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THE IMMUNE FUNCTION OF DEVELOPMENTAL STAGES OF *MYTILUS EDULIS* AND EFFECTS OF STRESSORS

HELEN ELIZABETH PARRY

Submitted to the University of Wales in fulfilment of the requirements for the Degree of Doctor of Philosophy

University of Wales Swansea

May 2007

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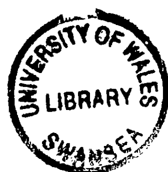
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Summary

The Immune Function Of Developmental Stages Of *Mytilus edulis* And Effects Of Stressors

Helen Elizabeth Parry

The aim of this thesis was to assess immune function in developmental stages of the marine mussel, *Mytilus edulis*. Light and electron microscopy studies were carried out to examine haemocytes of larval, postlarval and adult mussels. The susceptibility of both larval and postlarval mussels to several common marine bacteria were examined. Three species of bacteria that have been linked with disease outbreaks were studied; namely *Vibrio tubiashii*, *Vibrio alginolyticus* and *Alteromonas haloplanktis*. Interactive effects of pollutants (copper and phenanthrene) and food availability on the susceptibility of postlarvae to bacteria was also examined.

In summary, it was found that larval *M. edulis* were more susceptible to the bacteria examined than postlarvae. The susceptibility of postlarvae to bacteria altered with the introduction of stressors. Food deprivation and co-exposure to phenanthrene increased the susceptibility of postlarvae to *V. tubiashii*. However, co-exposure to copper decreased the susceptibility of the postlarvae to *V. tubiashii*. There were shown to be strong seasonal effects that further altered the susceptibility of postlarvae to bacteria, these effects were thought to be due to nutritional status of the postlarvae as opposed to reproductive effects as the postlarvae were reproductively immature. The ontogeny of *M. edulis* haemocytes was examined. Blood cells were not detected in electron micrographs of D-shell larvae but were identified in both *in situ* in postlarval sections processed for electron microscopy as well as in haemolymph samples taken from postlarvae. A previously undescribed blood cell type was reported in postlarvae, lending support to the model put forward by Mix (1976) stating that all mussel haemocytes derive from a single cell line.

DECLARATION

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

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This thesis is the result of my own investigations, except where other wise stated. Other sources are acknowledged by footnotes giving explicit references. A bibliography is appended.

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Contents

Title page i

Summary ii

Declaration and statements iii

Contents iv

List of tables vii

List of figures viii

List of acronyms x

Acknowledgements xi

Chapter 1: Introduction 1

1.1 Mussels 1

1.2 Bivalve Immune System 3

1.2.1 Cellular defences 6

1.2.1.1 Blood cell types 8

1.2.1.2 Phagocytosis 12

1.2.1.3 Encapsulation 16

1.2.1.4 Wound repair and inflammation 18

1.2.2 Humoral defences 18

1.2.2.1 Lysins 19

1.2.2.2. Cytotoxicity reactions 20

1.2.2.3 Respiratory burst 20

1.2.2.4 Agglutinins (with lysins collectively known as opsonins)........ 21

1.2.2.5 Antimicrobial factors 23

1.3 Immunotoxicology 25

1.4 Aims and objectives of the thesis 28

Chapter 2: Microscopy of postlarval haemocytes 29

2.1 Introduction 29

2.2 Materials and methods 31

2.2.1 Light microscopy of postlarval and adult haemocytes 31

2.2.2 *In situ* electron microscopy of postlarval haemocytes 31

2.2.3 Electron microscopy of haemocytes from postlarval blood samples 32

2.3 Results 32

2.3.1 Light microscopy of postlarval and adult haemocytes 32

2.3.2 Electron microscopy of postlarval haemocytes *in situ* 33

2.3.3 Electron microscopy of haemocytes from postlarval blood samples 37

2.4 Discussion 43

Chapter 3: Susceptibility of larvae to bacteria 47

3.1 Introduction 47

3.1.1 Developmental stages of mussels 47

3.1.2 Bacteria associated with mussels 50

3.2 Development of exposure method 53

3.2.1 Bacterial viability in exposure vessels 53

3.2.2 Quantification of larval viability 56

3.3 Electron microscopy of D-shell larvae 64

3.3.1 Materials and methods 64

3.3.2 Results 64

3.3.3 Discussion 66

3.4 Exposure of larvae to bacteria 69

3.4.1 Materials and methods 69

3.4.2 Results 70

3.4.2.1 Trochophore exposures 70

3.4.2.2 D-shell exposures 73

3.4.3 Discussion 78

Chapter 4: Susceptibility of postlarvae to bacteria 84

4.1 Introduction 84

4.2 Exposure of postlarvae to bacteria: development of exposure method 86

3.2.1 Exposure vessel selection 87

3.2.2 Indigenous bacterial contamination 92

4.3 Bacterial exposures 95

3.3.1 Materials and methods 95

3.3.2 Results 96

4.4 Discussion 100

Chapter 5: Effects of food availability on postlarval disease susceptibility 107

5.1 Introduction 107

5.2 Materials and methods 110

5.3 Results 111

5.4 Discussion 116

Chapter 6: Effects of copper on postlarval disease susceptibility 122

6.1 Introduction 122

6.2 Materials and methods 127

6.3 Results 129

6.4 Discussion 133

Chapter 7: Effects of phenanthrene on postlarval disease susceptibility 141

7.1 Introduction 141

7.2 Materials and methods 146

7.3 Results 147

7.4 Discussion 151

Chapter 8: General discussion 157

8.1 General discussion........................ 157

Chapter 9: Bibliography 165

List of Tables

Table 1.1	The immune system of *Mytilus edulis*	7
Table 1.2	Morphological classification of bivalve haemocytes	9
Table 3.1	Criteria for the classifying shellfish harvesting areas	51
Table 3.2	Results of bacterial viability in exposure vessels containing Marine Broth after 24 hours	55
Table 4.1	Comparison of the levels of mortality (percentage) in experiments run for 7 days with or without a depuration period after antibiotic treatment	96
Table 6.1	Metallothionein expression in metal exposed bivalves	135

List of Figures

Figure 1.1	Summary of the stages associated with phagocytosis of foreign particles by haemocytes........	14
Figure 2.1	Postlarval *M. edulis* haemocytes, differentially stained using Wrights stain...	34
Figure 2.2	The occurrence of blood cell types in differentially-stained haemolymph samples taken from postlarvae and adult *M. edulis* ranging from 0.73 to 2.67 cm in shell length........	35
Figure 2.3	TEM of *M. edulis* postlarval haemocytes in situ, 5,000 x magnification. Scale bar = 5µm........	36
Figure 2.4	TEM of *M. edulis* postlarval tissues showing agranular haemocyte in situ, 5,000 x magnification. Scale Bar = 5µm........	36
Figure 2.5A-D	Adult *M.edulis* haemocytes, ultrathin sections stained using lead citrate and uranyl acetate........	38
Figure 2.5E-H	Adult *M.edulis* haemocytes, ultrathin sections stained using lead citrate and uranyl acetate........	39
Figure 2.5I-L	Postlarval *M.edulis* haemocytes, ultrathin sections stained using lead citrate and uranyl acetate........	40
Figure 2.5M-P	Postlarval *M.edulis* haemocytes, ultrathin sections stained using lead citrate and uranyl acetate........	41
Figure 2.5Q-T	Postlarval *M.edulis* haemocytes, ultrathin sections stained using lead citrate and uranyl acetate........	42
Figure 3.1	Light micrograph of D-shell larva exposed to Eosin Y solution	61
Figure 3.2	Light micrograph of a trochophore larva exposed to Eosin Y solution	61
Figure 3.3	Light micrograph of D-shell larvae exposed to fluorescein diacetate	62
Figure 3.4	Light micrograph of live D-shell larvae	62
Figure 3.5	Light micrograph of fixed D-shell larvae	63
Figure 3.6	Composite TEM of 48h D-shell larva	65
Figure 3.7	Trochophore larval viability after 48 hour exposure to *Vibrio tubiashii*	72
Figure 3.8	Trochophore larval viability after 96 hour exposure to *Vibrio alginolyticus* ..	72
Figure 3.9	D-shell viability after 48 hour exposure to *Vibrio tubiashii*	76
Figure 3.10	D-shell viability after 5 day exposure to *Vibrio tubiashii*	76
Figure 3.11	D-shell viability after 5 day exposure to *Vibrio tubiashii*	77
Figure 3.12	D-shell viability after 48 hour exposure to *Vibrio alginolyticus*	77
Figure 4.1	Tissue culture vessel postlarval exposure equipment	89
Figure 4.2	Tissue culture vessel postlarval exposure equipment: detail showing hypodermic needle entry	89
Figure 4.3	Biopsy box postlarval exposure equipment	90
Figure 4.4	Postlarval viability after 4 day exposure, using tissue culture vessel method, to *Vibrio tubiashii*	91
Figure 4.5	Postlarval viability after 7 day exposure, using biopsy box method, to *Vibrio tubiashii*	91
Figure 4.6	TCBS agar plates following exposure to postlarvae	93
Figure 4.7	Postlarval viability after 7 day exposure (01/11/99) to *Vibrio tubiashii*	97
Figure 4.8	Postlarval viability after 7 day exposure (21/03/00) to *Vibrio tubiashii*	97
Figure 4.9	Postlarval viability after 7 day exposure (04/04/00) to *Vibrio alginolyticus* ..	99
Figure 4.10	Postlarval viability after 7 day exposure (18/04/00) to *Alteromonas haloplanktis*	99

