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Identification of cost-effective measures to improve holding conditions and husbandry practices for the horseshoe crab *Limulus polyphemus*

A Scientific Report by

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Contents

1. Introduction

1.1 Distribution and life history of the American Horseshoe crab, *Limulus polyphemus*

The American or Atlantic Horseshoe crab (HSC), *Limulus polyphemus*, is one of only four extant species of HSC worldwide. While wild populations of *L. polyphemus* are located on the Atlantic coast of North America (from the Gulf of Mexico to Maine; Smith *et al*, 2002a), the three other species: *Tachypleus tridentatus*, *Tachypleus gigas* and *Carcinoscorpius rotundicauda*, are found in the coastal waters of Asia, with distribution extending from India to Japan as well as the Philippine Islands (Walls *et al*. 2002; Smith and Berkson 2005). The largest *L. polyphemus* populations are located in Delaware Bay and Chesapeake Bay (Faurby *et al*, 2010; Shuster, 1982). Despite the name these chelicerate arthropods are more closely related to spiders and scorpions than crustaceans and insects (Walls *et al*. 2002; Gerhart 2007). While there is some debate regarding the exact classification of *L. polyphemus* within sub-phylum Chelicerata (Smith *et al*, 2002b), they are currently classified as members of Class Merostomata, Subclass Xiphosura, Order Xiphosurida, Suborder Limulina, Superfamily Limulacea and Family Limulidae (Walls *et al*, 2002).

L. polyphemus are regarded as environmental generalists, being eurythermic and euryhaline, able to tolerate temperatures ranging from -5 to 35°C and salinities between 5 and 35ppt (Nolan and Smith, 2009). The species is poikilothermic and are regularly found buried in the sediment, which may be a behavioural adaptation to avoid lower temperatures (Frankel, 1960). It is a theory supported by the presence of suitable coastal habitat to the north of their range indicating that temperature is the likely limiting factor in their distribution (Sekiguchi and Shuster, 2009).

Typically opportunistic omnivores, *L. polyphemus* have been shown to preferentially predate upon bivalves and polychaetes, indeed bivalves have been demonstrated as comprising over 80% of some animals diet (Botton, 1984; Ruppert & Barnes, 1994; Botton *et al*, 2003; Tzafrir-Prag *et al*, 2010). Indeed captive Horseshoe crabs show a preference toward thin shelled clams such *Mulinia lateralis* and *Mya arenaria* (Botton, 1984). Further studies have also shown that *L. polyphemus* require sources of particulate organic matter (POM), such as algae, to supplement their diets (Gaines *et al*, 2002; Carmichael and Valiela, 2005), whilst Botton (1984) determined that 90% of animals sampled were found to have vascular plant material within their digestive systems. Natural predators of *L. polyphemus* include molluscs, crustaceans, fish, leopard sharks, eels, birds and sea turtles; additionally they have also been fished by humans for both bait and haemolymph collection (Walls *et al*, 2002).

Adult *L. polyphemus* over-winter beyond the subtidal zone in waters between 5 and 20 metres in depth (Shuster, 1982). Stimulated by the lengthening photoperiod they begin to migrate shoreward in late spring, appearing in the intertidal zone around April (Shuster, 1985). Breeding appears to be primarily governed by the lunar cycle with activity peaking during spring tides while declining during neap tides (Widener & Barlow, 1999). The height of the breeding season occurs around the spring tides of May and June (Shuster, 1985). The end of breeding activity is signalled by the movement of the adult animals back to deeper water (Shuster, 1985).

Females must move up onto the beach to spawn, typically accompanied by a male, who 'hitches a ride' by clasping her terminal spines using modified front legs (Cohen & Brockman, 1983; Brockman & Penn, 1992; Walls *et al*, 2002). These breeding pairs may remain attached to one another for weeks, returning to the beach for repeated spawning (Brockman & Penn, 1992). It is common for such pairs to be accompanied up the shore by other males, in an attempt to fertilise the female's eggs before the attached male is able to do so (Brockman and Penn, 1992). The females typically lay 5-7 clusters of green, ovoid eggs (2-3mm in diameter) during each spawning event, with each cluster containing approx 3,650 eggs (Shuster, 1950). A female may lay around 88,000 eggs over the course of a breeding season (Shuster, 1982). After fertilisation by the male/s the female covers the eggs with sand to a depth of between 5 and 30 cm (Brockman, 1990). After 2-5 weeks the majority of the eggs hatch into trilobite larvae (approx. 1cm in length), which remain in the sand for several weeks (Rudloe, 1979; Rudloe, 1981a). However, in some instances a proportion of eggs will remain within the sand and not hatch until the following spring (Botton *et al*, 1992).

At the post-trilobite stage the larval horseshoe crabs moult into the first instar stage at which point they cease the nocturnal swimming behaviour of the trilobite larval stage and begin crawling across the sediment surface (Rudloe, 1981b). The first and all subsequent instar stages resemble the adult, the only major difference in outward appearance being telson length.

L. polyphemus can live up to 20 years and typically moult around 18 times, taking 9 to 12 years to reach maximum size (Shuster, 1985; Shuster & Sekiguchi, 2003). Moulting occurs during the warmer summer months and becomes more time consuming and difficult as the animals age (Shuster, 1982). Females reach maturity a year later and so generally moult at least once more than their male counterparts, as a consequence females are significantly larger than males at maturity (Shuster, 1955). Indeed the mean prosomal width of a mature male is around 75 to 79% that of a mature female (Shuster, 1982).

1.2 Anatomy of *Limulus polyphemus*

1.2.1 External anatomy

L. polyphemus possess a rigid, external exoskeleton primarily composed of the polysaccharide, chitin (Ruppert & Barnes, 1994). The animals are dorsoventrally flattened with a carapace divided into three sections (Figure 1): the prosoma, opisthosoma and tail-like telson (Smith and Berkson, 2005). The opisthosoma possess distinctive, flexible, terminal spines, which the male grasps during breeding (Cohen and Brockman, 1983; Brockman and Penn, 1992; Walls *et al*, 2002). While the telson is typically utilised by *L. polyphemus* to right the animal when on its back (Ruppert & Barnes, 1994; Smith and Berkson, 2005).

L. polyphemus possess 14 pairs of ventral appendages (Figure 1) (Ruppert & Barnes, 1994). These comprise a pair of modified chelicerae, six pairs of segmented pedipalps (the first four pairs of which are chelated), a pair of chilaria and six pairs of branchial appendages (the first pair of which have fused to form the operculum and the remained five adapted to form book gills) (Ruppert & Barnes, 1994; Smith and Berkson, 2005). In males the first

pair of pedipalps are modified and are commonly referred as 'boxing gloves' (Figure 2). These are utilised to clasp the female during mating; an adaptation that allows easy differentiation between the sexes (Walls and Berkson, 2000; Brockman and Penn, 1992). The fifth pair of pedipalps are not involved in locomotion, but bear a lateral, spatulate process known as a flabellum, which is utilised to direct water currents into the respiratory chamber of the opisthosoma (Ruppert & Barnes, 1994). The fifth pair of walking legs (sixth pair of pedipalps) are not chelated, but instead possess four leaf-like processes attached to the end of the first tarsal segment; typically used for burrowing, to clear away mud and silt (Ruppert & Barnes, 1994).

Vision is provided by a pair of laterally located compound eyes and a central ocellus, or simple eye, located on the dorsal surface of the prosoma (Smith and Berkson, 2005). In addition, *L. polyphemus* also possess a series of photoreceptors on their opisthosoma and telson (Schuster 1982).

The mouth is centrally located on the ventral surface, at the confluence of the pedipalps (Ruppert & Barnes, 1994). When a food item is detected by the chelicerae, the claws at the ends of the pedipalps grasp it and pass it back to the gnathobases; spiny areas on the median side of the coxa of the first four pairs of pedipalps (Ruppert & Barnes, 1994). The gnathobases act as primitive teeth and jaws, macerating and transporting fragments of food anteriorly (Ruppert & Barnes, 1994). These fragments are conveyed into the crab's mouth with assistance from the chelicerae and chilaria. Waste is excreted through the anus located on the ventral surface of the opisthosoma, slightly anterior to the telson.

1.2.2 Internal anatomy

L. polyphemus possess a large dorsally-located tubular heart; from which a well developed arterial system extends and connects to an open vascular system via tissue sinuses (Schuster 1982; Ruppert & Barnes, 1994). The heart has eight pairs of ostia, each having two valves through which oxygenated haemolymph enters the heart from the pericardial chamber (Ruppert & Barnes, 1994). Once in the heart the haemolymph is pumped forward and escapes through three pairs of aortae, one pair of cerebral arteries, and a single frontal artery (Schuster 1982). Deoxygenated haemolymph from the tissues collects in two large ventrally located, longitudinal sinuses (Ruppert & Barnes, 1994). The haemolymph then flows from these ventral sinuses into the book gills, the beating of which not only results in external water circulation, but also act as a pump driving blood through the lamellae (Ruppert & Barnes, 1994). The haemolymph then returns to the pericardium. For the purposes of bleeding, the cardiac sinus can be accessed through the arthrodial hinge located between the prosoma and opisthosoma (Smith and Berkson, 2005).

Horseshoe crabs possess a basic digestive system comprising a mouth, which opens into a cuticle-lined esophagus (Figure 1). This turns backwards into the proventriculus, which is composed of a 'crop' and 'gizzard' (Figure 1). The crop can expand considerably to accommodate ingested food. The food then passes into the gizzard, which has the thickest muscular wall of the entire intestinal tract, where food is reduced to a pulp. Undigestible material, such as fish bones or larger shell pieces are regurgitated. The pulverised food then passes into the midgut, through the pyloric valve that protrudes from the end of the gizzard, where it is digested. The hindgut is a short muscular tube that opens through the slit-like anus.

L. polyphemus has a rudimentary nervous system, displaying a high degree of fusion (Ruppert & Barnes, 1994). It comprises a ventrally situated cerebral ganglion, or 'brain', linked to a ventral nerve cord possessing five smaller ganglia (Ruppert & Barnes, 1994). Radiating from these are a pair of optic nerves, running directly to the two lateral eyes, 8 pairs of haemal nerves extending into the prosoma as well as numerous lateral nerves branching into the opisthosoma (Ruppert & Barnes, 1994).

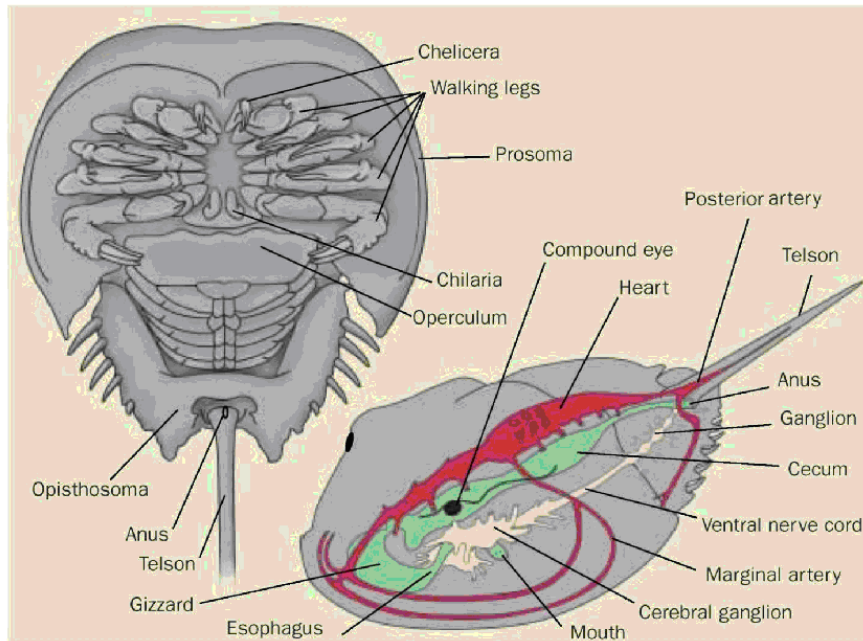Figure 1: Diagram of the external and internal anatomy of the American Horseshoe crab (*Limulus polyphemus*). (From http://what-when-how.com/animal-life/subclass-merostomata/).

Figure 2: Photograph showing the modified front pedipalp of a male American horseshoe crab (*L. polyphemus*). From:http://www.flickr.com/photos/artour_a/1534830634/

1.3 Haemolymph

1.3.1 Function and structure

The two predominant components of *L. polyphemus* haemolymph are the copper-containing respiratory protein haemocyanin and a single amoebocyte cellular component (Toh *et al.* 1991; Ruppert & Barnes, 1994). Haemocyanin fulfils the requirement for an oxygen transporter while the primary role of the amoebocytes involves blood clotting (Ruppert & Barnes, 1994). *L. polyphemus* haemocyanin contains two copper atoms and corresponds closely to the minimum molecular weight, of approximately 74 kDa, of typical arthropod haemocyanin (Sullivan *et al*. 1974). However, unlike most forms of haemocyanin and haemoglobin, oxygen binding by *L. polyphemus* haemocyanin exhibits a reverse Bohr effect (Sullivan *et al*. 1974).

