus, A. skirrowii,* and *A. cibarius* (Collado *et al.*, 2008) with *A. mytili, A. butzleri*, *A. cryaerophilus, A. skirrowii,* and *A. cibarius* isolated from European waters. *Arcobacter* like spp. have been previously associated with crustacea. Goffredi *et al.*, (2008) isolated *Arcobacter* from the carapace of Yeti crab (*Kiwa hirsute*) from deep sea hydrothermal vents whilst Gugliandolo *et al.*, (2008) isolated *Arcobacteri* from copepods in the Mediterranean. However no publications have proven that *Arcobacteri* isolated from crustaceans are pathogenic.

This study showed that there was a significant increase in granulocytes in lobsters with an increase in time spent in captivity. Histology did not show enough data to conclude that animals showed systemic pathologies throughout the trial.

Long term storage (28 days) during the trial did not appear to have an adverse affect on the lobster. However, the study did show the presence of a bacterial film on both animals and the holding system. Existing literature (Ayres and Wood, 1977; Evans and Jones, 1999; Estrella, 2002; Beard and McGregor, 2004; Jacklin, 2007) on recommended storage conditions did not consider biofilms. Recommendations by Jacklin (2007) concentrated on the design of re-circulatory systems, emphasising issues caused by macro organism biofouling from natural seawater sources on the mechanics and design and not the livestock. However, Jacklin (2007) did highlight the use of UV filtration in destroying bacteria in order to 'promote stock health' in recirculation systems but did not associate the UV filter in preventing sessile bacterial films. The use of chemicals to reduce sessile bacteria may have adverse effects on the nitrifying bacteria used in biofilters. Furthermore, the use of chemicals on products used for the human food chain generally results in their removal from market for a minimum of 30 days.

This study identified a stromatolite bacterial biofilm. It is suggested that accumulating biofilms on lobsters are reduced and prevented by increasing the water flow rate within the system and methodically transfering the animals into a secondary system to allow the primary system to be cleaned.

4.0 GENERAL SUMMARY

Brown crab and lobster fisheries are important to rural and island communities, with brown crab being the most commercially valuable shellfish species within Ireland (Tully *et al.*, 2006a). Lobster and brown crab landings and live exports continue to increase resulting in more animals being subjected to stressors such as handling and transportation.

Disease is considered to be one of the most limiting factors in crustacean fisheries (Subasinghe, 1997). As a result, there has been an increase in the use of disease resistant brood stock (Smith *et al,* 2003, Sung, *et al,* 1992) and prophylactic treatments using immunostimulants, probiotics (Hauton, *et al,* 1997) and antibiotics (Scholz, *et al,* 1999). Whilst these methods are useful in cultured species such as shrimp, the cost of treatment may be greater than that of the live product value, especially with brown crab. Furthermore, antibiotics result in removal of animals from the marketing chain for a set period, typically 30 days and possibly affect the natural bacteria of the aquaria, which act as a probiotic. The flow through design of many holding facilities makes the use of such treatments improbable as they would allow chemicals to enter natural environments. Basic husbandry and reduced handling may help prevent infections and disease. However, the quality of live crustaceans for human consumption is still often considered unsatisfactory with animals having reduced survivability. This results in animals being discarded in the later stages of the marketing chain (Barrento *et al.,* 2008; Barrento *et al.,* 2010).

Little information is available on the microfuana and flora of crab or lobster. Moreover, there is no information available on the pathological and microbial affects of nicking on brown crab from the Irish fishery. This study provides records on the differences that occur to the physiology of nicked crab compared to un-nicked crab and the effects of long term storage on lobster.

The consequences of this research questions the current chelae retention method of nicking brown crab within the Irish fishery and the data provides previously unknown information regarding the status of Irish brown crab physiopathology during post harvest processes. Furthermore, it provides scientifically driven data on the effects of storage on European lobsters in the Irish fishery.

This study identifed the typical microfauna and flora of brown crab and provides a baseline to which the microfauna and flora of nicked brown crab could be compared. The study also aimed to evaluate the progression of potential pathogens in Irish brown crab which have undergone the typical fishing practice of nicking by assessing numeration of viable bacteria and through histopathology.