Figure 5.1	Postlarval viability after 7 day exposure (20/12/99) to *Vibrio tubiashii*	112
Figure 5.2	Postlarval viability after 7 day exposure (21/02/00) to *Vibrio tubiashii*	112
Figure 5.3	Postlarval viability after 7 day exposure (07/03/00) to *Vibrio tubiashii*	113
Figure 5.4	Postlarval viability after 7 day exposure (21/02/00) to *Vibrio alginolyticus* ..	113
Figure 5.5	Postlarval viability after 7 day exposure (21/02/00) to *Alteromonas haloplanktis*	115
Figure 6.1	Postlarval viability after 7 day exposure (27/9/00) to *Vibrio tubiashii* and 0.02ppm copper........	130
Figure 6.2	Postlarval viability after 7 day exposure (10/10/00) to *Vibrio tubiashii* and 0.02ppm copper........	130
Figure 6.3	Postlarval viability after 7 day exposure (18/05/01) to *Vibrio tubiashii* and 0.02ppm copper........	132
Figure 6.4	Postlarval viability after 7 day exposure (07/06/01) to *Vibrio tubiashii* and 0.02ppm copper........	132
Figure 7.1	Postlarval viability after 7 day exposure (26/10/2000) to *Vibrio tubiashii* and 0.2ppm phenanthrene........	149
Figure 7.2	Postlarval viability after 7 day exposure (04/05/01) to *Vibrio tubiashii* and 0.2ppm phenanthrene........	149
Figure 7.3	Postlarval viability after 7 day exposure (19/06/01) to *Vibrio tubiashii* and 0.2ppm phenanthrene........	150

List of Acronyms

CFU	Colony forming units
ECP	Extra-cellular products
NCIMB	National Collections of Industrial and Marine Bacteria
PAH	Polycyclic aromatic hydrocarbon
PBS	Phosphate buffered solution
TEM	Transmission Electron Micrograph
TCBS	Thiosulfite citrate bile sucrose
UV	Ultra-violet

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1. Introduction

1.1 Mussels

The blue mussel, *Mytilus edulis*, is a member of the Phylum Mollusca and belongs to the Class Bivalvia. Molluscs are found in marine, freshwater and terrestrial environments and, in terms of numbers of species, are second only to the arthropods (Fish & Fish, 1989). As the name implies, bivalve molluscs have a shell comprising of two valves, which are held together by an elastic ligament, the adductor muscle. Bivalves are common members of the intertidal fauna (Fish & Fish, 1989) and tend to occur in high densities. They are grouped into a large number of families with mussels belonging to the Family Mytilidae.

Bivalves, such as mussels, are dominant members of coastal and estuarine communities and have a wide geographical distribution (Widdows & Donkin, 1992). The Mytilidae are a dominant component of rocky shore communities in cooler waters of the northern and southern hemispheres, with the blue mussel, *M. edulis,* being found from mild subtropical regions to the Arctic (Gosling, 2003). *M. edulis* is widespread and common in northwest Europe where it is found attached to hard substrata from the middle shore to the shallow sublittoral. Physiological intolerance to temperature extremes and desiccation represent the most important factors in determining the upper limits of *Mytilus* populations in intertidal sites (Seed & Suchanek, 1992). Growth of animals is restricted at the upper limits due to reduced feeding times. The lower limits are under strong influence from biological factors, especially predators such as starfish, crabs and gastropods. Their distribution may also be controlled by physical factors such as burial in sand. *M. edulis* is also found in estuaries and will tolerate salinities down to 4-5% (Fish & Fish, 1989).

Mussels and other bivalve molluscs are filter feeders, extracting particulate food from the water that surrounds them. Food is collected and processed by the gills, which have become greatly enlarged (Gosling, 2003). Water enters through the inhalant siphon, circulates through the mantle cavity passing over the gills where fine particles, such as phytoplankton, are filtered out before being expelled through the exhalent siphon.

Mussels settle on a wide variety of substrates, from rocks and pebbles to shells and wood, provided that the substrate is firm enough for secure anchorage. They will also settle on manmade structures such as dock pilings, oil platforms and the hulls of boats. Mussels attach to the substrata via byssus threads, a bundle of tough threads of tanned protein (Gosling, 2003), that are secreted by a gland in their foot providing extremely strong anchorage. In suitable habitats, mussels tend to form large dense beds; newly settled individuals known as spat are often removed from these beds and cultivated, removing the need for hatchery production. The mussels' natural propensity to live in dense beds in the wild makes it readily adaptable to the high population densities necessary for an economically viable farming system (Hickman, 1992). The increase in farming of molluscs and crustaceans to meet the increasing consumer requirements is also driving the increased interest in invertebrate immunology. The spatially intensive nature of this farming frequently leads to outbreaks of disease, it is now recognised that an understanding of the immune function of the stock would assist in preventing and overcoming these outbreaks.

Further interest in mussels has been sparked by the increase in their aquaculture. For centuries, mussels, mainly *M. edulis* and *Mytilus galloprovincialis*, have been cultured on the coasts of the Netherlands, France and Spain. About 350BC Aristotle mentions the cultivation of oysters in Greece, and Pliny describes commercial holding ponds for oysters near Naples, Italy around 100BC (Gosling, 2003). Methods of growing shellfish range from bottom culture with dredge harvesting in the Netherlands to intertidal bouchot systems in France and off-bottom raft culture in Spain (Hickman, 1992). There are two main categories of aquaculture, the first being extensive aquaculture where animals can be cultivated by allowing individuals to feed on suspended particulate matter, or prey naturally present in their habitats. In contrast, intensive aquaculture provides artificial diets for organisms cultured throughout their entire life cycle, as seen in fish farming (Gosling, 2003).

Bivalve culture is an example of extensive aquaculture. Mussel spat are generally collected from the wild whereas oyster spat may be raised from broodstock in a hatchery. The spat are then grown on to marketable size in cages or on ropes, feeding on the seston, suspended particulate matter, present in seawater. The number of

bivalve species being cultured is increasing annually with more and more developing countries becoming involved (Gosling, 2003). In 1989, production of bivalves from fisheries and aquaculture was over 3.2 million metric tonnes worldwide, by 1999 this had risen to over 8.8 million tonnes with a monetary value of over $9.3 billion (FAO, 2001). China is by far the biggest contributor, producing 6.77 million of this total 8.8 million tonnes. In the UK, molluscan shellfish production was around 28,000 tonnes in 2004 (DEFRA, 2004). The production of mussels in Scotland has risen from 708 tonnes in 1993 to 3236 tonnes in 2002, an increase of more than 4 fold (Scottish shellfish farm production survey, 2002).

1.2 *Bivalve immune system*

In order to survive, all animals have evolved some kind of response, mediated by the immune system, to discriminate between self and non-self. Once material has been recognised as foreign to the animal, the immune system then acts to neutralise, eliminate or metabolise non-self without destroying self.