L. polyphemus amoebocytes (Figure 3) are responsible for the animals immune response to microbial invasion (Muta *et al*, 1993). The coagulation system employed by Horseshoe crabs is unique, involving a serine protease zymogen cascade initiated by the presence of bacterial endotoxins and various lipopolysaccharides (Muta *et al*, 1993). Once triggered, clotting factors stored in granules within the amoebocytes are released into the haemolymph via degranulation (Toh *et al*, 1991). The clot formed through activation of the cascade is highly effective in immobilising invading microorganisms, thereby allowing their neutralisation by antimicrobial substances, such as protein inhibitors, antimicrobial peptides and lectins, also secreted by the amoebocytes (Miyata *et al*, 1989; Armstrong, 1991; Kouno *et al*, 2008). Consequently, this coagulation system is critical for both haemostasis and the microbiological defence of *L. polyphemus* (Muta *et al*, 1993).
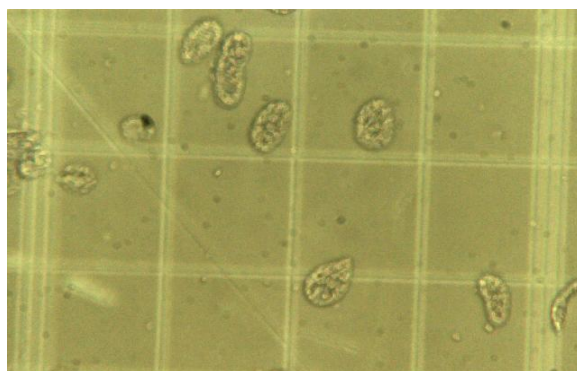

Figure 3. *L. polyphemus* amoebocytes. (Scale, 1 square = 1mm^2)
(Micrograph courtesy of Asma A. Ben Ahmeid)

1.3.2 Limulus amoebocyte lysate (LAL)

It is the unique qualities of *L. polyphemus* haemolymph that make it extremely useful to both the medical and pharmaceutical sectors. The high sensitivity of the *L. polyphemus* serine protease zymogen cascade is utilised in detecting bacterial contamination of medical and pharmaceutical products, such as intravenous drugs, vaccines and medical implements (Walls *et al*, 2002).

The phenomenon was first noted in 1955 by Dr. Frederik Bang whilst working at the Marine Biological Laboratory in Woods Hole, Massachusetts. Bang observed that an injection of marine bacteria into the bloodstream of *L. polyphemus* resulted in massive clotting (Bang, 1956). Through later research it was determined that the clotting was triggered by endotoxins present on the surface of the bacteria (Levin & Bang, 1964a). The active component of the clotting process was subsequently localised to the amoebocytes with the reaction being reproducible *in vitro* utilising cell-free amoebocyte lysate (Levin &

Bang, 1964b, Novisky, 1984). The Limulus amoebocyte lysate, or LAL, assay, received FDA approval for use in the testing of drugs, blood products, intravenous fluids and disposable pharmaceutical devices, in the 1970's (Wall *et al*, 2002). The LAL assay has the capacity to detect one millionth of a billionth of a gram of endotoxin in approximately 45 minutes (Mikkelsen, 1988, Wall *et al*, 2002).

Harvesting of Limulus amoebocyte lysate involves drawing haemolymph from the cardiac sinus of live adult *L. polyphemus* using a large bore needle (Walls *et al*, 2002). Typically, 100-300 ml of haemolymph is extracted from each animal, the amount dependent on size, sex and the period between bleedings (Rudloe, 1983). Aquarium studies have suggested that a bled individual can restore its blood volume in 3-7 days; however, it may take up to 4 months for it to regain its pre-bled amoebocyte levels (Novisky, 1984).

Post collection, the haemolymph is centrifuged at low speed, resulting in separation of the cellular component (amoebocytes) from the cell-free supernatant (Novisky, 1984). The supernatant is discarded allowing the cellular component to be first homogenised and then centrifuged at high speed. Centrifugation separates the cellular debris, while the required clotting factors remain suspended in the resultant supernatant, referred to as the Limulus amoebocyte lysate (LAL) (Novisky, 1984). For storage purposes the LAL is then frozen in its raw, inactive form. It is converted to the active form via the addition of ions in the form of sodium, calcium or magnesium salts before a patented solvent extraction is performed to improve the reagent's sensitivity prior to freeze-drying for long-term stability (Novitsky, 1984; Wall *et al*, 2002).

There are several methodologies utilised for the performance of LAL sterility tests, however, only the three most widely used are described below. All three share the same initial step involving the mixing, in a glass dish, of the reagent and test solution in equal proportions (Wall *et al*, 2002). The first method then incubates the mixture at 37°C for approximately 1 hour before examining for evidence of clot formation, which would indicate the presence of endotoxin or pyrogen (Novitsky, 1984). The second method involves assessment of the rate of turbidity of the mixture prior to the formation of a clot (Wall *et al*, 2002). The final method, known as the chromogenic substrate method, involves a colour change (yellowing) of the reagent post mixing with the degree of colouration after a fixed period used to indicate the presence of endotoxin or pyrogen (Wall *et al*, 2002).

Since its discovery the LAL assay has been a significant and widely utilised tool in the pharmaceutical industry, providing a rapid and cost effective alternative to the use of live animals and animal products in testing for bacterial contamination (Mikkelsen, 1988; Walls *et al*, 2002). In addition to its primary use in the testing of pharmaceutical products, new applications for LAL continue to be found, such as the detection of bacterial contamination in meat, fish, and dairy products, including frozen items (Wall *et al*, 2002).

1.3.3 Effects of bleeding-induced stress on *L. polyphemus*

Aquatic invertebrate health is affected by a variety of factors, of which physical or physiological stress is one of the most significant (Evans *et al*. 1999). Both short and long-term stressors can lead to alterations in respiratory function and structure as well as changes

to metabolism, osmotic balance, haemolymph volume and tissue pH (Paterson and Spanogh, 1997). The cumulative effects of such stress may ultimately lead to reductions in metabolic rate, animal growth and survival (Allender *et al*, 2010).

Stress has been shown to impact on crustacean haemocyanin function as well as altering haemolymph composition, varying parameters such as pH and L-lactate concentration (Carlsson and Gäde, 1986; Burnett, 1992). Gäde (1983) discovered that respiratory and heart rates were suppressed in some arthropod species after exposure to a low oxygen environment. In addition, during seasonal hypoxia increases in haemolymph L-lactate levels have been observed (Whiteley *et al*, 1990). The increased L-lactate could be a result of anaerobic glycolysis utilised to produce ATP under low oxygen conditions. (Hill *et al*. 1991). During periods of air exposure, horseshoe crabs experience respiratory acidosis (a decrease in haemolymph pH) due to CO_2 accumulation, resulting in a shift of the oxygen equilibrium to the left (Burnett *et al*. 1988). Furthermore, it was determined that such acidosis could lead to a disturbance of enzymatic pathways and potentially increase animal mortality (Burnett *et al*. 1988).

During the typical collection and bleeding processes employed by researchers and pharmaceutical workers, horseshoe crabs are exposed to a number of stressors, which may include handling, air and sun exposure, temperature variations and of course blood loss. To date the vast majority of *L. polyphemus* bled commercially for LAL production have been wild capture animals. As a consequence limited work has been conducted examining stress parameters and mortality post bleeding. However, there is evidence indicating that commercial bleeding results in an increase in mortality of between 8% and 15% in wild caught animals (Rudloe, 1983; Wall *et al*, 2000; Wall *et al*, 2002 and Hurton *et al*, 2009). Rudloe (1983) showed that bleeding resulted in increased mortality in animals in the wild by 10% in the first 12 months and by 11% in the second year. *L. polyphemus* mortality has been observed to increase from 2.6% in unbled animals to 8% in bled individuals under exposure to low and high impact stressors, such as air exposure and temperature variation (Hurton *et al*, 2009). Consequently, elevated mortality likely results from synergistic interactions between such stressors (Schisler *et al*. 2000; Hatch & Blaustein 2003). Therefore, efficient and effective methods are required to assess the physiological state of *L. polyphemus*. Such methods would work to minimise mortality by allowing any overstressed or weakened animals to be selected out prior to bleeding.

1.4 Culture of *L. polyphemus*

Due to the increasing pressures on wild stocks of *L. polyphemus* measures are clearly needed to prevent a significant and potentially irreversible population decline. However, management of wild stocks is difficult due to the many different stakeholders involved. Foremost amongst these are the biomedical/pharmaceutical industry and conservation bodies both of whom wish to see population levels maintained, but for very different reasons (Berkson & Shuster, 1999). It is this strong collective will, however, that has led to the development of management strategies and to an increasing interest in aquaculture as a means of both restocking and providing an alternate supply of animals for bleeding (Wall *et al*, 2002; Tzafrir-Prag *et al*, 2010).

To date *L. polyphemus* have primarily been held in captivity for research purposes rather than as stock for biomedical bleeding (Schreibman & Zarnoch, 2009). Schreibman and

Zarnoch (2009) regarded the production of mature *L. polyphemus* in aquaculture systems as unfeasible due to their slow maturation rate and suggested instead that the enhancement of wild stocks with cultured juveniles was a better strategy. However, given that they were able to hatch and maintain a population of animals for 7+ years indicates that a biomedical industry subsidiary should be able to breed and sustain large numbers of animals in captivity for the purposes of blood extraction. This section does not detail the system setup and culture conditions employed by the projects industrial partner, Marine Biotech Ltd., as these are described in detail in the appendices.

As with the majority of commercially cultured invertebrate species one of the primary issues affecting the aquaculture of *L. polyphemus* is the potential loss of stock due to disease outbreaks. Indeed, stock loss due to disease is considered to be the primary restriction in the development of the aquaculture sector (Defoirdta *et al*, 2007). The risk of such outbreaks increases significantly in intensively mono-cultured animals kept at high stocking densities (Kautsky *et al*, 2000). Other significant factors in the culture of *L. polyphemus* are; diet composition, water quality and maintenance of abiotic parameters (Smith & Berkson, 2005; Schreibman & Zarnoch, 2009; Carmichael *et al*, 2009). Poor water quality and insufficient waste removal leads to overloading of metabolites and environmental degradation, this puts the animals under stress and increases their susceptibility to pathogens. Excessive fluctuations in abiotic factors such as dissolved oxygen levels, salinity and temperature also increase stress and vulnerability to disease (Smith & Berkson, 2005).

Culture systems are home to a diverse bacterial community, many of which are potential pathogens which cannot be eliminated entirely by water treatment (Meyer, 1991; Schulze *et al*, 2006). While these potential pathogens are also present in wild populations they rarely cause mass mortality (Sommerset *et al*, 2005). However, this is not the case in cultured populations where unnaturally high stocking densities coupled with other potential stressors, such as suboptimal culture conditions conspire to produce in ideal environment for disease (Hansen & Olafsen, 1999; Verschuere *et al*, 2000; Winton, 2001). Bacteria are continually added to culture systems via feed, as well as through translocation of infected stock, a problem particularly common in mollusc culture (Paillard *et al*, 2004).

There is relatively little information available regarding the bacterial diseases of *L. polyphemus*, largely due to the fact that, to date, all commercially viable stocks were wild and no large scale disease outbreaks of note have been observed and investigated. What is known about the bacterial strains associated with *L. polyphemus* is summarised here. The earliest recorded observation of bacterial disease in *L. polyphemus* occurred in 1955; where Gram negative bacteria were isolated from the haemolymph of moribund animals (Bang, 1956). It was also observed that the haemolymph of the crabs in question did not appear to clot and exhibited a lack of circulating amoebocytes (Bang, 1956). Since these initial observations other potential pathogens have been isolated. *Oscillatoria* spp and *Beggatoa* spp are causative agents in gill infections, although the latter is less invasive and less pathogenic (Leibovitz & Lewbart, 2004; Smith & Berkson, 2005). Members of genera *Leucothrix*, *Vibrio*, *Flavobacterium*, *Pseudomonas* and *Pasturella* have all been found associated with external lesions on *L. polyphemus* (Smith & Berkson, 2005; Nolan & Smith, 2009). *Vibrio alginolyticus* in particular is thought to be a pathogen of *L. polyphemus* (Bosniak & Armstrong, 2004). However, no records of further investigation or descriptions of the pathogenicity of these bacteria to *L. polyphemus* can be found.

As with all health issues, prevention is better than cure, therefore good animal husbandry is vital in maintaining a healthy, productive captive *L. polyphemus* population (Smith & Berkson, 2005; Schreibman & Zarnoch, 2009). This is further emphasised by the lack of targeted antibiotic treatments specifically formulated for *L. polyphemus* (Smith & Berkson, 2005). Indeed for a time there was general consensus that the animal's highly sensitive clotting response rendered them essentially impervious to systemic bacterial infection. This has now been discounted and the testing of *L. polyphemus* haemolymph for the presence of bacteria is now advocated as a method of disease detection (Nolan & Smith, 2009).

Good aquatic animal husbandry requires regular monitoring of both animals and environmental conditions within the culture system. Ideally, injured or diseased animals should be isolated from the general population and examined more frequently for changes in health status. Daily system checks should include the monitoring of water quality parameters such as levels of; NH_4, NO_2, and pH, as well as abiotic factors such as dissolved O_2, temperature, salinity and redox potential. Also key is the timely removal of any solid waste material that has accumulated within the system. Although suspended particulate material is generally removed by filters vigilance is required to prevent the accumulation of larger debris, particularly in holding tanks. Such matter typically takes the form of faeces and unconsumed feed, but may also occasionally include dead animals. This waste will increase turbidity as well as provide a growth substrate for potentially harmful bacteria and algae. Poor water quality and insufficient waste removal leads to overloading of metabolites and environmental degradation, putting the animals under stress and increasing their susceptibility to pathogens (Beristain, 2005). Excessive fluctuations in abiotic factors such as dissolved oxygen, salinity and temperature also increase stress and vulnerability to disease (Beristain, 2005).

1.5 Project Aims

- o Identification of cost-effective measures that may improve and optimise holding conditions and husbandry practices for *L. polyphemus,* and thereby reduce stock loss.