It was hypothesised that there would be no difference in granulocyte counts, viable bacteria counts or difference in tissue degradation between nicked crab and non-nicked crab. It was also predicted that lobsters in long term storage would show no difference in granulocyte counts, nor would they show any pathologies throughout the trial.

The study showed that nicking effects the physiology of brown crab, resulting in elevated levels of haemolymph glucose, lactate and granulocytes. Nicking also resulted in more pathologies such as melanised nodules. Melanised nodules are a consequence of an immune response to an infection (Söderhäll and Ajaxon, 1982; Johnson, 1987; Factor and Beekman, 1990; Vanderbraak *et al.,* 2002). Therefore the increase levels of melanised nodules in nicked crab suggests that they may be more susceptible to infections.

The investigation also showed that whilst long term storage does not have major implications on lobster tissues it does result in elevated circulating granulocytes. Furthermore, long term storage may make the animals more susceptible to bio-films which can have the potential to cause secondary issues. Such issues can include bio-films smothering the gill lamella, resulting in reduced oxygen uptake.

During this investigation histology, microbiology, physiological parameters and PCR were used to compare nicked and non-nicked crab subjected to commercial practices. Generally nicked crab showed elevated results compared to non-nicked crab. Significantly higher numbers of pathologies were observed in the muscle of nicked crab. Glucose and lactate levels and colony forming units from haemolymph samples were also greater in nicked crab. Furthermore, though not significant, granulocyte counts and muscle bacteria levels were elevated in nicked crab.

Barrento *et al.,* (2010) showed that $_{D}$-Glucose increased due to anaerobic metabolism resulting in an accumulation of $_{L}$-lactate. Barrento *et al.,* (2010) demonstrated that the post capture processes of un-loading non-immersed crab as well as poor water quality and high densities were the most important stressors. The results from this study showed that the initial 48 hours of storage had the greatest affect on the physiological variables of crab, both nicked and non-nicked. The lactate, glucose and RI levels declined with a reduction in salinity, pH and Nitrite whilst ammonia and nitrate levels stayed comparative. Therefore, unlike Barrento *et al.,* (2010) *et al,* it is not possible to conclude that water quality had an affect on the physiology of the crab. Interestingly, Barrento *et al.,* (2010) showed that the position of an animal in densely packed tanks also had an effect on the physiology. It was not feasible to look at the effects of such a variable in this study due to limited sample size and consequent densities. However, alterations in densities throughout the trial period must be considered. It would be assumed that with a reduction in densities there would be a reduced chance of injury and infection. In addition, densities during this study were not as high as those observed in the fishery.

Ninlanon and Tangkrock-Olan (2008) indicated that chelae tying could reduce metabolic activity and stress in mud crab (*Scylla serrata*). This could delay quality and physiological degradation during emersed storage. Ninlanon and Tangkrock-Olan (2008) found that retaining chelae through tying, resulted in a greater increase in lactate during emersion. However, in crab that had been subjected to pre-cooling (kept at 5^{0}C for 5 minutes prior to emersion) lactate increased to greater levels in crab without chelea retention. During this study, lactate levels were constantly higher in crab which had their chelae immobilised by nicking. Moreover, Ninlanon and Tangkrock-Olan (2008) showed that a reduction in muscle yield was greater losses in crab which had their chelae retained. Crab with no chelae restraints had a reduced meat yield of circa 20%, whilst crab with immobilised chelae had meat yield loss of circa 90%. It is probable that meat yield losses in crab that have been nicked would be greater.

Storing of live lobster has been increasingly used to regulate and stabilize the market. The Irish lobster fishery has a history of fluctuations in price/kg and landings (Browne *et al,* 2001). These fluctuations are habitually due to imports of American lobster, fuel prices, national and international economy status and supply & demand in the market. Some re-circulatory storage systems have the capacity to hold >20 tonne of live lobster (www. [4].),

requiring minimal staff and do not necessitate a coastal location. Host undergoing stressors such as storage may create environments which favour specific pathogens. Introduction of a pathogen could have significant economical implications due to the high densities at which the animals are kept and re-circulatory nature of the systems utilized. Fotedar *et al.*, (2006) noted that extended storage times can increase stress, starvation, and aggression in live stored animals. Whilst there have been many documents recommending optimum holding conditions, none hold legal status. This study is the first to applpy histology, PCR and physiological variables to samples of live stored lobster from the Irish fishery.