The first lines of defence against foreign agents are the physicochemical barriers (or constitutive mechanisms of defence), which, when intact are almost impenetrable to microorganisms. These physiochemical barriers include the tests and shells that are seen, for example, in insects and molluscs, as well as the mucus produced by annelid worms and many other invertebrates. Cilia and mucus provide a physical barrier on the more susceptible membranes lining the inner surfaces of the body, acting as a protective barrier to prevent the adhesion of pathogens to the cell walls.

If a pathogen does enter the body then the immune system will respond. In 1882, Élie Metschnikoff became one of the first scientists to study the immune response of invertebrates, piercing the larva of the common starfish with a rose thorn. The larva was transparent, allowing examination using a microscope, which revealed that the rose thorn had been surrounded by mobile cells (Metschnikoff, 1883). Metschnikoff called these mobile cells, phagocytes, from Greek words meaning "devouring cells". In 1908, Metschnikoff received jointly, with Paul Ehrlich, the Nobel Prize for his studies of cellular defences in mammals and invertebrates. Ehrlich worked on the side chain theory, which stated that cells in the immune system released "side-chains" (now called antibodies) which were receptors that recognise the foreign molecule (the antigen)

(Ehrlich, 1900). When a body is infected with a pathogen, the resultant antigens stimulate the release of side chains from the immune cell. The two scientists, however, belonged to opposing camps supporting what were then considered mutually exclusive theories. Ehrlich worked with Robert Koch and Emil von Behring on "The Humoral Theory" which, stated that a soluble substance in the blood was the essential unit of immunity. The opposing Metschnikoff camp challenged this view of humoral immunity and supported "The Cellular Theory" which stated that cells, not a soluble substance, were the principal units of immunity. We now know that both cellular and humoral arms of the immune response are critical in resisting and fighting infection, with the immune serum acting together with the phagocytic cells to increase phagocytosis and to mediate other immune reactions.

The central role of the immune system is recognition, recognising self and discriminating against non-self elements, against which a response is mounted for destruction and elimination. The immune response involves cellular and humoral components that can be separated into specific and non-specific responses:

- The non-specific immune response (innate immunity) is generally mediated by phagocytes with the ability to recognise foreign material non-specifically, and has a very limited or no memory component.
- The specific immune response (adaptive immunity) is mediated by lymphocytes and macrophages. It is directed against, and specific for, an eliciting agent (antigen), and has a strong memory component.

The adaptive immune system is able to recognize differences between molecular patterns of pathogens in a highly specific manner and to raise antibodies and cellular reactions against these antigens. In contrast, the innate immune system relies on the detection of patterns which are common to a large range of pathogens. Both systems enable protection, but only adaptive immunity leads to long-term immune memory. With innate defence, each phagocytic cell or humoral factor could respond to a range of different antigens. In adaptive immune responses, we see the development of cells and antibodies that are each only able to target one, and only one, antigen. So there are lymphocytes that only target infected or dying self cells. These lymphocytes and other

cells produce cytokines that mediate these processes. Certain antibody classes only target bacteria while other classes specialise in defending against parasites. This has required the expansion of the defence system and the use of many more cells to ensure that all antigens can be recognized by each individual. Different parts of the adaptive immune system therefore have different functions.

Invertebrates do not posses lymphocytes or any other type of cell capable of adaptively producing molecules directed against a specific invader. They lack immunoglobulins, T cell receptors and a major histocompatibility complex so do not exhibit immune memory in the strict sense. Recently, however, evidence has been emerging pointing to the existence of an adaptive immune system, with immunological memory, in invertebrates such as the shrimp *Penaeus vannamei* (Alabi et al., 2000). This research is in its early stages and the evidence is still fairly limited and controversial. Kurtz and Franz (2003) have reported the presence of an invertebrate defence system that may be capable of specific memory. They found that the success of reinfection of the marine copepod, *Macrocyclops albidus*, by a parasitic tapeworm, *Schistocephalus solidus* was dependant on the antigenic resemblance between consecutively encountered parasites. Prior exposure to related (sibling) parasites resulted in less secondary infection than occurred after exposure to unrelated parasites. They did not investigate the mechanism for this species specific memory but postulated that it may be a result of binding by lectins. Memory has also been reported in the shrimp, *Penaeus japonicus,* challenged twice by the same fungal constitutive antigens. The shrimps mounted a 5% increase in haemocyte proliferation rate after the second fungal antigenic challenge (Arala-Chaves & Sequeira, 2000). The mealworm beetle, *Tenebrio molitor*, has also been shown to possess a form of adaptive immunity, in that it exhibits a long-lasting immune response that provides increased resistance to later infections (Moret & Siva-Jothy, 2003). Moret and Siva-Jothy (2003) experimentally mimicked a primary immune insult (pre-challenge) by dosing mealworm larvae with LPS. LPS, lipopolysaccharides, are highly immunogenic, non-pathogenic surface molecules that are a major constituent of the cell walls of Gram negative bacteria, in this case the LPS was derived from *E. coli.* The larvae were subsequently exposed (4 or 7 days post pre-challenge) to the entomopathogenic fungus *Metarhizium anisopliae.* The LPS-treated larvae showed better survival to fungal infection, this enhanced resistance to infection was correlated

with elevated antimicrobial activity in the haemolymph for the period of the fungal inoculations (Moret & Siva-Jothy, 2003).

As invertebrates have not yet been shown conclusively to posses any capacity for specific immune response, the invertebrate immune system has often been regarded as second-rate to that of the vertebrates and hence progress in research on invertebrate immunity has been much slower than that on vertebrates (Ratcliffe, 1985). They are also considered to be short-lived and hence not in need of a complex, efficient immune system. The fresh water pearl mussel, *Margaritifera margaritifera*, however, lives for up to 120 years, several times longer than many vertebrates, the shrew *Sorex areneus*, for example, only lives for one year. Thus, from the viewpoint of the species survival, the defence system of invertebrates should be no less efficient than that of the vertebrates (Yakovleva et al., 2001). Recent studies on vertebrate immunity have highlighted the crucial role of the innate system in signalling-acquired effector mechanisms with information for the nature of alien antigens and for the induction and direction of adaptive immune responses (Rinkevich, 1999). This, along with the discovery of novel defence reactions and compounds, has led to the reassessment of the classification of innate immunity as "an evolutionary rudiment whose only function is to contain the infection until the real, or adaptive immune response can be induced" (Rinkevich, 1999).

Although both cellular and humoral defences interact to maintain efficient elimination of foreign bodies and immune function, for ease the two systems will now be considered individually. An overview of the immune system of *Mytilus edulis* is given in Table 1.1.

1.2.1 Cellular defences

The blood cells of invertebrates play a prominent role in the defence against potential pathogens. The main mechanisms of neutralization and elimination of foreign bodies in molluscs are phagocytosis and encapsulation, serving to engulf the foreign body.

Bivalves possess an open circulatory system, which includes a heart, comprising a single muscular ventricle and two thinner walled auricles. The open system bathes the

	Immune response	Mediated by:	Reference:
CELLULAR DEFENSES	Phagocytosis	Large granular cells	Pipe et al., 1997
	Encapsulation	Large granular cells	Ratcliffe et al., 1985
	Wound repair/inflammation	All haemocytes	Bubel et al., 1977
HUMORAL DEFENSES	Lysins	Lysosomal enzymes	Moore & Lowe, 1977
	Cytotoxic reactions	Cytotoxic molecules	Wittke & Renwrantz, 1984
	Respiratory burst	Superoxide anion; hydrogen peroxide	Pipe, 1992
	Aglutinnins	Lectins	Renwrantz & Stahmer, 1983
	Antimicrobial factors	Antibacterial and antifungal peptides	Hubert et al., 1997.

Table 1.1. The immune system of *Mytilus edulis*.

tissues continuously in haemolymph. There are no capillaries joining arterial and venous vessels; instead, haemolymph pumped from the heart into the arteries seeps into sinuses (Cheng, 1981). The haemolymph then returns from the sinuses to the veins where it is carried to the kidneys for purification. In *Mytilus,* some of the haemolymph from the kidney network enters the gills travelling over them before returning back to the kidneys (Gosling, 2003). The haemolymph then returns back to the auricles of the heart. The haemolymph of most bivalves contains cells that float in a colourless plasma, although some bivalves, such as *Scapharca inaequivalvis*, do possess cells with respiratory pigments (Holden et al., 1994). The haemocytes are not confined to the haemolymph system but move freely out of the sinuses into surrounding connective tissue, the mantle cavity and gut lumen (Gosling, 2003).