- o To investigate the effect of the withdrawal of haemolymph on the physiology of *L. polyphemus* and to identify any methods that may provide greater yields of haemolymph in good condition for the extraction of the necessary biochemical compounds (LAL).

- o Investigation of the culturable bacterial flora found within the different parts of the recirculating aquaculture system (RAS) employed by Marine Biotech Ltd. and the identification of any potential problem areas requiring action.

- o Investigation of the culturable bacterial flora associated with adult *L. polyphemus* held at the Marine Biotech Limited facility, with emphasis on the identification of potential pathogens.

2. <u>Materials & Methods</u>

2.1 Physiological stress parameters

2.1.1 Experimental design

Twelve adult male *L. polyphemus* (average weight 1106 g; average prosomal width 219 mm) were randomly selected to examine the effects of air emersion and haemolymph withdrawal on five physiological parameters regarded as potential indicators of stress. These animals were divided into three experimental groups of four individuals: group one, where haemolymph was withdrawn and the animals were immediately returned to water and groups two and three where the animals were exposed to air (at 17°C) for a further 1 and 2 hours respectively, post bleeding. The animals were re-sampled after 2 hours to evaluate short-term effects and again after a 2 week recovery period to evaluate any longer term effects.

The extracted haemolymph was analysed, with measures taken of temperature, pH, protein concentration, L-lactate level and the total number of circulating haemocytes.

2.1.2 Haemolymph extraction

After surface sterilisation with 70% ethanol solution, haemolymph was withdrawn via a sterile 19 Gauge needle inserted through the arthroidial membrane at the base of the telson into the cardiac sinus. The haemolymph was collected in a sterile Universal tube, samples from which were then used for the determination of haemolymph protein concentration, L-lactate level, temperature, and pH. For the total haemocyte counts, haemolymph was drawn into a sterile 10 ml syringe containing 10 % neutral buffered formalin (NBF) solution (see appendix) at a ratio of 1:2 or 1:3, blood to NBF.

2.1.3 Temperature and pH of haemolymph

Immediately upon extraction the temperature and pH of the haemolymph was determined using a Jenway Model 3510 pH, conductivity/TDS and temperature meter (calibrated using pH standards prior to sampling).

2.1.4 L-lactate level

Haemolymph L-lactate was measured using an Accutrend portable lactate analyser and BM-Lactate test strips (Roche Diagnostics Ltd., West Sussex, UK) as per manufacturer's guidelines. The limit of detection of this analytical system was 0.8 mmol/l.

2.1.5 Haemolymph protein

Haemolymph protein concentration was determined using a standard, hand-held clinical refractometer calibrated for serum protein (0-12g/dl). Two to three drops of fresh haemolymph was placed on the lens of the refractometer using a disposable plastic pipette before a reading of the protein concentration in g/dl was taken via the eyepiece.

2.1.6 Total haemocyte counts

Total haemocyte counts (THCs) were performed on the Haemolymph/NBF solutions using a Neubauer haemocytometer and a Leitz Wetzlar light microscope. Haemolymph/NBF solution (25 µl) was pipetted onto the haemocytometer, with counts performed under x40 magnification. Duplicate counts were performed for every sample.

2.1.7 Statistical analysis

Using the statistical package Minitab 16, standard statistical procedures were applied to compare the data sets. An ANOVA test for parametric data and a Kruskal-Wallis test for non-parametric data were used to measure differences among the three data sets. Further comparisons between the treatment means were made using a Tukey´s range test, and a t-test was used to compare the mean difference between two data sets. Significance was accepted at $p<0.05$.

2.2 Environmental bacteriological analysis

2.2.1 Sample Collection

Water samples and biofilm swabs were collected from the recirculating, seawater, aquaculture system at the facility of Marine Biotech Limited, Callander, Scotland.

Water samples, in triplicate, were collected from both male and female (brood stock) holding tanks (each containing approx. 100 animals), as well as from holding tank and protein skimmer outflow pipes. Further water samples were collected from the system's biofilter, each containing between 1 and 3 biofilter beads. Tank and biofilter water samples were obtained by submerging, then opening, a sterile, 50ml Universal tube, while the outflow pipe samples were obtained by holding an open Universal tube under the flow of water.

Biofilm samples were collected, in triplicate, from the two animal holding tanks, biofilter and the system sump. In addition, swabs were also taken from the surface of one of the bag filters located within the sump. Swabs from the BBL™ CultureSwab™ Collection & Transport system were utilised to gather biofilm from an area equivalent to 1 cm², below the waterline.

The surface bacterial microflora of *L. polyphemus* was sampled using similar methodology. Swabs of the dorsal carapace, between the lateral eyes, and the gills of 3 males (animals 22, 33 & 67) and 3 females (animals 104, 106 & 139) were collected. The carapace samples were gathered as per the system biofilm, whereas the gill samples were collected via the insertion and rotation of a swab between adjacent leaves of the gill booklets. Any lesions or wounds present on the selected animals (22 & 106) were sampled in the same fashion as the gills. In addition, a further swab was taken from a significant carapace lesion present on a female, number 183.

Haemolymph from the same animals was also collected for bacteriological analysis. Haemolymph was extracted as described in Section 2.1.2 and immediately spread plated (2-3 drops), in duplicate, onto Marine agar (Difco™ 2216), Tryptic (Trypticase) Soy Agar

(TSA) (Oxoid[TM] CM0131) with 3% NaCl and Thiosulfate-Citrate-Bile salts-Sucrose (TCBS) agar (Difco[TM] 265020). The plates were then sealed and upon arrival at the University of Glasgow, incubated at 15°C under aerobic growth conditions. Despite the lack of aseptic technique during plating, all effort was made to minimise potential contamination of the haemolymph samples.

Processing of the water and CultureSwab[TM] samples was undertaken, using aseptic technique, upon return to the facilities at the University of Glasgow.

2.2.2 Sample processing

All water samples (excluding biofilter samples) underwent a ten fold serial dilution, (1ml of sample into 9ml of sterile 3% NaCl solution; previously optimised) prior to plating. A volume of 25μl was then pipetted and spread homogeneously onto 90mm plates of Marine agar, TSA (with 3% NaCl) and TCBS agar. The plates were then incubated aerobically, at 15°C.

The biofilter samples were processed by transferring the plastic beads collected, individually, to Universal tubes containing 5ml sterile 3% NaCl solution. These tubes were then vortexed for 30 seconds in order to resuspend bacteria adhering to the bead. A 25μl volume of each sample was then plated using the methodology previously described for the water samples. The plates were then incubated aerobically, at 15°C.

CultureSwab[TM] samples were processed as follows; the tips were aseptically removed and placed into 1.5ml Eppendorf tubes containing 500μl of sterile 3% NaCl solution. Each tube was then vortexed for 30 seconds to resuspend any adhered bacteria. A 25μl volume of each sample was then plated using the methodology previously described before being incubated aerobically, at 15°C. Colony counts were performed on all plates after 24, 48 and 168 h growth time. After incubation period of 48 hours the relative proportion of *Vibrio*-like strains in a given sample was then determined using the relative number of colony forming units that developed on the TCBS agar and TSA (3% NaCl) plates. The TSA (3% NaCl) as opposed to the Marine agar counts were selected due to the similar growth characteristics of the TCBS and TSA media.

2.2.3 Selection and identification of isolates

After 48 h growth all visually-distinct colonies, i.e. those with differing gross colony morphology, were picked and quadrant streaked onto fresh plates of the same media. Each isolate was assigned an identifying number (01-41) with a record of its origin and a digital image of its gross appearance. The plates were then incubated aerobically at 15°C and checked after 48 and 72 hours. After sufficient growth to determine the presence of a monoculture, single colonies were picked and continuously streaked onto fresh plates (in duplicate) for strain identification. These plates were incubated aerobically at 15°C and 20°C, respectively, this ensured the presence of young, fast growing colonies required for Gram staining and API[®] 20E[TM] testing. In addition, the isolates were also incubated on slopes of appropriate media and stored at 5°C.

The nature of the cell wall of the isolates, i.e. Gram type, was determined via Gram staining (Colomé *et al*, 1986). Identification of the isolates to species level was performed using the API® 20E™ test system (Biomerieux, Marcy l'Etoile, France). The API® 20E™ is a standardised identification system used to identify *Enterobacteriacaea* and other non-fastidious, Gram negative bacteria. The test system was utilised as per manufacturer's instructions, with the exception of incubation temperature. An incubation temperature of $20^{o}C$ was selected to better reflect the growth requirements of the isolates. All test strips were initially incubated for 24 h (as per manufacturer's instructions); however; those strips displaying less than three positive reactions after this period were incubated for a further 24h to make allowance for the lower incubation temperature (i.e. slower growth). Furthermore, any strips exhibiting zero positive reactions after 48 h incubation were re-run in order to differentiate between true negative results and growth failure of the isolate. Results for each isolate were recorded on the sheets provided and species identification obtained (along with a percentage accuracy of the I.D.) using the Biomerieux API® software and online database. Any identification with an accuracy of less than 50% resulted in the isolate in question being re-tested.

3. <u>Results</u>

3.1 Physiological stress parameters

3.1.1 Haemolymph temperature

The bleeding procedure and associated air emersion (1 and 2 hours exposure) had no statistically significant effect on the temperature of *L. polyphemus* haemolymph. Indeed the results support the literature regarding the ectothermic nature of *L. polyphemus* in that the temperature of its haemolymph reflected the prevailing ambient water and air temperatures.

3.1.2 Haemolymph pH

The mean haemolymph pH values are shown in Figure 4. Baseline (mean) haemolymph pH was 7.5, with a statistically significant decrease in the mean of 0.3 (p= 0.010 and F= 7.83) observed in the 1 hour post bleed samples (Figure 4; **A**). After the 2 week recovery period haemolymph pH returned to a (mean) value of 7.4, determined as not significantly different from the baseline value. Statistically significant deviation from the baseline pH value was observed in both the 1 hour and 2 hour air-exposed groups (Figure 4; **B**). Mean pH dropped to 7.0 for the 1 hour group and to 7.1 for the 2 hour exposed group (Kruskal-Wallis test: H= 11.86, p= 0.003). However, no statistically significant difference was observed in pH between the 1 hour and 2 hour air-exposed animals.
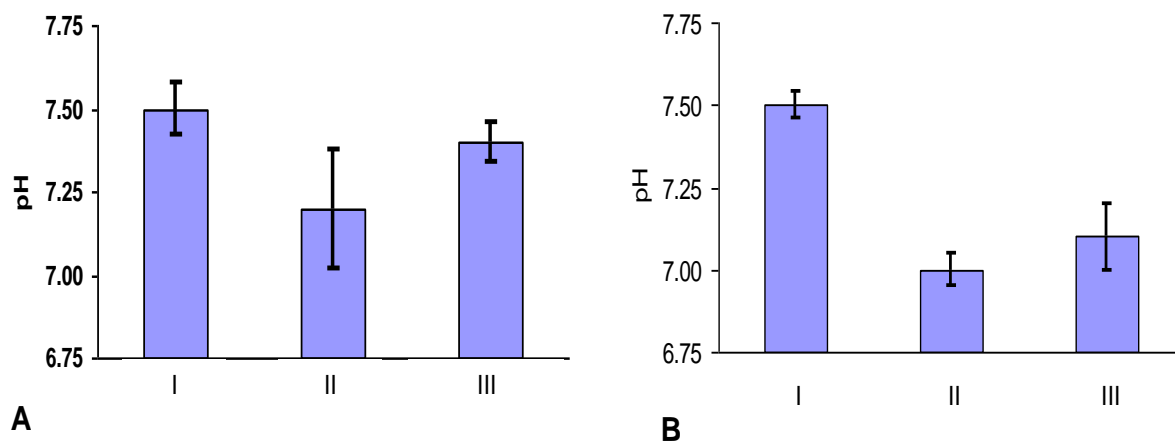
Figure 4. Haemolymph pH values; **A** – pH values at: time of bleeding, i.e. baseline value (**I**), after 1 hour recovery (**II**) and after 2 weeks recovery (**III**). **B** – pH values at: time of bleeding i.e. baseline value (**I**), after 1 hour of air exposure (**II**) and after 2 hours air exposure (**III**). Mean values ± SEM, Baseline, n = 8; Experimental groups, n = 4.

3.1.3 L-lactate level

Of the 24 haemolymph samples analysed only five possessed L-lactate concentrations exceeding the detection threshold of the analyser (0.8 mmol/l). The samples in question and their L-lactate values are displayed in Table 1. Given the size of this sample set it is not possible to draw any viable conclusions from this data regarding the effects of bleeding/handling stress on *L. polyphemus* blood lactate levels, only that *L. polyphemus* possesses haemolymph L-lactate levels that are lower than those of previously tested crustacea.

Table 1. L-lactate concentration of those fresh haemolymph samples that registered on the Accutrend portable lactate analyser. Detection threshold, 0.8 mmol/l.

	Number of animals sampled	Haemolymph L-lactate concentration (mmol/l)
Baseline	2	0.9 & 0.8
1 hour air exposed	1	0.9
1 hour air exposed	1	1.0
2 hour air exposed	1	0.8

3.1.4 Haemolymph protein

Despite the appearance of a decline in haemolymph protein concentration with increased air exposure, no statistically significant variation was observed between the haemolymph protein concentrations of the baseline samples and those obtained for the experimental groups (Table 2).

Table 2. Protein concentration of fresh haemolymph obtained from *L. polyphemus* – baseline samples, after 1 hour and 2 weeks recovery and after 1 hour and 2 hours air exposure. (P & H values as indicated; n = 4, excluding air exposed baseline where n = 8).