The results from the study showed a highly significant increase in granulocytes during the storage period. Whilst there were too few observed pathologies to permit statistical analysis, tissue abnormalities were recorded on most sample days. In addition, PCR carried out on a biofilm sample from the holding system tested positive for 16s rRNA and was later identified as *Proteobacterium* (identity score >98%), *Epsilonbacteria* (identity score >90%), *Arcobacter* identity score >80%) by a BLAST search.

Fotedar *et al.*, (2006) showed the effect of holding duration on the haemocytes of western rock lobster (*Panulirus cygnus*). After eight days of storage in a commercial re-circulation system the western rock lobster showed a significant increase in hyaline cells. However, overall there was no significant difference between days one and eight. Granulocyte numbers decreased overall, with a significant peak on day four, whilst semi-granulocytes increased overall. Other experiments by Fotedar *et al.*, (2006) showed that semi granulocyte and hyaline cell numbers in western rock lobster decreased during holding. It was suggested that the increase was a result of an increased release of haemocytes from a storage site increasing the total haemocyte count (Fotedar *et al.*, 2006). Fotedar *et al.*, (2006) hypothesised that the fluctuation in counts between days one and eight were due to an unidentified environmental influence and differences between experiments showed that results were not solely due to batch variation. Whilst western rock lobster belong to a different family than European lobster haemocytes counts have been used to assesses health status in many crustaceans (Patterson and Spanoghe, 1997; Johansson *et al*, 2000; Ridgeway *et al*, 2006). This study showed that there was a highly significant difference in granulocytes over time. Mean granulocyte counts increased over time in holding with the greatest increase in mean granulocyte counts occurring between day 13 and day 19. There

was a small decline in granulocytes between day 0 and day 6. Unlike Fotedar *et al* (2006), the results from this study were consistent with no significant fluctuations.

Future studies may be enhanced by including other parameters such as meat yield. Also, the quantification of colony forming units from water samples would aid in assessing when the animals are exposed to the greatest amounts of stressors. Furthermore, application of the experiments to other Irish crustacean stocks would provide a better understanding of general microfauna.

5.0 CONCLUSIONS

This study presents baseline information on the Irish crustacean fishery regarding the effects of nicking on brown crab and the long term storage of European lobster. Results showed that:

- Nicking has a negative effect on the physiology of brown crab. Nicking results in elevated glucose and lactate levels in the haemolymph.
- Nicking of brown crab results in elevated levels of circulating granulocytes and a slight increase in haemolymph refractivity.
- Nicked crab have higher levels of bacteria within the muscle tissue and haemolymph.
- Nicked crab show more pathologies than non-nicked crab.

- Lobster do not show an overall increase in pathologies whilst being stored long term.
- Long term storage has an effect on the number of circulating granulocytes in lobster haemolymph with overall numbers increasing with time in storage.
- Lobsters are colonised by bio-films whilst in long term storage facilities.

From the results and discussion the following is to be recommended for

A) The Irish brown crab industry:

- Continued use of claw restraints to prevent inter-species damage during post harvest processes.
- Research and development to be undertaken to source an alternative, non-invasive method to nicking as a means of claw retention.

B) The Lobster industry:

- Continued screening of lobsters and the holding system for biofilm accumulation.
- Increase flow rates are so as to prevent stromatolite biofilms.
- Regularly remove and power wash system trays using clean artificial seawater or freshwater, so as to prevent build up of biofilms.

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Appendix

Appendix I . Global disease of commercial crustaceans with particular reference to those which have economic implications.