1.2.1.1 Blood cell types

Most invertebrates have free circulating unpigmented blood cells known as haemocytes or coelomocytes depending on the nature of the body cavity (Ratcliffe et al., 1985). They are of course some exceptions, such as *Scapharca inaequivalvis*, which have circulating red blood cells containing a form of haemoglobin (Holden et al., 1994). These red blood cells function in the same way as vertebrate red blood cells, carrying oxygen around the body. Red blood cells in invertebrates are not restricted to bivalve molluscs, they are found in organisms ranging from sea cucumbers (*Caudina arenicola*), brine shrimp (*Artemia salina*) to the midge (*Chironomus thummi thummi*), and gene sequences that code for red blood cells are even found in yeast and bacteria (Weber & Vinogradov, 2001).

Work on classification of the blood cells of invertebrates may have begun in the 1800's but only in the mid 1900's were the blood cells definitively classified into types on the basis of their morphology as determined using electron microscopy (Table 1.2). Confusion still arises regarding nomenclature of these blood cells as they are sometimes referred to using vertebrate terms such as granulocyte and lymphocyte, or referred to as amoebocytes, macrophages and phagocytes. In general, the differences in terminology are due to different researchers using their own descriptive terminology. Blood cells can be classified on the basis of their function or on the basis of their morphology. In the case of morphology, cells are split into 2 forms, granular and agranular (or hyaline), agranulocytes containing few, if any, cytoplasmic granules, and granulocytes, showing

BIVALVE SPECIES	HAEMOCYTES REPORTED	REFERENCE:
Lamellibranchiate molluscs	Round granular cell Nucleated cell devoid of contents Minute globules filled with opalescent fluid	Williams, 1852
	Agranulocytes Granulocytes	Feng et al., 1977
Crassostrea gigas	Granulocytes Hyalinocytes	Bachère et al., 1988
	Granulocytes Agranulocytes Vesicular cells	Chang et al., 2005
Crassostrea virginica	Hyalinocytes Granulocytes Fibrocytes	Foley & Cheng, 1972
	Granulocytes Hyalinocytes	Cheng, 1975
	Large granulocytes Small granulocytes Agranulocytes	Ashton-Alcox & Ford, 1998
Mercenaria mercenaria	Granulocytes Hyalinocytes	Cheng, 1975
	Agranulocytes Small granulocytes Large granulocytes	Moore & Eble, 1977
Mytilus edulis	Granule cells Nucleated cells	Wharton-Jones, 1846a,b,c
	Lymphocytes Macrophages Granulocytes	Moore & Lowe, 1977
	Granulocytes Agranulocytes	Rasmussen et al., 1985
	Agranular cells Granular cells containing large granules Granular cells containing small granules	Pipe, 1990
Scrobicularia plana	Granular haemocytes Agranular haemocyes	Wootton& Pipe, 2003

Table 1.2. Morphological classification of bivalve haemocytes.

numerous granules of different sizes (Feng et al., 1977). These two basic cell types can be further subdivided on the basis of staining and biochemical properties (Pipe et al., 1997). If blood cells are classified according to their function then there are five main groups: progenitor cells, phagocytic cells, haemostatic cells, nutritive cells and pigmented cells (Ratcliffe et al., 1985).

Molluscs have both agranular and granular cells. There are apparently differences in the roles that granular and agranular cells play in any organism, and those roles are not necessarily the same for each species (Fisher, 1986). Indeed, there is often conflict between researchers as to the types of cells present within specific species let alone the roles of these cells. Cheng (1975) studied the haemocytes of *Mercenaria mercenaria* and concluded that there were two cell types, granulocytes and hyalinocytes. However, Moore and Eble (1977) studied the same organism and concluded that there were three distinct haemocyte types: agranulocytes, small granulocytes and large granulocytes. Conflicting findings are not restricted to different research groups, perceptions of cell types can alter within research groups over a period of time, especially if different methods are used to investigate the cells. In 1972, Foley and Cheng recognised three cell types in the oyster, *Crassostrea virginica*: large cells designated as either granulocytes or fibrocytes and smaller agranular cells designated hyalinocytes. However, further examination of the fibrocytes using electron microscopy resulted in the discovery that fibrocytes are actually granulocytes that have undergone degranulation and are carrying out intracellular digestion (Cheng, 1975). The study and characterisation of blood cells from bivalve molluscs is still an active area of research, although most researchers now acknowledge that classification on the basis of light microscopy alone is unsatisfactory generating equivocal results. Wootton and Pipe (2003) have recently studied the blood cells of the estuarine bivalve mollusc *Scrobicularia plana* using both light and electron microscopy. *Scrobicularia* were found to have three types of haemocyte: eosinophilic granular haemocytes, basophilic granular haemocytes and basophilic agranular haemocytes. The terms basophilic and eosinophilic simply relate to staining properties: basophilic substances stain readily with basic dyes whereas eosinophilic substances stain readily with eosin or other acid dyes.

The haemocytes of the marine mussel *Mytilus edulis* have been well studied. Moore and Lowe (1977) recognised three cell types in *M.edulis*: small basophilic cells

(hyalinocytes) or lymphocytes as they termed them, larger basophilic cells or macrophages, and eosinophilic granular haemocytes or granulocytes. Moore and Lowe (1977) also studied the enzyme cytochemistry of the blood cells looking for the presence of lysosomal hydrolases such as indoxyl esterase and β-glucuronidase. These enzymes are known to be important in the destruction of ingested non-self material (Wootton et al., 2003a) and were found in the basophilic cells of *M.edulis* which affirms their roles as macrophages. Pipe (1990a) also studied the haemocytes of *M.edulis* using electron microscopy to examine the differential binding of lectins. In doing so, he confirmed the findings of Moore and Lowe (1977), classifying the haemocytes into three categories: hyalinocytes and two forms of granulocyte differentiated by the size of the granules. The large granule cells are eosinophilic whilst the small granule cells and agranular cells are basophilic (Pipe,1990a). The large granule eosinophilic cells are capable of phagocytosis, release of reactive oxygen metabolites and have the potential for the release of degradative enzymes, however specific functions for the agranular and basophilic cells are unclear (Pipe, 1990a).

Moore and Lowe (1977) suggest that, in *M. edulis,* the small basophilic cells (hyalinocytes) are in fact stem cells that are part of a developmental series of basophilic cells, the small basophilic cells mature into the larger basophilic macrophages. This pattern of development is supported by the gradual appearance of the lysosomal enzymes as the basophilic cells increase in size. There have been several attempts to establish models of haemocyte generation and maturation, however, there is not a great deal of information on cytogenesis in bivalves (Auffret, 1988). Cheng (1981) proposed that there are two distinct cell lines, hyalinocytes and granular haemocytes, each originating from a unique stem cell. The hyalinocytes originate from a hyalinoblast and mature into largely agranular cells with a large nuclear to cytoplasmic ratio (basophilic). Granuloblasts develop into the granulocytes which are large actively phagocytic cells containing numerous acidophilic, cytoplasmic granules (eosinophilic). In contrast, Mix (1976) proposed that haemocytes originate from one common stem cell, the leucoblast, which develops into the hyalinocytes. The hyalinocytes are considered as an undifferentiated cell that can then proliferate into other cell types.

1.2.1.2 Phagocytosis

The primary mechanism of internal defence for invertebrates is phagocytosis of small particulates while larger foreign bodies are encapsulated. From the viewpoint of evolution, phagocytosis is probably the oldest of immune response, traceable even to the protozoans so that phagocytic cells are ubiquitous (Cooper, 2001).

Since Metschnikoff's initial work on phagocytosis (Metschnikoff, 1883), many investigators have described phagocytosis as a defence mechanism of invertebrates. A vast range of microorganisms including bacteria, viruses, fungi and protozoans have been shown, both *in vivo* and *in vitro*, to be phagocytosed. The mechanisms of phagocytosis in oysters, *Crassostrea* (*Ostrea*) *virginica*, were first delineated by Stauber in 1950. This early work formed the basis of our understanding of the mechanisms of phagocytosis, with subsequent work elucidating the finer details such as the mechanisms by which the particles clumped and the phagocytic cells migrated towards the foreign particle.