	Mean (g.100ml^{-1}) ± SD	P-value	H-value
Baseline	6.1 ± 2.4		
1 hours recovery	6.2 ± 2.5	} 0.790	} 0.48
2 weeks recovery	5.8 ± 1.2		
Baseline	6.0 ± 2.3		
1 hour air exposed	5.8 ± 2.5	} 0.829	} 0.38
2 hour air exposed	5.4 ± 1.2		

3.1.5 Total haemocyte counts

No statistically significant differences were observed between the baseline total haemocyte (amoebocyte) counts of any of the experimental groups (Table 3). All groups exhibited a high degree of variance between individual animals, illustrated by the large standard deviation values, when compared to the mean, displayed in Table 3.

Table 3. Total haemocyte (amoebocyte) counts (THC) of fresh haemolymph obtained from *L. polyphemus* – baseline samples, after 1 hour and 2 weeks recovery and after 1 hour and 2 hours air exposure. (P & H values as indicated; n = 4, excluding air exposed baseline where n = 8).

	Mean THC ± SD (x10^{6})	P-value	H-value
Baseline	11.8 ± 5.3		
1 hours recovery	12.1 ± 5.4	} 0.105	} 4.50
2 weeks recovery	14.9 ± 6.1		
Baseline	15.2 ± 6.1		
1 hour air exposed	15.5 ± 8.4	} 0.309	} 1.29
2 hour air exposed	21.6 ± 6.1		

3.1.6 Long-term effects of air exposure

Further to the air exposure data displayed in sections 3.1.1 through 3.1.5, the animals tested were returned to the holding tank and re-sampled after a 2 week recovery period. This was undertaken in order to ascertain whether sustained air exposure had any longer term effects on the physiological parameters tested. L-lactate was not reassessed given the lack of useable data collected during initial sampling.

The only test parameter to exhibit statistically significant variation over the course of the trial as a result of air-exposure-induced stress was haemolymph pH (Table 4). In both the 1 hour and 2 hour air exposed groups blood pH displayed a statistically significant decline (increased acidity) of 0.5 and 0.4 units respectively, with the effect being reversed in the 2 week samples (Table 4). Haemolymph temperature also displayed statistically significant variation but only as a reflection of the ambient air/water temperature and not as a result of air exposure *per se*.

Table 4. Haemolymph temperature, pH, protein concentration and total haemocyte count data for the 1 hour air exposed and 2 hour air exposed groups after a 2 week recovery period (*); included for comparison are the baseline and immediate post exposure values. Mean values ± SD; n = 4. (P & H values as indicated).

	Temperature (^{o}C)	pH	Protein (g.100ml^{-1})	THC (x10^6)
Baseline	18.6 ± 0.2	7.5 ± 0.04	5.6 ± 2.1	12.1 ± 7.8
1 hour air exposed	17.8 ± 0.3	7.0 ± 0.05	5.8 ± 2.1	15.5 ± 8.4
1 hour air exposed *	18.2 ± 0.1	7.4 ± 0.10	5.3 ± 1.6	17.0 ± 1.9
P-value H-value	P-value H-value	P-value H-value	P-value H-value	P-value H-value
0.005 11.23	0.005 11.23	0.015 8.35	0.811 0.42	0.173 3.51
Baseline	18.4 ± 0.3	7.5 ± 0.03	6.0 ± 2.1	18.3 ± 2.7
2 hour air exposed	17.7 ± 0.2	7.1 ± 0.09	5.4 ± 2.1	21.6 ± 6.1
2 hour air exposed *	18.5 ± 0.2	7.4 ± 0.08	5.3 ± 1.7	16.1 ± 0.87
P-value H-value	P-value H-value	P-value H-value	P-value H-value	P-value H-value
0.001 15.75	0.001 15.75	0.03 7.36	0.887 0.12	0.328 2.23

3.2 Environmental bacteriological analysis

3.2.1 Colony forming units and the proportion of *Vibrio*-like bacteria

The numbers of colony forming units (CFU) present (May 2011) at the various sample locations within the Marine Biotech Ltd recirculating aquaculture system (RAS) are displayed in Figure 5 (complete data tables are given in Appendix 2). For clarity, all colony forming unit values for TSA (3% NaCl) are displayed in red, those for marine agar in blue and where applicable, those for TCBS agar in green.
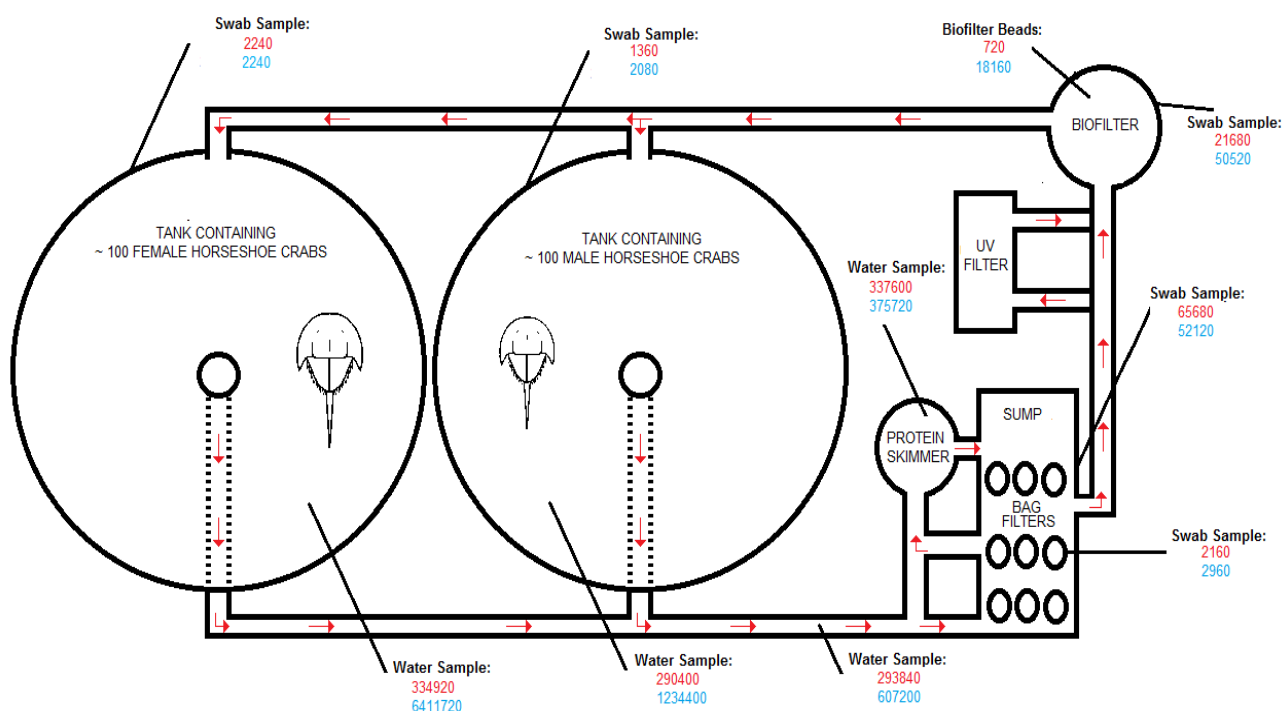
Figure 5. System schematic of the Marine Biotech Limited broodstock RAS, displaying average colony forming units per 1 ml of sample. Growth on TSA (red) and marine agar (blue).

The greatest number of colony forming units within the system was observed in the water samples obtained from the female and male holding tanks (6.41×10^6 & 1.23×10^6 CFU respectively – averaged after 72 hours growth on marine agar). The highest densities grown on TSA (3% NaCl) were observed in the protein skimmer outflow and female tank water samples (at 3.38×10^5 & 3.35×10^5 CFU respectively, after 72 h). CFU values for the haemolymph and animal surface swab samples could not be accurately determined due to fungal contamination or low CFU density, those colonies present, however, were included for API 20 identification.

Estimates of the relative percentage bacteria exhibiting *Vibrio*-like growth characteristics are displayed in Table 5. These percentages varied considerably between samples, ranging from 0.0% in the majority of samples, to 18.2% (protein skimmer outflow).

Table 5. Relative percentage of total CFU exhibiting *Vibrio*-like growth characteristics within the samples obtained from the Marine Biotech Ltd RAS.

Sample location													
Tank water		Tank Biofilm		Outflow pipes		Biofilter		Sump		Animal			
Female	Male	Female	Male	Tank	Protein Skimmer	Biofilm	Beads	Biofilm	Bag filter	Carapace	Gill	Blood	Lesions
4.76	5.26	0.00	0.00	5.32	18.18	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.88

3.2.2 Isolate identification

Forty one visually distinct bacterial isolates were selected from colonies arising from the colony count plates, for identification using the Biomerieux API® 20E™ test system. However, six of these remain unidentified as they could not be resurrected from their original cultures. Only those colonies with differing gross colony morphology were selected as the scope of testing was both time- and resource-limited. The successfully identified isolates, the location from which they were sampled, along with the percentage accuracy of the identification, are displayed in Figures 6 & 7 (full data relating to the identification of these isolates are given in Appendix 3). All forty one isolates were Gram negative. Ten strains were identified from the animal swabs; illustrated in Figure 6. The remaining 25 strains were isolated from the RAS water/swab samples and can be seen in Figure 7 on the next page.
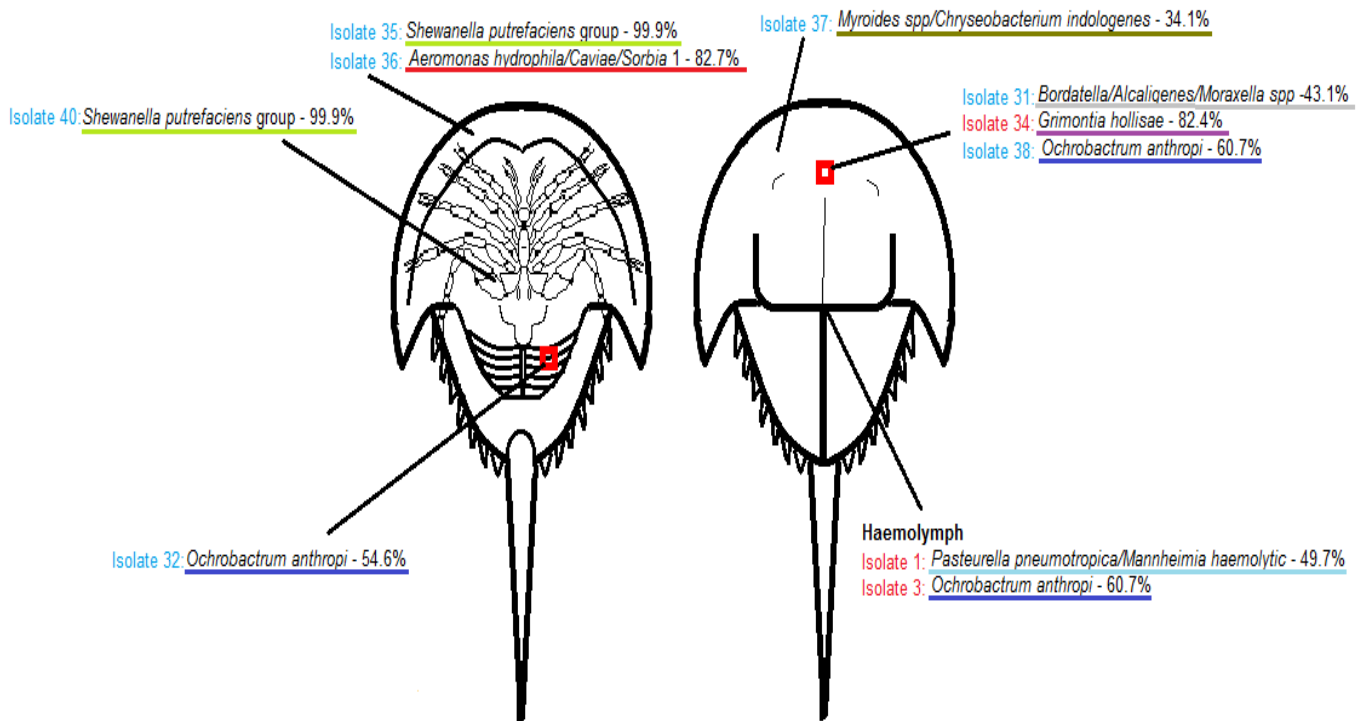