Phylum/Class	Name	Host	Geographic Distribution	Suspected Impacts	Control
Arthropoda Maxillopoda	*Rhizocephlan*	*Carcinus meanas, Callinectus sapidus, Lithodus, Paralithodus*	Global	Distruction of Muscle, alters moulting, intersex, stunting of growth	Removal and destruction of host.
Cilophora Oligohymenophorea	*Mesanophrys spp. (Ciliate)*	*Carcinus meanas, Callinectus sapidus,Cancer pagurus, Cancer irroratus, Pugettia producta, Chionoecetes bairdi, Lithodes aeqispina, Paralitithiodes platypus*	Europe Pacific Coast of North America	Found in wild and captive kept animals. Possibly opportunistic using wounds for entry. System tissue destruction. Mortalities	Reduced densities, handling and damage.
Nemertea Anopla	*Carcinonoemertes spp.*	*Cancer magister, Paralitjodes camtschatica, Chionoecetes bairdi, Callinectus sapidus*	Ubiquitous	Feed on eggs causing loss as well as fungal and bacterial film.	Dipping in freshwater.
Ascomycota Ascomycetes	*Trichomaris ivadens*	*Chionoecetes bairdi, Chionoecetes opilio, Chionoecetes tanneri*	Alaska, British Colombia, Canada	Increased granulocyctes and possibly opens wounds for opportunistic pathogens through hyphae penetration of the exoskeleton.	Non Known.
Protozoa Lobosa	*Paramoeba perniciosa*	*Callinectus sapidus, Cancer irroratus, Carcinus meanas* as well as *Homarus americaus*	East coast of North America	Tissue displacement, lysis and decreasedprotein, heamocyanin and glucose. Mortality	Non Known
Cercozoa Ascetosporea	*Haplosporidium*	*Callinectus sapidus*	East coast of North America	Haemocytic dysfunction. Mortaility	Non Known
Dinoflagellata Dinophyceae	*Hematodinium*	*Carcinus meanas, Cancer pagaurus, Portunus depurator, Necora puber, Callinectus sapidus, Callinectus similes, cancer irroratus, Cancer borealis, Ovalipes oscellatus*	Europe East Coast of North America.	Tissue degeneration, Milky heamolymph. Mortality.	Non Known
Virus	*Bi-facies virus*	*Callinectus Sapidus, Carcinus meanas, Carcinus mediterraneus, Macropipus depurator.*	East Coast of North America. France	Failure of haemolymph clotting.	Minimizing stress, particularly in captivity.

Table 14. Overview of Global disease found in Brachyura and some related species. Adapted from the Synopsis of Infectious Diseases and Parasites of Commercially Exploited Shellfish, Bower et al (1994-2005).

Phylum/Class	Name	Host	Geographic Distribution	Suspected Impacts	Control
Virus	*Rhabdo-like virus Rhabdo-like enveloped helical virus*	*Callinectus Sapidus, Carcinus meanas, Carcinus mediterraneus, Macropipus depurator.*	East Coast of North America. France		Removal and destruction of host.
Virus	*Picorna-like virus*	*Callinectus Sapidus, Carcinus meanas, Carcinus mediterraneus, Macropipus depurator.*	East Coast of North America. France	Destruction of bladder, epidermis, gill, gut and nervous system.	Reduced densities, handling and damage.
Virus	*Herpes-like virus*	*Callinectus Sapidus, Carcinus meanas, Carcinus mediterraneus, Macropipus depurator.*	East Coast of North America. France	Destruction of bladder and antennal gland.	Minimizing stress, particularly in captivity.
Virus	*Rickettsia-like*	*Carcinus mediterraneus, Paralithodes platypus*	Mediterranean, Baring Sea	Mortility in captivity. Necrosis, hepatopancreatic tubule encapsulation. Abnormal moulting, abnormal ovarian development.	Non Known
Virus	*Chlamydia-like*	*Cancer magister, Cancer irroratus, Cancer borealis*	Puget Sound, North East America	Mortality rate increases with time in captivity. Altered haemocyte morphology. Destruction of haemopatic tissue.	Non Known
Choanozoa Mesomycetozoea	*Psorospermium spp.*	Crayfish:- *Cherax tenuimanus, Cherax quadricarinatus,*	Australia	Orange spots on carapace, haemocytic encapsulation.	Non Known.