All haemocytes of *Crassostrea virginica* and *Mercenaria mercenaria* have been shown to be phagocytic, however, the granulocytes were the most active (Foley & Cheng, 1975). The percentage of cells involved in phagocytosis can be affected by a number of factors such as season, temperature, pH and salinity (Parry & Pipe, 2004). In addition, the number of circulating haemocytes may rise and fall due to a stimulation of their release from tissues into circulation and vice versa.

If the process of phagocytosis were to be sub–divided it could be summarised in four steps (Figure 1.1):

- Attraction: the foreign particle stimulates a chemotactic response from the haemocytes.
- Adherence: the haemocyte attaches to the foreign particle.
- Internalisation: the foreign particle is ingested.
- Intracellular digestion or exocytosis: the ingested foreign particle is then digested or eliminated.

Chemotaxis and the attachment of foreign particles to the haemocyte surface constitutes the vital initial recognition phase of the cellular defences (Ratcliffe et al., 1985). Chemotaxis is the movement of a cell along a chemical gradient and, in the case of cellular defence, it serves to increase the frequency of encounters between phagocyte and the material to be phagocytosed. Chemotaxis has been well documented in mammals and vertebrates, however, little is known of this process in molluscan species (Renwrantz, 1990). Cheng and Howland (1979) found that haemocytes of *Crassostrea virginica* were attracted to live bacteria but not to heat-killed ones, suggesting that the live bacteria release a substance stimulating the chemotactic response. Chemotaxis has also been demonstrated in *M. edulis*, with haemocytes showing positive chemotactic responses to lipopolysaccharides from the Gram negative bacteria, *Serratia marcescens* and *Escherichia coli* (Schneeweiβ & Renwrantz, 1993).

Once contact, either randomly or by chemotaxis, has been made between the foreign invader and the haemocyte surface, then the attachment phase is initiated (Ratcliffe et al., 1985). Attachment in many invertebrates is promoted by factors in the serum (opsonins) that coat the foreign particle and bind it to receptors on the cell surface. These factors will be considered later as part of the humoral immune system. These opsonins are also present on the cell membrane of the haemocytes and present in the cytoplasm of the cells. Renwrantz and Stahmer (1983) demonstrated the presence of membrane bound lectin molecules at the surface of haemocytes from *M. edulis*. Vasta et al. (1984) also demonstrated the presence of lectins on the surface of the haemocytes of *Crassostrea virginica* using antibodies raised against the serum lectin involved in the agglutination of red blood cells. Antibodies raised against a lectin from the serum of the pond snail, *Lymnaea stagnalis*, bound to the surface of haemocytes from this gastropod (Van der Knaap et al., 1981). Although phagocytosis can proceed in the absence of haemolymph serum, and its associated opsonins, its efficacy is greatly reduced.

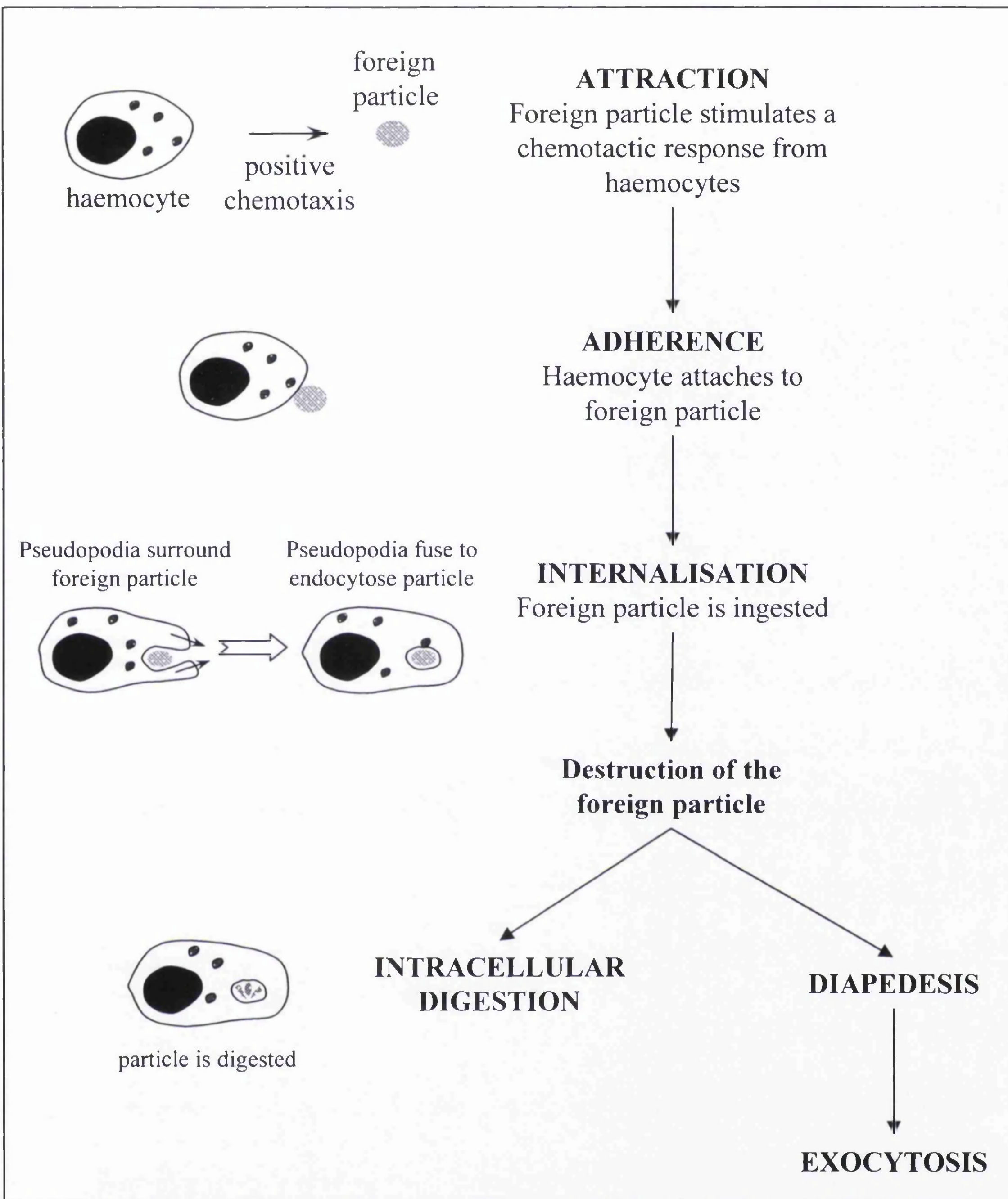


Figure 1.1. Summary of the stages associated with phagocytosis of foreign particles by haemocytes.

Following attachment, the foreign particle is then ingested in a number of different ways. There are three different mechanisms reported in bivalves (Cheng, 1981), usually pseudopods form at the cell surface and engulf the foreign particle enclosing it in a phagocytic vacuole which is withdrawn into the cytoplasm. This mechanism was reported by Bang (1961) who detailed the adherence of bacteria to filopods of haemocytes of *Crassostrea virginica,* they were then seen to glide along the surfaces of the filopodia and become engulfed in the cytoplasm. These observations have been confirmed by Renwrantz (1990) who studied the uptake of latex particles by the haemocytes of *M. edulis*. A second mechanism has been reported by Cheng (1975), who reported that contact between bacteria and granulocytes of *Crassostrea virginica* resulted in the development of invaginations of the cell surface and the bacteria were taken up into endocytotic vacuoles without the formation of filopodia. The third mechanism was reported by Renwrantz et al. (1979) studying the uptake of rat erythrocytes by *Crassostrea virginica* haemocytes which were commonly seen to form a single pseudopod that became greatly enlongated into a funnel-like structure. This funnel-like structure could reach a length equivalent to the diameter of the phagocyte itself and was directed towards the target cell. The target cell then glided along this funnel into the interior of the phagocyte, the funnels then retracted and closed. This mechanism has subsequently been recorded in *Mytilus* haemocytes (Renwrantz, 1990) and granulocytes of the gastropods *Biomphalaria glabrata* and *Bulinus truncatus* (Cheng, 1981).