Figure 6. Animal derived isolates with % accuracy and sample location. Isolates 35, 36, 37 & 40 were obtained from lesions at the locations indicated. [Growth media; TSA = red, TCBS agar = green, Marine agar = blue].
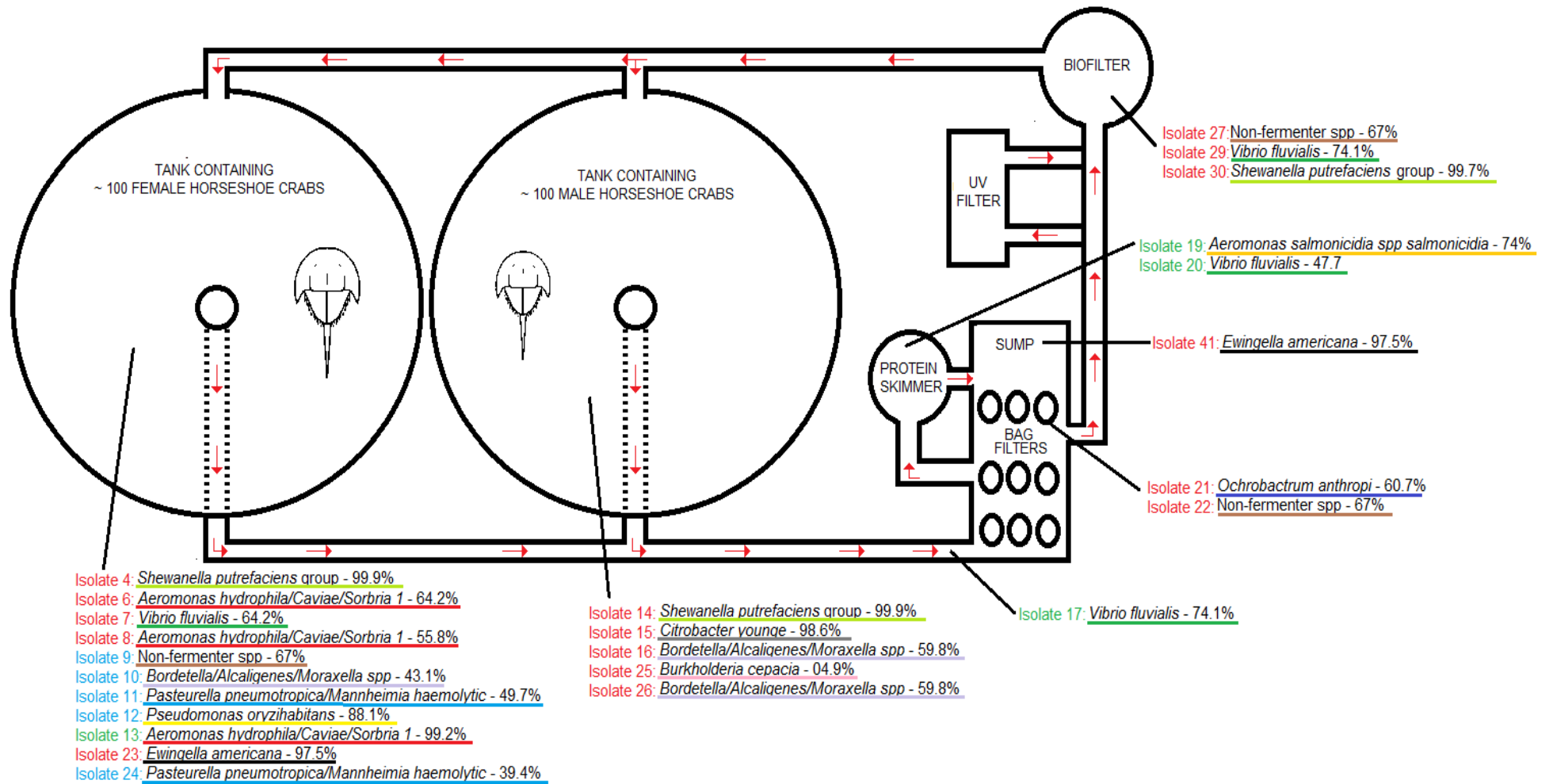
Figure 7. Isolates derived from the Marine Biotech Limited RAS, with % accuracy and sample location.
[Growth media; TSA = red, TCBS agar = green, Marine agar = blue].

4. Discussion

Of the physiological parameters examined, only haemolymph pH exhibited any significant variation as a result of bleeding and air-emersion-induced stress. Numerous studies have established that when exposed to air HSC maintain dissolved haemolymph oxygen levels of only ca. 36% that of their normal (submerged) levels (McDonald *et al*. 1979; Allender *et al*. 2010). Given this and the fact that the animals were observed to cease gill movement upon removal from the holding tanks, it is likely that the decline in pH is a result of respiratory acidosis, due to the accumulation of dissolved CO_2 in the haemolymph, rather than to metabolic acidosis due to the accumulation of acidic end products (such as lactate) from anaerobic metabolism (Defur *et al*, 1980, Mangum, 1986). Similar findings of respiratory acidosis have been reported by Allender *et al.* (2010) following long-term (24 h) emersion of HSCs, and by Burnett and McMahon (1987) following air exposure in certain terrestrial crab species.

The fact that haemolymph pH can be determined both cheaply and efficiently in the field (through the use of commercially available pH paper test strips) makes this parameter an ideal means of assessing air emersion stress in *L. polyphemus*. It would be prudent to monitor and minimise such air emersion stress in *L. polyphemus,* as it has been shown to interfere with the enzymatic and osmoregulatory functions (Caillouet 1968, Prosser 1973, Haupt *et al.* 2006).

The inability to accurately assess haemolymph lactate levels during these trials does not negate the viability of this parameter as a physiological indicator of stress in *L. polyphemus*. The literature indicates that *L. polyphemus* blood lactate concentrations are an order of magnitude lower than those observed in crustacea (Gäde et al., 1986; Allender *et al*. 2010). In addition, *L. polyphemus* has been shown to produce D-lactate through exposure to environmental anoxia rather than L-lactate (Carlsson & Gäde 1986). Although these both have the same chemical formula, they are optical isomers and can behave differently enzymatically. However, the Accutrend analyser did provide above threshold readings for some of the samples, indicating that the low concentration of lactate rather than optical isomerism was responsible. Similarly low values of lactate in stressed HSC were obtained by Allender *at al.* (2010) using another field instrument, a 'point-of-care' analyzer.

Since haemolymph lactate concentration is widely regarded as an excellent indicator of physiological stress in crustaceans (Vermeer, 1987; Taylor & Waldron, 1997; Haupt *et al*. 2006; Ridgway *et al.* 2006) it is a parameter that certainly merits further investigation in *L. polyphemus*. Consequently, it is recommended that any future study should re-examine haemolymph lactate concentration using a more sensitive laboratory assay than provided by the Accutrend portable lactate analyser (as in Ridgway *et al.* 2006).

The data support the conclusion that bled *L. polyphemus* appear fully recovered two weeks post-bleeding, and can tolerate at least two hours air emersion with no apparent long-term detrimental effects.

The greatest bacterial loading observed within the Marine Biotech Ltd recirculating aquaculture system was exhibited by the holding tank containing female *L. polyphemus*. Animal numbers in both holding tanks were approximately equal (*ca*. 100); however females are approximately 20-25% larger than males (Shuster, 1982). This size difference results in a skewing of the biomass distribution within the system, and consequently the

greater biomass present in the female tank may explain the higher bacterial load. A further factor may be the difference in hormone balance between male and female, which can also cause localised variation in bacterial populations. An improvement in overall water quality could be achieved by reducing the system load, either by increasing the volume of the biofilter and/or sump, or via a reduction in system biomass. A more regimented diet and feeding regime would also aid in improving water quality. Any improvements in water quality/ abiotic conditions would likely reduce stress considerably in the animals.

Biofilm (swab) samples contained lower than expected numbers of CFU (Costerton *et al* 1995) likely due to the sampling methodology employed. Given that bacteria present in the biofilm probably have efficient mechanisms of surface attachment, any future trials will require a more vigorous method of homogenisation to better resuspend the sampled microorganisms.

With regard to the bacterial flora of the animals, very few CFU were recovered from the carapace or gill samples, as is often the case in marine arthropods such as *L. polyphemus*. Indeed other species of horseshoe crab (*Tachypleus gigas* and *Carcinoscorpius rotundicauda*) have been shown to demonstrate highly effective biochemical mechanisms to prevent bacterial biofouling (Ismail *et al*, 2011). As anticipated, greater numbers of bacteria were found associated with the various lesions. Very little bacterial growth was observed on the haemolymph plates; however, many of these plates were found to have been contaminated by mould.

The proportion of *Vibrio*-like strains within the system fluctuated between 0% (majority of locations) and 18.2% (protein skimmer outflow water). Given that *Vibrio* species make up a very high proportion of the bacteria found in the marine environment (up to 80% in some cases) (Tsukamoto *et al*, 1993; Lavilla-Pitogo *et al*, 1998) it is unusual to find such low concentrations in a seawater RAS. This may be explained by the exclusive use of artificial seawater at the facility, coupled with a purposefully low water temperature (sub 18°C). It is generally accepted that *Vibrio* spp. are less successful at lower temperatures (Kelly, 1982; Choi & Peters, 1992; Liu *et al*, 2010). For example, the optimal growth temperature for *Vibrio harveyi* and *Vibrio* (*Beneckea*) *vulnificus* is 28°C; also *V.* (*Beneckea*) *vulnificus* is rarely isolated from waters below 20°C (Kelly, 1982; Choi and Peters, 1992; Liu *et al*, 2010).

Of the 35 bacterial strains identified via the API® 20 E™ identification system, only four could potentially behave pathogenically toward *L. polyphemus*. These are *Aeromonas hydrophila/caviae/sobria*, *Aeromonas salmonicida*, *Ewingella americana* and *Vibrio fluvialis*. Two further strains were identified as the potential human pathogens, *Pseudomonas oryzihabitans* and *Pasteurella multocida*. A full review of the species identified from the Marine Biotech Limited facility can be found in the appendices.

Although bacterial loading within the system is not of a level to cause concern at this time the presence of several strains, that could be potentially harmful to humans and *L. polyphemus*, does merit attention. Currently, the animals housed within the system appear well able to tolerate the presence of these strains, however, were conditions to deteriorate these organism could become a significant problem. This issue could be addressed by the relocation of the UV sterilisation unit from its current position to one between the biofilter and the holding tank feeder pipe. Also the effectiveness of such UV treatment is directly proportional to its degree of use. Consequently, its continual usage is recommended for maximum effectiveness in the control of bacterial numbers.

5. <u>Conclusions</u>

o   Haemolymph pH appears to provide an effective and practical physiological indicator for air- exposure-induced stress in adult *L. polyphemus*.

o   THC, haemolymph protein concentration and blood temperature exhibited no statistically significant variation in stressed (air exposed) versus unstressed animals.

o   Haemolymph L-lactate concentrations were, in all but five instances over the trial, below the detection threshold of the Accutrend™ portable lactate analyser. Consequently, further investigation of this parameter is required using a more sensitive assay method before any conclusion can be made regarding its viability as an indicator of stress in *L. polyphemus*.

o   All indicators point to adult *L. polyphemus* being capable of tolerating 2 hours of air exposure (sufficient for bleeding purposes) with no long-term negative effects.

o   Bacterial loading within the various components of the recirculating aquaculture system (RAS) currently employed by Marine Biotech Ltd were deemed, at the time of sampling, within tolerable limits.

o   Of the 35 bacterial strains identified via the API® 20 E™ identification system, only four could potentially behave pathogenically toward *L. polyphemus*. These are *Aeromonas hydrophila/caviae/sobria*, *Aeromonas salmonicida*, *Ewingella americana* and *Vibrio fluvialis*. Two further strains were identified as the potential human pathogens, *Pseudomonas oryzihabitans* and *Pasteurella multocida*.

6. <u>Recommendations</u>

Improvements in husbandry practices

o   The implementation of a carapace lesion monitoring system, via the use of a digital camera, to allow comparison of wounds and lesions over time (i.e. during routine examination or bleeding) as a means of assessing health status and identifying animals that may require isolation and/or treatment.

o   Improved management of the bacterial flora of the Marine Biotech Limited RAS system through the relocation of the UV sterilisation unit from its current position to one between the biofilter and the holding tanks, as well as its continual usage.

o   Improvement of overall water quality and reduction of animal stress, either via an increase in system volume (i.e. size of the biofilter and sump) or a reduction in system biomass, as well as a more regimented diet and feeding regime.

o The introduction of protocols for hand washing after animal handling/contact with culture water and the use of alcohol gel and gloves for staff suffering injuries with the potential to become infected.

Further scientific studies

o Further assessment of haemolymph L-lactate concentration as a potential indicator of stress in *L. polyphemus*.

o Investigation of the potential of metabolic profiling (metabolomics) as a means of examining, in greater detail, the biochemical and physiological impact of stress on *L. polyphemus*.

o Investigation into the cumulative effect/s of repeated air exposure and commercial haemolymph extraction on the health status of captive *L. polyphemus*.

o Identification of an optimum recovery period post bleeding as well as the determination of an optimum blood volume to mass ratio for commercial extraction.

<u>Acknowledgements</u>

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Appendices

Appendix 1

Marine Biotech Limited Recirculating Aquaculture System (RAS)

RAS employed at the Marine Biotech Limited facility near Callander, Scotland. Arrows indicate direction of water flow. Photographs; a) and b) show the filtration system, c) the two holding tanks, d) female animals in their holding tank.

The system comprises two 3.8m diameter, circular, fibreglass holding tanks, protein skimmer, sump with bag filters, UV sterilisation unit and a suspended bead column biofilter. Water exits each holding tank via centrally located drain pipes, passes through the protein skimmer then drains into the sump (via the numerous bag filters). After this the water is pumped into the suspended bead column biofilter, either directly or via the UV sterilisation unit. Water exits the biofilter through the return pipe which subsequently divides to supply both holding tanks.

Culture water is artificial seawater comprising Open Ocean™ mix dissolved in tap water (left to settle for 48h with air stone prior to mixing). Total system volume is approximately 14,000 litres; each tank contains ca. 5700 litres, thereby leaving around 3,600 litres in the pipes and filter system. 15% system volume (ca. 2000 litre) water exchanges occur every 4 - 8 weeks (dependant on ammonia levels).

Each holding tank contains ca. 100 adult *L. polyphemus* (segregated by sex). These are fed a variable diet of sprat, sand eel, squid and mackerel (processed into segments 2-4cm in size) at a rate of 750 – 1000 grams every 3 - 4 days. Abiotic culture conditions are as follows:

- Temperature; 14 - 16°C
- Salinity; 25 - 30 ppt
- Dissolved Oxygen; minimum 70%
- pH; 7.8 - 8.6
- Ammonia and nitrate; low as possible
- Photoperiod; 11 hours (8am – 7pm)

Neutral buffered formalin (NBF) solution (10 %)

Formaldehyde (37-40%)	100 ml
NaH_2PO	44.0 g
Na_2HPO_4 (anhydrous)	6.5 g
Distilled water	900 ml
Mix to dissolve.	