Table 15. Overview of Global disease found in Brachyura and some related species. Adapted from the Synopsis of Infectious Diseases and Parasites of Commercially Exploited Shellfish, Bower et al (1994-2005).

Phylum/Class	Name	Host	Geographic Distribution	Suspected Impacts	Control
Cilophora	*Anophryoides haemophila*	*Homarus americanus* (closely related species infect crab and shrimp)	East USA, East Canada	Destruction of haemocytes, High Mortalities. Prevalent in aquaria and ponds.	Possible prevention is lower salinity (8.0 ppt), increased temperature (>5^{0}C) and esposure to formaline (50mg/l)
Acanthocephala Archiacanthocephala Polymorphus botulus	Acanthocephalan Larvae/cystacanth infection	*Homarus americanus* crabs	Eastern Canada and coast of New England	No major effect	No Methods known.
	Bacterial Shell disease/ Epizootic shell disease	*Homarus americanus, Panulirus argus* crabs and shrimps	Ubiquitous on the east coast of USA. Rhode Island and Long island sound	Pitted and darkened shell. Melanisation of cuticle. Prevalent in aquaria and ponds.	Removal of infected animal. Reduce densities, improved nutrition and improved holding conditions.
Ascomycota Sordariomycetes Possibly *Fusarium solani*	Burn spot disease	Homarus americanus Also been reported in shrimp and prawns	Reported in rearing facilities in New york.	Gill dysfunction. Mortality.	Non-known. Reduce densities, improved nutrition and improved holding conditions.
	Fungal Shell disease	*Homarus americanus, Homarus gammarus, Palinurus elephas* most penaeid shrimp and most crusteacea	USA, UK, Japan, Italy	Degradation of shell, lesions, penetration to internal tissues. Mortality	Removal of infected animal. Reduce densities, improved nutrition and improved holding conditions.
Firmicutes *Bacilli Aerococcus viridians homari/Gaffkemia homari*	Gaffkemia/ Blood diease / Pink disease	*Homarus americanus, H. gammarus, H.vulgaris*	USA, Europe.	Mortalities caused by as few as 5 cells/kg of body weight.	Reduced temperature, wound prevention, culling of weak animals, Antibiotics and disinfection of facilities.
Apicomplexa	Gregarine Parasitism	*Homarus americanus Nephrops norvegicus*	East Canada, Atlantic and Mediterranean coast of France.	Gregarines.	Non Known
Proteobacteria Gamma Proteobacteria	*Vibrio Spp/* Juvanile Vibriosis	Homarus gammarus	Ubiquitous	Mortalities. No external signs.	Improve husbandry.

Table 16. Overview of Global disease found in Clawed lobsters and some related species. Adapted from the Synopsis of Infectious Diseases and Parasites of Commercially Exploited Shellfish, Bower et al (1994-2005).

Phylum/Class	Name	Host	Geographic Distribution	Suspected Impacts	Control
Dinoflagellata Dinophyceae	*Hematodinium*	*Carcinus meanas, Cancer pagaurus, Portunus depurator, Necora puber, Callinectus sapidus, Callinectus similes, cancer irroratus, Cancer borealis, Ovalipes oscellatus*	Europe East Coast of North America.	Tissue degeneration, Milky heamolymph. Mortality.	Non Known
Heterokontophyta Oomycota	*Lagenidium sp.* Fungus Disease	*Homarus americanus, H. gammarus, H.vulgaris* Shrimps, prawns and crabs	California (cultured larval lobsters)	White opaque appearance caused by mycelia. Mortalities.	Improved husbandry
Protozoa Lobosa	*Paramoeba*	*Homarus americaus* and crabs.	East coast of North America	Tissue displacement, lysis and decreasedprotein, heamocyanin and glucose. Mortality	Non Known
Nicothoe asteci	Parasitic copepods	*Homarus gammarus (vulgaris)*	Scotland	Attaches to the gills, feeds on haemolymph.	Non Known
Nemertareans	Pseudocarcinone mertes homari	Homarus americanus	North east USA and Canda	Feed on eggs causing upto 100%begg loss	Briefly exposing to fresh water.
Ameson sp	Microsporidian	*Panulirus ornatus Panulirus cygnus*	South west Australia New Guinea	Milky muscle due to degradation.	Non known
Nematoda Cystidicolidae	Nematodes/Ascar ophis sp.	Homarus americanus	East Canada, South Cape-cod, USA	Encysts rectum, stomach wall and lumen.	Non known
Platyhelminthes Trematode	metacercariae/ *Stichocotyle nephropis*	*Homarus americanus Nephropis norvegicus* and species of crab	Scotland, East Canada, South Cape Cod	No Major effect.	Non Known