After ingestion, the phagosomes, regardless of the mechanism used in their formation, soon interact with the lysosomal system and a range of antimicrobial factors are discharged onto the ingested microorganisms. The phagocytosed, or encapsulated (see later 1.2.1.3), agent is then intracellularly digested by one of a repertoire of killing mechanisms.

These killing mechanisms are thought to have evolved from digestive processes as the intracellular degradation process is the same whether it is foodstuffs or engulfed foreign molecules or organisms that are being digested (Feng 1988). The haemocytes of molluscs have been shown to be rich in lytic enzymes (Fletcher & Cooper-Willis, 1982). The hydrolytic enzyme released from lysosomes include acid phosphatase, non-

specific esterases, peroxidases, lysozyme and β-N-acetyl-glucosaminidase (Ratcliffe, 1985). These killing mechanisms result from degranulation of lysosomes and storage granules during phagocytosis to bring about the degradation of the endocytosed pathogen. After enzyme digestion is complete, certain molecules, such as monosaccharides and fatty acids, apparently diffuse through the phagosomal membrane into the cytoplasm (Cheng, 1981). Glucose can then be synthesised into glycogen and stored in glycogen granules in the cytoplasm. The degree of intracellular destruction will depend on the susceptibility of particles to the lysosomal enzymes contained within the cell. Not all particles are destroyed, some intracellularly sustained microorganisms that can grow and multiply within the cytoplasm of host blood cells have, through evolutionary adaptation, become mutalists of their hosts (Cheng, 1981), such as the intracellular zooxanthellae of the marine bivalves *Hippopus* sp. and *Tridacna* sp. Any indigestible foreign particles can be disposed by diapedesis of the blood cell and exocytosis of the undigested particle. Diapedesis is the passage of blood cells from blood sinuses and into the surrounding tissue, while exocytosis is the process of discharging material from a cell. Diapedesis of laden haemocytes through the digestive organs was recorded by Stauber (1950) in *Crassostrea (Ostrea) virginica* after the pericardial injection of Indian ink.

1.2.1.3 Encapsulation

If the object is larger than the phagocyte, the phagocyte will extend over the surface of the foreign particle and more phagocytic cells will accumulate, resulting in encapsulation. The factors involved in the recognition of a particle as nonself are thought to be similar to those involved in phagocytosis, detailed above (Ratcliffe et al., 1985). The nature of the invertebrate encapsulation response is highly species-specific, but the basic mechanisms, involving opsonins and other humoral components as well as non-specific physicochemical factors such as electrostatic forces, are potentially common to all encapsulation reactions (Wootton et al., 2006).

If a particle too large to be engulfed is surgically inserted into a mollusc, haemocytes will swarm around it. They form into layers of cells, called lamellae, around the particle entirely encapsulating it and the outer layers remaining rounded whilst the inner layers become progressively flattened. The process of capsule formation has been well described at both the light and electron microscopic level (Tripp, 1961; Sparks & Chew,

1966; Rifkin et al., 1969). The nature of the response varies according to the kind of foreign body or parasite being encapsulated with the number of cell layers and degree of flattening of the inner cell layers varying greatly. Once encapsulated, the parasite or foreign particle may be destroyed passively or actively.

Passive destruction of the parasite results from the inner layers of the capsule dying off to form an impenetrable wall. The parasite is then effectively entombed. This mechanism is very useful for protecting against large parasites. The tomb stops the parasite from obtaining nutrients from the host resulting in starvation; it may also asphyxiate the parasite (Ratcliffe et al., 1985). The capsule may then remain within the animal as an inert nodule with the capsule itself serving to stop the immune system from being further stimulated by the foreign antigenic material. Alternatively the capsule may be exocytosed.

Active destruction results from the action of haemocytes on the inner surface of the capsule releasing lysosomal enzymes. As the contents are destroyed and taken up by the haemocytes the capsule regresses (Cheng & Garrabrant, 1977). The role of lysosomal enzymes in causing parasite death has not been conclusively demonstrated, however, elevated levels of lysosomal enzymes have frequently been reported in capsules. Elevated levels of β-glucuronidase, amylase, and lipase have been reported in the capsules formed in *Biomphalaria glabrata* (Rodrick & Cheng, 1974). Another mechanism by which blood cells can kill pathogens during phagocytosis is a respiratory burst, which involves the release of highly reactive oxygen metabolites (Pipe et al., 1993), see section 1.2.2.3. The haemocytes are also able to resume their haemocyte-like appearance (from their flattened form) and infiltrate the foreign parasite or tissue within the capsule (Fletcher & Cooper-Willis, 1982).

Parasite death does not inevitably follow encapsulation, some parasites use the capsule to protect themselves from further host response and continue their life cycle. The larvae of the parasitic nematode, *Angiostrongylus,* successfully complete their metamorphosis in *Biomphalaria* despite rapid encapsulation by haemocytes which give a strong staining reaction for acid phosphatase, β-glucuronidase, and non-specific esterases (Harris & Cheng, 1975).

Additionally, in their natural molluscan hosts, most metazoan parasites elicit no cellular response and are not recognised as nonself (Fletcher & Cooper-Willis, 1982). Little or no reaction was noted in eastern oysters, *Crassostrea virginica*, infected with sporocysts of the trematode *Bucephalus* sp. (Cheng & Burton, 1965). Wootton et al. (2006) found that only one third of metacercarial cysts of the trematode *Himasthla* sp. were encapsulated in the foot of the *Cerastoderma edule*. Targets (foreign bodies) with a positive electrostatic charge stimulate the most vigorous response in *C. edule*, with non-specific electrostatic forces and humoral plasma factors having a synergistic role in haemocyte attachment and the encapsulation response (Wootton et al., 2006).

1.2.1.4 Wound repair and Inflammation

Injury to marine bivalve molluscs typically evokes an inflammatory response in which haemocytes infiltrate the area of injury and phagocytose or sequester the injurious agent, the inflammatory exudate, and necrotic tissue debris (Sparks & Morado, 1988). The initial response to wounding in *M. edulis* involves the infiltration of the wounded area by numerous haemocytes. Bubel et al. (1977) found that all haemocyte cell types (small basophils, macrophages and granulocytes) were involved in this initial response, although after 7 days the number of basophils decreased. The second stage of the response begins 12 to 24 hours later and is characterised by intensive phagocytosis with both intra- and extracellular digestion (Sparks & Morado, 1988). This continues to clear all dead and damaged tissue and any opportunistic microorganisms until the wound is repaired and dead or damaged tissue is replaced by healthy tissue. The wound is closed by new epithelium which originates from the adjacent undamaged tissue by mitosis (Ruddell, 1971; Bayne et al., 1979). The numerous haemocytes that infiltrate the wound site also aggregate to form a plug filling the wound (Bubel et al., 1977; Feng, 1988).

1.2.2 Humoral defences

Cell-free haemolymph of different invertebrate species contains a variety of biologically active molecules (Leippe & Renwrantz, 1988). Invertebrate serum, although lacking in the immunoglobulins seen in vertebrates, has a range of factors which mediate lytic, agglutinating and antimicrobial activities against various biological agents (Ratcliffe, 1985). The main cellular defence reactions of phagocytosis and encapsulation are

closely related to the activity of humoral factors, such as agglutinins, opsonins, and lysins (lyososmal enzymes).

1.2.2.1 Lysins

Lysozyme activity has been demonstrated in many invertebrate species (Chu, 1988) from annelids to insects, molluscs and insects (Ratcliffe et al., 1985). Lysozyme is an important part of antibacterial defence, particularly against Gram-positive bacteria, both inside and outside haemocytes. It acts by hydrolysing the bond between N-acetylmuramic acid and N- acetylglucosamine, thus cleaving an important polymer of the cell wall of many bacteria. Other lysosomal enzymes have been reported in the haemolymph of bivalve molluscs. These lysosomal enzymes include acid phosphatases, nonspecfic esterases, β-glucuronidase, amylase, arylsulphatase and lipase and have been demonstrated in a wide variety of bivalve molluscs such as *Crassostrea gigas* (Feng et al., 1971), *Mercenaria mercenaria* (Cheng & Rodrick, 1975), *Scapharca inaequivalvis* (Holden et al., 1994), *Mytilus edulis* (Moore & Lowe, 1977) and *Scrobicularia plana* (Wootton & Pipe, 2003). These lysozymes are active against many species of bacteria, β-glucuronidase from the northern quahog (*Mercenaria mercenaria*) was found to hydrolyse mucopolysaccharides which are found in bacterial cell walls (Chu, 1988). This, together with the evidence of enhancement of lysosomal enzyme serum levels following bacterial challenge, has led to the theory that they serve as non-specific, humoral defence molecules (Pipe, 1990b).