Appendix 2

Bacterial colony counts (complete data)

Sample:	Location:	Thiosulphate Citrate Bile Salts Agar:			Tryptone Soya Agar:			Marine Agar:		
		24hrs:	48hrs:	1 Wk:	24hrs:	48hrs:	1 Wk:	24hrs:	48hrs:	1 Wk:
BB1	Biofilter	0	0	0	0	0	600	0	200	9200
BB2	Bead*	0	0	0	0	120	840	0	80	2700
W4		0	0	0	2880	7120	9880	4320	5600	9160
W4*		0	400	400	3200	8400	12400	4800	9200	436400
W5	'Female'	0	0	0	0	0	2120	0	0	3200
W5*	Tank	0	0	0	0	4400	9600	5600	8800	338400
W6		0	0	0	0	5120	7840	3320	6600	11400
W6*		0	0	0	0	5200	8400	4000	112000	163200
W7		40	40	40	1400	3240	4280	2280	5040	19400
W7*	'Male'	0	0	0	1600	4800	8400	1600	6800	132400
W8	Tank	0	120	120	1480	3720	4520	160	2280	3320
W8*		0	400	400	0	7600	8800	2400	8800	11200
W9		0	0	0	1640	4560	5960	0	3560	7240
W9*		0	0	0	800	6800	11600	2400	6000	11600
W10		0	0	0	100	3560	5680	40	3960	7120
W10*	Tank	0	0	0	2000	3600	6400	1200	2400	3600
W11	Outflow	40	200	200	840	3760	4920	1560	3360	5360
W11*		0	0	0	3200	4400	8400	0	4800	10000
W12		0	40	40	0	4480	6280	240	5160	9800
W12*		0	400	400	0	7600	12400	0	8000	55200
W13		0	160	160	800	5840	7280	1360	4160	6800
W13*	Protein	400	400	400	0	8400	12800	0	4800	10400
W14	Skimmer	40	200	200	0	3480	5720	480	640	5080
W14*	Outflow	400	800	800	0	4400	9600	0	5600	12400
W15		0	120	120	1720	5720	8440	320	3920	9280
W15*		0	0	0	400	3200	6800	5200	4800	12400

CFUs per ml derived from <u>neat</u> and 10 fold dilutions (*) of RAS water ('W') and biofilm bead ('B') samples after 24 hours, 48 hours and 1 week of incubation at 15°C.

Sample:	Location:	Thiosulphate Citrate Bile Salts Agar:			Tryptone Soya Agar:			Marine Agar:		
		24hrs:	48hrs:	1 Wk:	24hrs:	48hrs:	1 Wk:	24hrs:	48hrs:	1 Wk:
S1	Biofilter	0	0	0	0	720	62960	0	0	135360
S2	Tank	0	0	0	0	0	2000	0	0	16160
S3	Biofilm	0	0	0	0	0	80	0	0	0
S4	'Female'	0	0	0	0	40	2200	0	0	1640
S5	Tank	0	0	0	0	40	2720	0	80	2720
S6		0	0	0	0	160	1800	0	0	2440
S7	'Male'	0	0	0	0	120	1400	0	0	960
S8	Tank	0	0	0	0	120	2480	0	0	3920
S9		0	0	0	40	120	200	0	0	1240
S10	Sump	0	0	0	0	200	52320	0	80	50920
S11	Tank	0	0	0	0	0	7800	0	80	19760
S12	Biofilm	0	0	0	0	360	136900	0	80	85720
S13	Bag Filter	40	40	40	0	0	840	0	0	1040
S14	Biofilm	0	0	0	0	0	3280	0	0	5120
S15		0	0	0	0	0	2360	0	0	2680

CFUs per ml derived from RAS swab samples after 24 hours, 48 hours and 1 week of incubation at 15°C.

Sample:	Location:	Thiosulphate Citrate Bile Salts Agar:			Tryptone Soya Agar:			Marine Agar:		
		24hrs:	48hrs:	1 Wk:	24hrs:	48hrs:	1 Wk:	24hrs:	48hrs:	1 Wk:
SC22A	Carapace	0	0	0	0	0	3200	0	40	3320
SC22B	Carapace	0	0	0	0	0	400	0	0	440
SC22C	Carapace	0	0	0	0	80	600	0	0	440
SG22	Gill	0	0	0	0	0	0	0	0	600
SL22	Lesion	0	0	0	0	0	12960	0	1760	15360
SC33	Carapace	0	0	0	0	0	0	0	0	0
SG33	Gill	0	0	0	0	0	40	0	0	40
SC67	Carapace	0	0	0	0	0	0	0	0	200
SG67	Gill	0	0	0	0	0	0	0	0	0
SC104	Carapace	0	0	0	0	0	0	0	0	40
SG104	Gill	0	0	0	0	0	0	0	0	0
SC106	Carapace	0	0	0	0	0	0	0	0	0
SG106	Gill	0	0	0	0	0	0	0	0	0
SL106	Lesion	0	40	80	2360	4560	45520	2720	9160	77320
SC139	Carapace	0	0	0	0	0	0	0	0	0
SG139	Gill	0	0	0	0	0	0	0	0	0
SL183	Carapace	0	0	0	40	1720	52800	0	33040	68400

CFUs per ml derived from *L. Polyphemus* swab samples after 24 hours, 48 hours and 1 week of incubation at 15°C.

Sample:	Thiosulphate Citrate Bile Salts Agar:			Tryptone Soya Agar:			Marine Agar:		
	24hrs:	48hrs:	1 Wk:	24hrs:	48hrs:	1 Wk:	24hrs:	48hrs:	1 Wk:
H22A	0	0	0	0	0	0	0	0	0
H22B	0	0	0	0	0	2	0	0	0
H33A	0	0	0	0	0	0	0	0	0
H33B	0	0	0	0	0	0	0	0	0
H67A	0	0	0	3	0	0	0	0	0
H67B	0	0	0	0	0	26	0	0	0
H104A	0	0	0	0	0	0	0	0	0
H104B	0	0	0	2	1	1	0	0	0
H106A	0	0	0	0	0	0	0	0	0
H106B	0	0	0	0	0	0	0	0	0
H139A	0	0	0	0	0	0	0	0	0
H139B	0	0	0	0	0	0	0	0	0

CFUs derived from haemolymph samples after 24 hours, 48 hours and 1 week of incubation at 15°C.
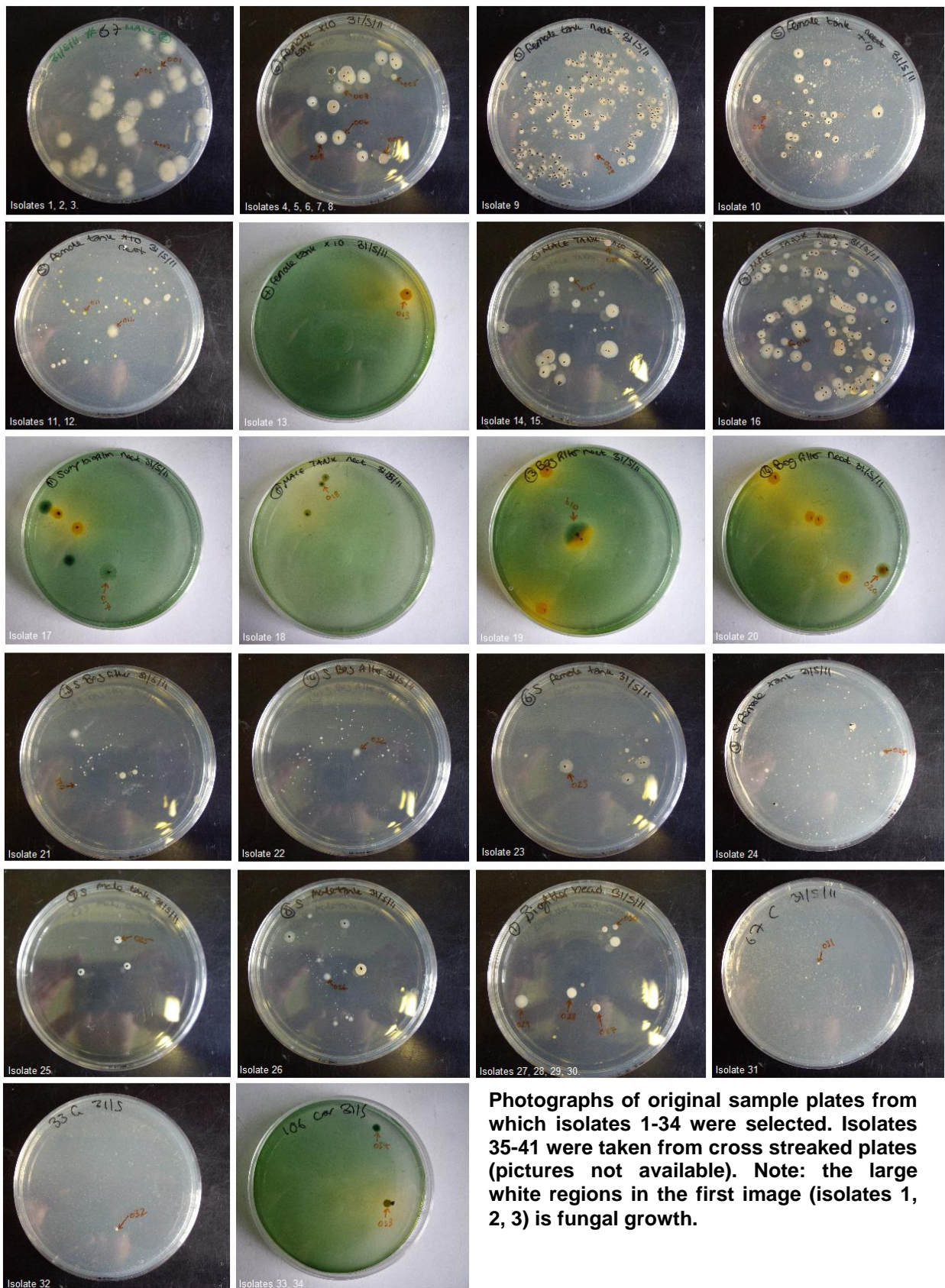
Appendix 3

Isolate identification

Isolate Number:	Sample Number:	Original Location:	Media	Isolate Number:	Sample Number:	Original Location:	Media
1	H22B	Haemolymph (22)	TSA	22	S14	Bag Filter	TSA
2	H22B	Haemolymph (22)	TSA	23	S6	'Female' Tank	TSA
3	H22B	Haemolymph (22)	TSA	24	S5	'Female' Tank	MAR
4	W6*	'Female' Tank	TSA	25	S9	'Male' Tank	TSA
5	W6*	'Female' Tank	TSA	26	S8	'Male' Tank	TSA
6	W6*	'Female' Tank	TSA	27	BB1	Biofilter Bead	TSA
7	W6*	'Female' Tank	TSA	28	BB1	Biofilter Bead	TSA
8	W6*	'Female' Tank	TSA	29	BB1	Biofilter Bead	TSA
9	W6*	'Female' Tank	MAR	30	BB1	Biofilter Bead	TSA
10	W5*	'Female' Tank	MAR	31	SC67	Carapace (67)	MAR
11	W5	'Female' Tank	MAR	32	SG33	Gill (33)	MAR
12	W5	'Female' Tank	MAR	33	SC106	Carapace (106)	TCBS
13	W4*	'Female' Tank	TCBS	34	SC106	Carapace (106)	TCBS
14	W7*	'Male' Tank	TSA	35	SL183	Lesion (183)	MAR
15	W7*	'Male' Tank	TSA	36	SL106	Lesion (106)	MAR
16	W7	'Male' Tank	TSA	37	SL106	Lesion (106)	MAR
17	W11	Tank Outflow	TCBS	38	SC22	Carapace (22)	MAR
18	W8	'Male' tank	TCBS	39	W6*	'Female' Tank	MAR
19	W13	Protein Skimmer	TCBS	40	SL22	Lesion (22)	MAR
20	W14	Protein Skimmer	TSA	41	S10	Sump Biofilm	TSA
21	S15	Bag Filter	TSA				

Isolates selected for API 20E identification, included; sample plate code, sample location and growth media (TSA – red, TCBS – green, marine agar – blue).

Isolate:	Gram type:	Isolate:	Gram type:	Isolate:	Gram type:	Isolate:	Gram type:
1	Negative	12	Negative	22	Negative	32	Negative
3	Negative	13	Negative	23	Negative	34	Negative
4	Negative	14	Negative	24	Negative	35	Negative
6	Negative	15	Negative	25	Negative	36	Negative
7	Negative	16	Negative	26	Negative	37	Negative
8	Negative	17	Negative	27	Negative	38	Negative
9	Negative	19	Negative	29	Negative	40	Negative
10	Negative	20	Negative	30	Negative	41	Negative
11	Negative	21	Negative	31	Negative		

Gram type of isolates; growth media indicated by colour (TSA – red, TCBS – green, marine agar – blue).

Photographs of original sample plates from which isolates 1-34 were selected. Isolates 35-41 were taken from cross streaked plates (pictures not available). Note: the large white regions in the first image (isolates 1, 2, 3) is fungal growth.