Table 17 Overview of Global disease found in Clawed lobsters and some related species. Adapted from the Synopsis of Infectious Diseases and Parasites of Commercially Exploited Shellfish, Bower et al (1994-200).

Appendix II:- Sequence Alignment with full *Arcobacter butzleri* genome.

Align.	DB:ID	Source	Length	Score	Identities	E
1	EM_PRO:CP000361	CP000361.1 STD:Arcobacter butzleri RM4018, complete genome.	2341251	1754	87.7	7.0E-68
2	EM_PRO:CP000361	CP000361.1 STD:Arcobacter butzleri RM4018, complete genome.	2341251	1754	87.7	7.0E-68
3	EM_PRO:CP000361	CP000361.1 STD:Arcobacter butzleri RM4018, complete genome.	2341251	1754	87.7	7.0E-68
4	EM_PRO:CP000361	CP000361.1 STD:Arcobacter butzleri RM4018, complete genome.	2341251	1754	87.7	7.0E-68
5	EM_PRO:CP000361	CP000361.1 STD:Arcobacter butzleri RM4018, complete genome.	2341251	124	65.0	0.47
6	EM_PRO:CP000361	CP000361.1 STD:Arcobacter butzleri RM4018, complete genome.	2341251	123	58.1	0.52
7	EM_PRO:CP000361	CP000361.1 STD:Arcobacter butzleri RM4018, complete genome.	2341251	112	76.6	1.4
8	EM_PRO:CP000361	CP000361.1 STD:Arcobacter butzleri RM4018, complete genome.	2341251	112	66.7	1.4
9	EM_PRO:CP000361	CP000361.1 STD:Arcobacter butzleri RM4018, complete genome.	2341251	111	62.7	1.6
10	EM_PRO:CP000361	CP000361.1 STD:Arcobacter butzleri RM4018, complete genome.	2341251	101	64.1	3.8
11	EM_PRO:CP000361	CP000361.1 STD:Arcobacter butzleri RM4018, complete genome.	2341251	98	55.5	4.7
12	EM_PRO:CP000361	CP000361.1 STD:Arcobacter butzleri RM4018, complete genome.	2341251	101	51.2	5.0
13	EM_PRO:CP000361	CP000361.1 STD:Arcobacter butzleri RM4018, complete genome.	2341251	105	58.1	5.0
14	EM_PRO:CP000361	CP000361.1 STD:Arcobacter butzleri RM4018, complete genome.	2341251	94	74.3	6.3
15	EM_PRO:CP000361	CP000361.1 STD:Arcobacter butzleri RM4018, complete genome.	2341251	94	84.6	6.3
16	EM_PRO:CP000361	CP000361.1 STD:Arcobacter butzleri RM4018, complete genome.	2341251	92	66.7	7.2

Appendix II. 1. Sequence identity scores of the unidentified *Arcobacter* isolated from *Homarus gammarus* and the holding system compared to the full *Arcobacter butzleri* genome (EMBL strain RM4018; Miller *et al.,* 2007). DB:ID = EMBL bacterial database identification. Score = Maximum number of match score summaries reported in the result output (default value of 50). Identities = Percentage of similarity between the full *Arcobacter butzleri* genome and the unidentified Arcobacter sequence isolated from *Homarus gammarus* and the holding system. E= the standardized score for random variables that result in HSPs (high-scoring segment pairs) ie, how likely it is that a HSPs have arisen by chance. All sources were cross-referenced with EMBL Nucleotide Sequences, Protein Families, Literature, Ontologies and Protein Sequences. FASTA version 35.04 Jan. 24, 2010 (W.R. Pearson & D.J. Lipman PNAS (1988) 85:2444-2448)