The origin of lysosomal enzymes is postulated to be in the lysosomes of granular haemocytes (Chu, 1988). The loss of cytoplasmic granules into the serum, which have been shown to be lysosomes, from granulocytes of *Mercenaria mercenaria* during the *in vitro* phagocytosis of *Bacillus megaterium* has been demonstrated semiquantitatively by Foley and Cheng (1977). Cheng et al. (1975) demonstrated that haemocytes of *Mercenaria mercenaria* released lysozyme into the serum when challenged by *Bacillus megaterium.* Cheng and Yoshino (1976) demonstrated that there is an increase in lipase activity in both the haemocytes and serum of *Mya arenaria* during *in vivo* phagocytosis of heat killed *Bacillus megaterium.* This process, designated as degranulation, represents the morphological basis of the release of lysosomal enzymes from granulocytes into serum associated with phagocytosis. External contact with certain foreign substances will result in hypersynthesis of intracellular lysosomal enzymes,

some of which are released from haemocytes into the serum where digestion of the foreign material, such as bacteria, is initiated (Cheng, 1981). It is thought that the roles of lysosomal enzymes as agents of defence and digestion are probably equally important (Chu, 1988). Lysosomal enzymes fulfil the purposes of both defence and digestion since bacteria are one of the foods of marine bivalves.

1.2.2.2 Cytotoxicity Reactions

Secretion of cytotoxic molecules has been reported for mussels. Wittke and Renwrantz (1984) have shown that *M. edulis* haemocytes are capable of secreting cytotoxic molecules into the serum which lyse human erythrocytes. They demonstrated that cells remain intact and that no apparent contact is required between the target and the effector cells, with killing appearing to be mediated by factors diffusing into the surrounding medium. Leippe and Rewrantz (1988) postulated that the secretion process is triggered when the haemocytes come into contact with a foreign surface. Not all mussel haemocytes were found to be capable of producing this lytic agent. Cytotoxic molecules extracted from *Mytilus* haemocytes possess a molecular weight of 72,000 daltons (72kDa), do not require free Ca^{2+} ions for their haemolytic activity and have strong esterase activity, indicating that they might be enzymes (Renwrantz, 1990). Hubert et al. (1996) have also identified cytotoxic activity in the plasma of mussels (*Mytilus galloprovincialis*) which was active against both vertebrate and protozoan cells. The protozoan parasite *Bonamia ostrea* was shown to be sensitive to the cytotoxic activity of this molecule, possibly explaining why mussels, in contrast to oysters, do not develop bonamiasis. These cytotoxic molecules were shown to be of molecular mass larger than 700kDa, and comprised of an inactive 320kDa proteinaceous base unit. After binding onto target cell membranes the polymerisation of this base unit induced lysis, due to the formation of transmembranal pores. The cytotoxic molecule is inducible and significant increases in the activity levels of this cytotoxic molecule were produced by simulation using erythrocyte (human group O red blood cells) and saline injections (Hubert et al., 1996).

1.2.2.3 Respiratory burst

The phagocytosing cells of mussels are also capable of a "respiratory burst", which involves the release of highly reactive oxygen metabolites. The generation of reactive oxygen metabolites, along with the release of degradative enzymes from lysosomes,

comprise a killing mechanism that complements the role of phagocytosis as an internal defence mechanism against potential pathogens. The haemocytes of molluscan species have been shown to produce a number of reactive oxygen metabolites including the superoxide anion radical (O_2^-) and hydrogen peroxide (H_2O_2) (Pipe, 1992). In order to minimise the potential for damage to adjacent tissues and cells, from reactive oxygen metabolites released during the respiratory burst, mussels possess a number of antioxidant enzymes (Pipe et al., 1993). These antioxidant enzymes include superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase.

1.2.2.4 Agglutinins (with lysins collectively known as opsonins)

There is confusion among the terms, lectin, agglutinin, and opsonins, although they are different by definition (Chu, 1988). This confusion arises, in part, from the adoption of mammalian and vertebrate terms in non-vertebrate and plant systems. In invertebrates, agglutinin is used as a term for a substance, other than a specific antibody, that causes organic particles to agglutinate. The term lectin (from the Latin legére: to choose) was originally coined for use in botany by William Boyd in 1954 (Sharon & Lis, 1987), to denote any of several plant glycoproteins that act like specific antibodies, in that they agglutinate specific compounds, but are not antibodies, in that they are not evoked by an antigenic stimulus. This term was generalised in 1972 (Sharon & Lis, 1987) to include all sugar-binding, cell agglutinating proteins of non-immune origin, from plants, animals or microorganisms. In invertebrates, agglutinins and lectins are generally used interchangeably to denote a substance that causes foreign particles to agglutinate. An opsonin is a substance that binds to the surface of a particle and enhances the uptake of the particle by phagocytes. In the vertebrate system, opsonins are antibodies in blood serum that attach to invading microorganisms and other antigens to make them more susceptible to the action of phagocytes. It is likely that many of the agglutinins found in bivalves are lectins, and can function as opsonins (Chu, 1988).

The organ systems in molluscs are bathed in circulating haemolymph. As a result, blood cells travel widely providing ample opportunity for chance encounters with foreign invaders, and this is the basic role of agglutinins and opsonins. The capability of the haemocytes to discriminate between self and non-self is based on the presence of lectins (or agglutinins) (Olafsen, 1988). Lectins may possess opsonising properties, that is, soluble factors that bind to the surface of a foreign body and thus mark it for

phagocytosis by the haemocyte, or alternatively lectins may occur as membrane bound recognition molecules on the surface of the haemocytes (Renwrantz, 1990).

Lectins have been identified in all taxa, from viruses, bacteria, yeasts, plants, invertebrates, vertebrates and as endogenous membrane components of cells (Sharon & Lis, 1988). Invertebrate lectins are believed to be involved in humoral defence reactions by interacting with non-self and augmenting the phagocytic response (Olafsen, 2001). Humoral lectins are found in the body fluids of most invertebrates, however, their origin and biological roles have not yet been firmly established. It may be that after combination of the lectin with bacteria, the lectin may undergo a molecular change so that different recognition sites on the molecule can combine with receptors on the haemocyte surface (Olafsen, 1988). This would allow the lectin to remain cryptic when not interacting with a bacterium and thus prevent unnecessary interactions with haemocytes. Increased *in vitro* phagocytosis has been demonstrated when bacteria are precoated with haemolymph factors or purified lectin (Olafsen, 1988). Renwrantz and Stahmer (1983) have demonstrated the opsonising properties of a lectin in *M. edulis*. *In vitro* phagocytosis assays demonstrated that only about 5% of washed haemocytes alone phagocytose yeast cells, compared to 50% in haemocytes suspended in haemolymph. When purified agglutinin was added to saline suspended yeast and washed haemocytes the phagocytosis rates returned to normal, thus demonstrating the opsonising properties of the agglutinin. Mullainadhan and Renwrantz (1986) further demonstrated the opsonising properties of lectins in that the induction of phagocytosis by four heterologous lectins only occurred in *M. edulis* when the lectin could bind to carbohydrate determinants on both the haemocyte surface and the target cell. Lectins that bound to only one of the surfaces failed to stimulate phagocytosis.

The horse mussel, *Modiolus modiolus*, has been shown to have natural agglutinins for pathogenic vibrios which react with purified lipopolysaccharide (LPS) from *V. anguillarum* and *V. salmonicida* (Tunkijjanukil et al., 1997). Increased lectin activity in Pacific oysters, *Crassostrea gigas*, as a result of *in vivo* exposure to bacteria in water suggests the involvement of these lectins in defence reactions. The increased activity occurred 6 hours after exposure to *Vibrio anguillarum* indicated activation or release of the lectin rather than *de novo* synthesis (Olafsen, 1988). Phagocytosis of *V. vulnificus* and *V. anguillarum* by *Crassostrea virginica* haemocytes was studied by scanning and

transmission electron microscopy by Rodrick and Ulrich (1984) and was found to increase 3 to 10 times in the presence of serum. When this serum was heat-treated and the haemocytes reconstituted with this treated serum, phagocytosis was depressed, indicating that the increased phagocytosis was due to a heat-labile factor in the serum.