Isolate	Strain Identity (Most likely):	%	Strain Identity:	%	Strain Identity:	%	Strain Identity:	%	Strain Identity (Least likely):	%
1	*Pasteurella pneumotropica/ Mannheimia haemolytic*	49.7	*Ochrobactrum anthropi*	42.1						
3	*Ochrobactrum anthropi*	60.7	*Bordetella/ Alcaligenes/ Moraxella spp*	27.8	*Pasteurella pneumotropica/ Mannheimia haemolytia*	8.5				
4	*Shewanella putrefaciens group*	99.7								
6	*Aeromonas hydrophila/ Caviae/Sobria 1*	64.2	*Vibrio fluvalis*	31.4	*Aeromonas hydrophila/ Caviae/Sobria 2*	24.2				
7	*Vibrio fluvialis*	91.0								
8	*Aeromonas hydrophila/ Caviae/Sobria 1*	55.8	*Aeromonas hydrophila/ Caviae/Sobria 2*	23.5	*Vibrio fluvialis*	20.6				
9	Non-fermenter spp.	67.0	*Bordetella/ Alcaligenes/ Moraxella spp*	10.6	*Myroides spp/ Chryseobacterium indologenes*	10.4	*Pseudomonas fluorescens/putida*	7.9		
10	*Bordetella/ Alcaligenes/ Moraxella spp*	43.1	Non-fermenter spp.	30.1	*Ochrobactrum anthropi*	23.8				
11	*Pasteurella pneumotropica/ Mannheimia haemolytic*	49.7	*Ochrobactrum anthropi*	42.1						
12	*Pseudomonas oryzihabitans*	88.1	*Bordetella/Alcaligenes/ Moraxella spp*	5.9	Non-fermenter spp.	3.0	*Ochrobactum anthropi*	2.5		
13	*Aeromonas hydrophillia/ Caviae/Sobria 1*	99.2								
14	*Shewanella putrefaciens group*	99.9								
15	*Citrobacter younge*	98.6								
16	*Bordetella/Alcaligenes/ Moraxella spp*	59.8	Non-fermenter spp.	22.7	*Ochrobactrum anthropi*	13.2	*Pseudomonas fluorescens/putida*	3.6		
17	*Vibrio fluvialis*	74.1	*Aeromonas hydrophillia/ Caviae/Sobria 1*	18.1	*Aeromonas hydrophillia/ Caviae/Sobria 2*	7.6				
19	*Aeromonas salmonicidia spp salmonicidia*	74.0	*Burkholderia cepacia*	11.7	*Chromobacterium violaceum*	4.2	Non-fermenter spp	4.1	*Pseudomonas fluorescens/putida*	2.6
20	*Vibrio fluvialis*	47.7	*Aeromonas hydrophillia/ Caviae/Sobria 1*	45.6	*Aeromonas hydrophillia/ Caviae/Sobria 2*	6.5				
21	*Ochrobactrum anthropi*	60.7	*Bordetella/Alcaligenes/ Moraxella spp*	27.8	*Pasteurella pneumotropica/ Mannheimia haemolytia*	8.5				

Isolate	Strain Identity (Most likely):	%	Strain Identity:	%	Strain Identity:	%	Strain Identity:	%	Strain Identity (Least likely):	%
22	Non-fermenter spp.	67	*Bordetella/ Alcaligenes/ Moraxella spp*	10.6	*Myroides spp/ Chryseobacterium indologenes*	10.4	*Pseudomonas fluorescens/putida*	7.9		
23	*Ewingella americana*	97.5	*Burkholderia cepacia*	1.7						
24	*Pasteurella pneumotropica/ Mannheimia haemolytic*	39.4	*Chyseobacterium meningosepticum*	20.3	*Burkholderia cepacia*	18.9	Non-fermenter spp.	17.7		
25	*Burkholderia cepacia*	94.9								
26	*Bordetella/Alcaligenes/ Moraxella spp*	59.8	Non-fermenter spp.	22.7	*Ochrobactrum anthropi*	13.2	*Pseudomonas fluorescens/ putida*	3.6		
27	Non-fermenter spp.	67	*Bordetella/ Alcaligenes/ Moraxella spp*	10.6	*Myroides spp/ Chryseobacterium indologenes*	10.4	*Pseudomonas fluorescens/putida*	7.9		
29	*Vibrio fluvialis*	74.1	*Aeromonas hydrophila/ Caviae/Sobria 1*	18.1	*Aeromonas hydrophila/ Caviae/Sobria 2*	7.6				
30	*Shewanella putrefaciens group*	99.7								
31	*Bordetella/ Alcaligenes/ Moraxella spp*	43.1	Non-fermenter spp.	30.1	*Ochrobactrum anthropi*	23.8				
32	*Ochrobactrum anthropi*	54.6	*Pasteurella pneumotropica/ Mannheimia haemolytia*	17.9	*Bordetella /Alcaligenes/ Moraxella spp*	13.3	*Myroides spp/ Chryseobacturium indolgenes*	8.5	*Chryseobactrum indologenes*	3.5
34	*Grimontia hollisae*	82.4	*Pasteurella multocida*	12.5						
35	*Shewanella putrefaciens group*	99.9								
36	*Aeromonas hydrophila/ Caviae/Sobria 1*	82.7	*Aeromonas hydrophila/ Caviae/sobria 2*	17						
37	*Myroides spp/ Chryseobacterium indologenes*	34.1	*Aeromonas salmonicida spp salmonicida*	24.5	*Chryseobacterium meningosepticum*	9.5	*Chryseobacterium indologenes*	6.6		
38	*Ochrobactrum anthropi*	54.6	*Pasteurella pneumotropica/ Mannheimia haemolytia*	17.9	*Bordetella/Alcaligenes/ Moraxella spp*	13.3	*Myroides spp/ Chryseobacturium indolgenes*	8.5	*Chryseobactrum indologenes*	3.5
40	*Shewanella putrefaciens group*	99.9								
41	*Ewingella americana*	97.5	*Burkholderia cepacia*	1.7						

Aeromonas salmonicida* spp *salmonicida - is a Gram-negative, non-motile bacterium (Herman, 1968). Causative agent of furunculosis in salmonids; a systematic disease responsible for significant losses within the aquaculture industry (Lund *et al*, 1991; Lutwyche *et al*, 1995). Some vaccines are available to prevent *A. salmonicida* outbreaks, however these are limited to organisms with specific immune systems (Evenberg *et al*, 1988). Virulent strains of *A. salmonicida* possess A-protein; while avirulent strains do not (Ishiguro *et al*, 1981). *A. salmonicida* was initially found within fish, and an assumption was made that these bacterial cells are unable to survive outside of the host, however Allen-Austin *et al* (1984) showed that the strain can survive in freshwater and cause disease outbreaks when salmonids come in contact with contaminated water.

A. salmonicida has been found to cause mortality in *Litopenaeus vannamei* and can be harboured by marine molluscs including the ebonyshell musssel, *Fusconaia ebena* (Buglione *et al*, 2010; Starliper, 2005). Furthermore, the *Aeromonas* group is typically chitinoclastic (Janda, 1985). There is no record of this strain detrimentally affecting *L. polyphemus*; however it is worth noting that one of the two locations that this potential strain was found was within a carapace lesion on animal 106. Given that this strain was also isolated from protein skimmer outflow water (isolate 019, 74%) it is likely free living within the culture system given the lack of an active UV filter.

***Bordetella* spp., *Alcaligenes* spp. & *Moraxella* spp.** - three genera of bacteria between which the API® 20 E™ database is unable to distinguish. Each will be discussed separately in this section.

- *Bordetella* spp: small, Gram negative, highly fastidious, obligate aerobes (Doern, 2000). Some strains are pathogenic to (largely immuno-compromised) humans, including *B. holmesii* (Guthrie *et al*, 2010). Has been found in samples from a freshwater aquaculture facility near London, but has not been associated with any aquatic disease outbreaks (Allen *et al*, 1983). However, *Bordetella bronchiseptica* has been identified as a contributing factor towards the mass mortality in Caspian seals, *Phoca caspica*, in 2000 indicating that some *Bordetella* species can be highly pathogenic (Kuiken *et al*, 2006).

- *Alcaligenes* spp: Strains from this group have been found in a marine RAS containing a variety of ornamental fishes (Raja *et al*, 2010). Some of the *Alcaligenes* strains are pathogenic to humans, including *Achromobacter (Alcaligenes) xylosoxidans* which cause respiratory tract infections in cystic fibrosis sufferers (Liu *et al*, 2002). *Alcaligenes denitrificans* was also isolated from the guts of healthy *L. vannamei* (Franco *et al*, 2010.).

- *Moraxella* spp: a genus of bacteria usually isolated from seawater as well as seaweed and decaying animals (Stolp, 1988). It has recently been isolated from various sampling sites in Mar Piccolo of Taranto (Ionian Sea, Italy) that are important for the farming of *Mytilus galloprovincialis* as well as on the mussels themselves, although no ill effects have been reported (Cavallo *et al*, 2009). *Moraxella* spp. have also been isolated from the hatchery samples of three sturgeon species, but again with no pathogenicity observed (Masouleh *et al*, 2005).

Therefore, it is unlikely that *Bordetella* spp., *Alcaligenes* spp. & *Moraxella* spp. strains are likely to present a problem to the HSCs or staff at Marine Biotech Limited.

Burkholderia cepacia complex - is a group of 17 closely related gram negative strains typically found in water and soil (Leitao *et* al, 2010; Vial *et al*, 2011). Originally named *Pseudomonas capacia*, but molecular analysis lead to the new genus of *Burkholderia* being created (Yabuuchi *et al*, 1992). This group can be pathogenic to humans and causes pneumonia in immuno-compromised individuals, especially those with pre-existing lung problems, particularly cystic fibrosis (Martin *et al*, 2011; Leitao *et al*, 2010). Although the *Burkholderia cepacia* complex is wide ranging, different strains predominate in different areas of the globe (Govan *et al*, 2007).

Caenorhabditis elegans, a nematode, is susceptible to pathogenic *B. cepacia* strains, with high levels of mortality found (Cooper *et al*, 2009). The complex is also pathogenic to mice and rats and some insects (Uhelinger *et al*, 2009). However, there appears to be no record of this strain affecting aquaculture systems or marine arthropods. This strain is not thought to be of great concern to the Marine Biotech Limited systems.

Chromobacterium violaceum - is a facultative anaerobic coccobacillus regularly found in water and soil in the tropics and sub-tropics (Chang *et al*, 2007). *C. violaceum* is produces an antimicrobial called Violacein (Hoshino, 2011). The strain rarely infects humans but on the rare occasion it does, can prove fatal (Chang *et al*, 2007).

This strain was a possible identification for isolate 019; however, the likelihood was only 4.2%, whilst the likelihood of this isolate being *Aeromonas salmonicida* was 74%. This combined with the limited pathogenicity of the strain suggest that its potential occurrence within the Marine Biotech Limited system is not of any concern.

Chryseobacterium indologenes - a non-fermentative, Gram negative bacillus; pathogenicity towards humans is rare although it has been found in diabetic children (Cascio *et al*, 2005). The *Chryseobacterium* group is ubiquitous in nature but is primarily found in soil and water (Kirby *et al*, 2004). *C. indologenes* is only rarely associated with infections in humans and appears to be mostly associated with infections caught by already immuno-compromised patients within a hospital environment or is transferred on internally implanted medical devices (Kirby *et al*, 2004).

There appears to be little or no information available on the occurrence of *C. indologenes* in marine organisms or culture systems and the records of this strain involved with humans suggests that it only causes problems in already immuno-compromised individuals and neonates, therefore it is unlikely that this strain will cause any problems at the Marine Biotech Limited Facility. Furthermore the likelihood of this strain being identified within the system is between 3.5% (isolates 32 and 38) and 6.6% (isolate 37).

Chryseobacterium meningosepticum - has been reclassified as *Elizabethkingia meningosepticum* (Kim *et al*, 2005). *E. meningosepticum* is found within infected wounds in humans as well as being associated with skin ulcers on cod (Grawinski *et al*, 2009). It has also been isolated from uncooked Ark shells, *Scapharca broughtonii*, (Yan *et al*, 2009). This strain is the most pathogenic of the group and has been associated with neonatal meningitis and up to 57% mortality in this condition as well as resulting in many long term post meningitis effects (Kirby *et al*, 2004).

This strain is obviously found within the marine environment and can be potentially pathogenic to both a variety of marine organisms and humans alike. The level of pathogenicity is minimal in adults, therefore should not cause any major problems to the animals or staff at Marine Biotech Limited as long as numbers are kept relatively low.

Citrobacter youngae - was first described by Brenner et al in 1993. *Citrobacter* spp are facultative anaerobic, motile Gram-negative, non-sporeforming bacillus (Borenshtein and Schauer, 2006; Kurtoglu *et al*, 2011). They are members of the Enterobacteriaceae and utilise citrate as a carbon source (Borenshtein and Schauer, 2006). *C. youngae* is considered pathogenic to humans and has been isolated from human blood and stool samples as well as meat scraps (Brenner *et al*, 1993). However, it is thought that immuno-competent adults are unlikely to develop *Citrobacter* infections (Kurtoglu *et al*, 2011). *C. youngae* has been isolated from drinking water/river water samples, whilst other species of *Citrobacter* have been isolated from the cloacal vent of green turtles, *Chelonia mydas*, (Boualam *et al*, 2003; Al-Bahry *et al*, 2011). Yathavamoorthi *et al* (2010) found Citrobacter species within the freshwater prawn, *Macrobrachium rosenbergii*, culture system in India and thought that the high proportion of *Citrobacter* and *Enterobacter* species may have significance to public health and indicate levels of water pollution. This strain is presumed pathogenic, but could be useful as a marker of water quality in the Marine Biotech Limited RAS. It can infect immuno-compromised humans but does not seem to be a major health threat to the *L. polyphemus* or staff members at Marine Biotech Limited.