Breakdown of alignment details between *Arcobacter butzleri* (EMBL strain RM4018) and unidentified *Arcobacter* isolated from *Homarus gammarus* and the holding system.

```
Unkno-                                CCCGGCGGCTGGCACGGAGTTAGCCGGTGC
                                      : :::: :::::::::::::::::::::::
EM_PRO TCCGAGTAACGCTTGCACCCTCCGTATTACCGCGGCTGCTGGCACGGAGTTAGCCGGTGC
       2275800   2275810   2275820   2275830   2275840   2275850

          420       410       400       390       380       370
Unkno- TTATTCATATGATACCGTCATTATCTTCTCATATAAAAGGAGTTTACGCACCGAAATGTG
       :::::::::: :::::::::::::::::: ::::::::::::::::::::::::::::::
EM_PRO TTATTCATATAATACCGTCATTATCTTCTTATATAAAAGGAGTTTACGCACCGAAATGTG
       2275860   2275870   2275880   2275890   2275900   2275910

          360       350       340       330       320       310
Unkno- TCATCCTCCACGCGGCGTTGCTGCATCAGAGTTCCCTCCATTGTGCAATATTCCCCACTG
       :::::::::::::::::::::::::::::: :: : ::::::::::::::::::::::::
EM_PRO TCATCCTCCACGCGGCGTTGCTGCATCAGACTTTCGTCCATTGTGCAATATTCCCCACTG
       2275920   2275930   2275940   2275950   2275960   2275970

          300       290       280       270       260       250
Unkno- CTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGACTGATCATCCTCTCAA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO CTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGACTGATCATCCTCTCAA
       2275980   2275990   2276000   2276010   2276020   2276030

          240       230       220       210       200       190
Unkno- ACCAGTTAAGTGTCATTGTCTTGGTGAGCCATTACCTCACCAACTAACTGATACTGTACA
       :::::::: : ::::: ::::::::  ::::::::: :::::::::::::::::  ::::
EM_PRO ACCAGTTATGCGTCATCGTCTTGGTAGGCCATTACCCCACCAACTAACTGATACAATACA
       2276040   2276050   2276060   2276070   2276080   2276090

          180       170       160       150       140       130
Unkno- GTCTCATCCCAAAGCCATAAATGTTTCCCTTGCTTACTTTTGTAATAAAGGCATATAGAG
       : :: ::: :  : : :::::: ::::::::  :  ::::::  : ::::: :::::  :
EM_PRO GGCTAATCTCTTACCAATAAATCTTTCCCTTTTTATCTTTTGATAAAAAGGAATATAAGG
       2276100   2276110   2276120   2276130   2276140   2276150

          120       110       100        90        80        70
Unkno- CATTACAAGTCGTTTCCAACAATTATTCTCTTCTTTGGGGCAGATTAACTATATATTACT
        ::::  : ::::::::::   :::: :  :  :  : :::: :::: ::::: ::::::
EM_PRO TATTAGCAATCGTTTCCAATTGTTATCCCTTAGTAAGAGGCATATTACCTATACATTACT
       2276160   2276170   2276180   2276190   2276200   2276210

           60        50        40        30        20         10
Unkno- CACCCGTGCGCCACTTAGCTGACAATATTAGCAAGCTAATATCCGTTCTCG-TCGACTTG
       ::::::::::::::::::::::::::  ::::::::::  ::::::::::: ::::::::
EM_PRO CACCCGTGCGCCACTTAGCTGACAATTATAGCAAGCTATAATCCGTTCTCGTTCGACTTG
       2276220   2276230   2276240   2276250   2276260   2276270

Unkno- CATGGTA
       ::::
EM_PRO CATGTGTTAAGCACGCCGCCAGCGTTCACTCTGAGCCAGGATCAAACTCTCCATAAATTT
       2276280   2276290   2276300   2276310   2276320   2276330
```