Ewingella Americana - is the only species of genus *Ewingella* (Grimont *et al*, 1983). *E. americana* has low pathogenic potential but very occasionally infects immuno-compromised patients, causing peritonitis (Pound *et al*, 2007). Most records of *E. americana* suggest that it is found within wounds and sputum of patients already within hospitals (Bear *et al*, 1986), however, there is a record of it possessing chitinolytic properties. This could result in the infection of *L. polyphemus* with wounds

or compromised carapaces (Inglis and Peberdy, 1997). Isolates 23 and 41 were potentially identified as being *E. americana*, (97.5% likelihood).

Grimontia hollisae (formerly ***Vibrio hollisae***) **-** is a member of family *Vibrionaceae* belonging to genus *Grimontia* (Thompson *et al*, 2003).
This strain was isolated on TCBS agar, however, the literature states that *G. hollisae* is unable to grow on TCBS agar (Thompson *et al*, 2003) indicating that this strain identification may be incorrect. The API® 20 E™ database identified the strain as *G. hollise* with an 82.4% likelihood. However, given the inability of this strain to grow TCBS media, isolate 34 is likely to be *Pasteurella multocida* (12.5% likelihood).

Ochrobactrum anthropi - first described in 1988 by Holmes *et al*; and named *anthropi* as most strains were isolated from human clinical specimens. *O. anthropi* are motile, using peritrichous flagella form locomotion. The colonies are typically circular, low convex and smooth; a description which fits with the appearance of isolates 03, 31 and 32, however, isolate 38 was a pale peach colour, suggesting that 038 may not be *O. anthropi.*
This strain is a common component of marine biofilms (Lee *et al*, 2003) and has been found on the spermatophores of the black tiger shrimp, *Penaeus monodon* (Nimrat *et al*, 2008). It is also an infectious agent in Mekong giant catfish, *Pangasianodon gigas* and has been isolated from nematodes in the Caribbean (Purivirojkul *et al,* 2005; Babic *et al*, 2000). Furthermore *O. anthropi* is starting to be recognised as a pathogen of humans, with some infections responsible for life threatening conditions such as endocarditis (Chain *et al*, 2011).
This strain should not necessarily be of concern to Marine Biotech Limited; however, it is important to note that the potential of this strain as a pathogen has only recently emerged (Chain *et al*, 2011).

***Myroides spp*.** – The *Myroides* genus contains various 'aerobic, yellow-pigmented, non-motile, non-fermenting, Gram-negative rods' (Benedetti *et al*, 2011). They are widely distributed but are particularly common in water, with strains such as *Myroides marinus* having been isolated from seawater (Benedetti *et* al, 2011; Sung-Heun *et al*, 2011).
Myroides spp are potentially pathogenic, especially to immuno-compromised patients, however infections have been seen in immunocompetent individuals (Benedetti *et al*, 2011). Infections in animals appear to be rare or not yet investigated. Therefore there appears to be limited threat to the animals and staff at Marine Biotech Limited.

Non-fermenter spp. - Non-fermenter species refers to any of a number of bacteria that are unable to catabolise glucose and subsequently cannot ferment. The test results did not provide the API® 20 E™ database with sufficient data to provide a more accurate identification.

***Pasteurella multocida* -** is a Gram negative coccobacillus and is a common oral bacterium of many animals, including cats and dogs and is the primary cause of infection in animal bite wounds (Weber *et al*, 1984). It can cause cellulitis, abscesses, osteomyelitis, septic arthritis and many other conditions (Weber *et al*, 1984). This strain is also responsible for hemorrhagic septicaemia in cattle, pneumonic pasteurellosis in sheep and goats, fowl cholera in poultry and atrophic rhinitis in pigs and snuffles in rabbits (Ranjan *et al*, 2011). Additionally, *P. multocida* was found to be one of the pathogenic strains responsible for a disease outbreak at the Laboratory of Marine Shrimp, UFSC, Sant Catarina State, Brazil, and has been found to cause mortality in *Litopenaeus vannamei* (Buglione *et al*, 2010).
Although it cannot be assumed that a shrimp pathogen would be harmful to *L. polyphemus*, the wide spread pathogenicity of this strain (which includes mammals, birds and arthropods as well as humans) makes it of concern. This strain is typically an opportunistic infectious agent in humans, gaining entry via open wounds, suggesting that any staff at Marine Biotech Limited with injuries should take precautions to prevent infection.

Pasteurella pneumotropica/Mannheimia haemolytica
- *Pasteurella pneumotropica*: is a short rod shaped bacterium; it is fairly ubiquitous and has been isolated from the sediment of a fish pond in Saudia Arabia (Sasaki *et* al, 2011; Al-Harbi and Uddin, 2006). It is also a strain commonly found associated with upper respiratory tract infections laboratory rats and mice(Sasak *et* al, 2011; Pritchett-Corning *et al*, 2009). The majority of information regarding the pathogenicity of this strain is focused on rodents. However, there appears to be very little, if any, threat to other animal groups. It is felt that this strain should not negatively impact *L.polyphemus*.
- *Mannheimia haemolytica*: is Gram negative bacterium found associated with infections of the upper respiratory tract of ruminants (Frank, 1989). It is responsible for a number of economically important cattle diseases (van Rensburgh *et al*, 2004). There appears to be no record of this strain within aquaculture or non-ruminant species.
P. pneumontropica and *M. Haemolytica* appear to be limited in their pathogenicity to rodents and ruminants, respectively. The highest likelihood of an isolate being either of these strains is 49.7% (isolates 01 and 11). Isolate 01 was obtained from the haemolymph of crab 22 and is likely the result of environmental contamination. Also given the relatively low I.D. accuracy and the strains' exclusivity to rodents and ruminants suggests that this may be an incorrect identification.

Pseudomonas fluorescens/putida - both species are Gram negative and rod-shaped, with *P. fluorescens* possessing multiple flagella (Klinge, 1959). Both of these strains are commonly isolated from soil samples (Cornelis, 2010). However Borges *et* al (2008) found *P. fluorescens* within the biofilter of a recirculating aquaculture system. The highest likelihood for these strains being the correct identification for any of the isolates was 7.9% (isolates 09, 22 and 27). However, it is not thought that these strains would present a problem to *L. polyphemus* or staff at Marine Biotech Limited.

Pseudomonas oryzihabitans - is a non-fementative, Gram negative bacterium, previously found associated with catheter-related infections in humans (Esteban *et al*, 2002). P. oryzihabitans was also found to be the causative agent in an ulcerous lesion on the forearm of a young boy resulting from a bite from an *Octopus vulgaris* (Aigner *et al*, 2011).The strain was determined to be an 88.1% match for isolate 12, a water sample from the 'female' holding tank. Given the resultant infection from the *O. vulgaris* bite it is recommended that staff at the Marine Biotech Limited facility take care to avoid allowing culture water to contact broken skin or mucus membranes (such as the mouth or eyes).
Shewanella putrefaciens group – *S. putrefaciens* is a facultative anaerobe previously isolated from marine environments and is thought to play an important role in the cycling of sulphur and trace metals in marine and freshwater habitats (Perry et al, 1993). The name '*putrefaciens*' is derived from the bacteria's association with purification. *S. putrefaciens* was found to be the dominant strain on *Penaeus vannamei* during cold storage (Zhao et al, 2011) and is considered to be a specific spoilage organism of marine cold-water fish during ice storage (Tryfinopoulou et al, 2007). The I.D. accuracy for *S. putrefaciens* was 99%, providing strong evidence to support that this strain is a component the RAS microflora. Two isolates (35 and 40) identified as *S. putrefaciens* were collected from carapace lesions on individuals 183 (female) and 22 (male). It is potentially of concern that *S. putrefaciens*, a bacteria primarily associated with putrefaction in marine organisms, was found within the lesions of at least two animals at Marine Biotech Ltd.

Vibrio fluvialis - Vibrios are ubiquitous to the marine environment with a corresponding proportion being pathogenic to marine organisms (Tall *et* al, 2003). *V. fluvialis* is a halophillic, slightly curved, rod-shaped, Gram negative bacterium. It has been identified in samples of seawater, animal faeces, human faeces, sewage and seafood products (Igbinosa and Okoh, 2010).
V. fluvialis causes cholera-like diarrhoea in humans and is resistant to many antimicrobial agents (Chowdhury *et al*, 2011). However, records of it pathogenicity in non-human hosts are limited (Tall *et al*, 2003). *V. Fluvialis* has been isolated from Hawaiian green turtles (*Chelonia mydas*) suffering from fibropapilloatosis, however, it was only found in 47% of animals tested suggested that it was not the causative agent (Aguirre *et al*, 1994). Tall *et al* (2003) implicated *V. Fluvialis* -like strains in

limp lobster disease; which causes lethargy, weakness and unresponsiveness to stimuli. Like most of the *Vibrionaceae*, *V. fluvialis* has been demonstrated to be chitinolytic (Osawa ann Koga, 2008). None of the isolates identified as *V. fluvialis* were directly associated with *L. polyphemus*. However, due to its chitinolytic properties and pathogenicity this strain should be viewed as a potential health threat to *L. polyphemus*. More significantly, this strain is known to cause cholera-like symptoms in humans and therefore could pose a health risk to staff at Marine Biotech Limited.

Appendix 4

Final Report Form
University Of Glasgow Innovation Network
First Step Award with Marine Biotech Ltd.

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PART ONE
(This section of the report will be made available for publication on the University of Glasgow website and for use by Scottish Enterprise and The Scottish Government.)

Project Description – max. 500 words – please state whether the original project description goals have been achieved, describe the tangible outcomes, commercial opportunities, and any unforeseen benefits.

The project assessed the effects of a simulated commercial bleeding procedure on several physiological indicators of stress in the American horseshoe crab, *Limulus polyphemus*. The main Indicators utilised were: total haemocyte count (THC), concentrations of haemolymph L-lactate and protein, blood pH and blood temperature. In addition, the bacterial loading and composition of the re-circulating aquaculture system and animal microflora were examined for the presence of potentially pathogenic strains, using both traditional culture methods and the Biomerieux API® 20 E™ bacterial testing system. The main findings obtained were:

- o Haemolymph pH appears to provide an effective and practical physiological indicator for stress induced by air exposure in adult *L. polyphemus*.

- o Adult *L. polyphemus* appear capable of tolerating at least 2 hours of air exposure in association with a bleeding procedure, with no long-term negative effects.

o Bacterial loading within the various components of the recirculating aquaculture system (RAS) currently employed by Marine Biotech Limited was, at the time of sampling, within acceptable limits.

o In total, 35 bacterial strains were identified via the API® 20 E™ testing system, and of these four were potentially pathogenic to L. polyphemus (*Aeromonas hydrophila/ caviae/sobria*, *Aeromonas salmonicida*, *Ewingella americana* and *Vibrio fluvialis*). In addition, two further strains were identified as the potential human pathogens, *Pseudomonas oryzihabitans* and *Pasteurella multocida*.

The project achieved all its original goals. Regarding tangible outcomes, measures have been identified to improve the holding conditions and to optimise husbandry practices for the culture of *L. polyphemus*, at Marine Biotech Ltd's facility. Implementation of these measures would reduce the likelihood of stock loss through environmental stress, bleeding procedures and disease. The main recommendations are:

o Increase control over the bacterial flora in the re-circulating seawater through modification of the recirculating aquaculture system. This could be achieved via the relocation of the UV filter unit from its current position to a position between the biofilter and holding tanks, as well as bringing it into continuous usage. In addition, an increase in biofilter and sump volume, as well as the implementation of a more regimented feeding regime, would also improve overall water quality and consequently reduce animal stress.

o Implement a carapace lesion monitoring system, via the use of digital camera images, to allow comparison of wounds and lesions over time (i.e. during routine examinations/bleeding). Such a system would be a useful indicator of health status and would aid greatly in the identification of animals requiring isolation and treatment.

o Investigate the cumulative effect(s) of repeated air exposure and haemolymph harvesting on the health status of *L. polyphemus*, as well as the determination of an optimum recovery period post-bleeding.

o Investigate the potential of metabolic profiling (metabolomics) as a means of determining in more detail the biochemical and physiological impact of bleeding stress on *L. polyphemus*.

Through implementation of the above recommendations the company could improve the holding conditions for brood stock, thereby reducing the occurrence of disease and mortality. Healthier brood stock typically provides better quality gametes, and these in turn increase the likelihood of the production of viable larvae. Such improvements in production would increase operational efficiency, and may also lead to new commercial opportunities for Marine Biotech Ltd.

PART TWO
(This section of the report is confidential and will not be made available for publication, and will only be made available to project partners.)

SME Contribution – Please detail the contribution made by the SME to the project.

The SME management aided in defining the remit of the project and provided information on areas of concern regarding animal holding conditions, through regular meetings with the scientific team. The SME provided access to their brood stock for experimental purposes, as well as facilities for sampling and limited consumables.

Future Plans – do you plan to take this work forward? Please describe plans for development(s) evolving from this project, has there been any intellectual property generated (or likely to be), have you applied for (or plan to apply for) any research grants to develop this work further?

The academic partner will honour the confidentiality agreement covering the work performed, and will seek permission from the company to present and publish the scientific outcomes of the work, where appropriate.

There is scope to extend this project to investigate further the responses of *L. polyphemus* to commercial bleeding, using metabolic profiling techniques. Such metabolomic analysis would be extremely useful in the determination of an optimum post-bleed recovery protocol.

In addition, the long-term goal of Marine Biotech Limited is the creation of a commercial scale *L. polyphemus* nursery facility. There is a great deal of research potential associated with such an endeavour as larval development has strict requirements with regard to environmental conditions and diet.

Funding for these possible future developments might be sought from KTP, SMART or SPARK awards, or other appropriate industry/science initiatives.