1.2.2.5 Antimicrobial factors

Since the discovery of inducible antimicrobial peptides in the moth, *Hyalophora cecropia,* by Boman and Steiner in 1981 (Steiner et al., 1981), over 400 peptides have been reported to participate in innate immunity, not only in insects but in all multicellular organisms that were investigated, including humans and plants (Hoffmann et al., 1999). Since the initial discovery of cecropin in the moth by Boman and Steiner. (1981) more than 170 antimicrobial peptides/polypeptides have been found in insects alone. In fact, at least 50% of the reported antimicrobials have been identified in invertebrates, predominantly within the insects (Bulet et al., 1999). Although these immune effectors share common characteristics (small size and cationic character) and similarities in structural patterns, one striking feature is their great diversity in terms of amino acid sequences, antimicrobial activities and their modes of action (Muňoz, et al., 2002). The 3D structure of mussel defensins reveals a close relationship, with arthropod defensins. However, although sharing the same name, invertebrate defensins are of totally different molecular structure to vertebrate defensins (Cooper, 2002). The peptides are involved in several levels of the anti-infectious response, a) intracellular on phagocytosed bacteria, b) extra cellular in a later systemic response.

In insects, septic injury results in a rapid synthesis of these peptides in the fat body (equivalent to the liver in vertebrates) which are released immediately into the haemolymph where they participate in a systemic response (Mitta et al., 2000a). Oysters, *C. gigas*, have been shown to continually express the defensin Cg-def in the mantle (Gueguen et al., 2006), the defensins Cg-defh1 and Cg-defh2 are continuously expressed in the haemocytes (Gonzalez et al., 2007). In other invertebrates, antimicrobial peptides are also reported in granules within haemocytes. Several antimicrobial substances active against both Gram-negative and Gram-positive bacteria have been found in both small and large granules within haemocytes of the horseshoe crab, *Limulus polyphemus* (Iwanaga & Kawabata, 1998). Upon microbial stimulation, these antimicrobial peptides are released from haemocytes by regulated exocytosis. The

horseshoe crab has also been shown to produce tachystatins that are not only effective against Gram-negative and Gram-positive bacteria but also have potent anti-fungal properties (Osaki et al., 1999). Penaeidins are a family of antimicrobial peptides that have been found to be constitutively produced and stored in haemocytes of penaeid shrimp (Muñoz, et al., 2002). It was found that the penaeidin mRNA and protein are restricted to the granular haemocytes of *Penaeus vannamei* and that, following microbial injection, haemocytes that migrated to the infection site showed increased penaeidin-transcriptional activity. In contrast to the release of antimicrobial peptides from haemocytes by exocytosis seen in horseshoe crabs, Muñoz, et al. (2002) saw no evidence of migration of granules towards the periphery of the haemocyte nor did they see any indication of degranulation of granule-containing penaeidin when haemocytes were challenged with *Vibrio in vitro*. The haemocytes were, however, noted to undergo changes in shape and morphology of their granules which was thought to be due to release of the contents of the granule into the cytoplasm. This was then followed by lysis of the cell and release of the cytoplasm content.

Mussels have been found to possess small antibacterial and antifungal peptides (Hubert et al., 1997), in addition to the large cytotoxic proteins with a broad range of recognitive specificities that have been reported (Hubert et al., 1996). These small, cationic, cystine-rich peptides have now been characterised from the haemolymph of mussels (Mitta et al., 2000a). Three groups of cationic, cystine-rich, 4kDa antimicrobial peptides have been identified: defensins, mytilins and myticins. In addition, a novel antifungal peptide, mytimycin, of 6.5kDa containing 12 cystines has been partially characterised from *M. edulis* plasma (Charlet et al., 1999). The mussel peptides have been shown to be synthesized as precursors in circulating haemocytes before being stored as mature forms in haemocyte granules (Mitta et al.,1999a,b, 2000b). Defensins and myticins are active against Gram-positive bacteria and much less active against Gram-negative bacteria and fungi. In contrast, mytilins display a wider spectrum of activity according to their isoforms (Mitta et al., 2000a). The fact that the different peptide families and isoforms possess different properties and activities against different pathogens may permit an increase in the antimicrobial capabilities of mussels (Mitta et al., 2000a). Confocal microscopy has revealed the presence of haemocytes containing only mytilins, only defensins, both peptides or neither peptide. Localisation of both peptides within the same cells, packed in different or in the same cell compartments has

been confirmed by electron microscopy (Mitta et al., 2000c). It is postulated that the different cell types have different roles in defence, with haemocytes containing mytilins alone being involved in the early phase response migrating to the infectious site and phagocytosing microorganisms. The second cell type containing both mytilins and defensins could be involved at a later stage by releasing the peptides that trigger a systemic response. A plasmatic increase in levels of both defensin and mytilin concentrations has been shown 24 hours after bacterial challenge (Mitta et al., 2000b).

Antimicrobial peptides target a fundamental difference in the design of membranes of microbes compared to multicellular organisms. Bacterial membranes are organised in such a way that the outermost leaflet of the bilayer, the surface exposed to the outer world, is heavily populated by lipids with negatively charged phospholipid headgroups (Zasloff, 2002). The antimicrobial peptide binds to the outer leaflet of the cell, displaces lipids and alters the membrane structure, permeabilising the cell membrane, resulting in the efflux of solutes (Hoffmann et al., 1999).

1.4 Immunotoxicology

Although the immune system was long considered to be autonomous in both regulation and action, over the past 20 years data has emerged suggesting that a significant reciprocal interaction occurs between the nervous, endocrine and immune systems (Koller, 1990). These links increase the immune system complexity and may make it more sensitive to environmental stressors. The immune system may be suppressed or enhanced as a result of exposure to chemicals or their metabolites. Immunosuppression may result in decreased resistance to opportunistic pathogens, whereas immunoenhancement could increase the risk of autoimmune reactions (Exon, 1990). The feeding habit of *Mytilus edulis,* filtering large quantities of water, and their near shore habitat (occuring to a maximum depth of 10 metres) exposes this species to many pollutants. They are long-lived and sedentary in nature, which serves to increase their exposure to pollutants and consequently they often accumulate a wide range of toxicants, so that tissue concentrations can increase by factors of 10 to 10^5 relative to the concentration in seawater (Widdows & Donkin, 1992). This bioaccumulation makes the measurement of trace contaminants easier to accomplish in tissues than in the seawater. Contaminants accumulated in mussel tissues are derived from suspended matter ingested as food, as well as by direct adsorption from fractions dissolved in sea

water. Measurement of the contaminants in the mussel gives an indication of their biological availability which cannot accurately be determined from direct measures of the environmental compartments (water, sediment or suspended particles). Many factors influence the relationship between the level of contaminants in the environment and the load in the mussel tissue. The degree to which the contaminants are accumulated depends upon both abiotic factors (eg. physicochemical properties of the contaminants and their speciation) and biotic factors (eg. pumping activity, growth, reproductive condition and metabolism/elimination) (Widdows & Donkin, 1992). Environmental variables, such as temperature and salinity, will also affect uptake of contaminants. Measuring levels of contaminants in mussels and assessing their biological effect gives an insight into the interactions that can occur between contaminant and organism.

More recently, emphasis has moved from chemical monitoring of contaminants in the environment onto biological monitoring to assess the effects of the presence of contaminants on individuals and populations of animals. Molluscs, particularly *M. edulis,* have been used as indicator organisms to monitor pollution in the marine environment, most notably as part of the "Mussel Watch" programme. The US "Mussel Watch Program" was initially conducted from 1976 to 1979 and used *M. edulis* to monitor trace metals, hydrocarbons and radionuclides at selected coastal sites. Comparisons of concentrations of various pollutants between sites and over time were used to indicate trends in contaminant input to the populations studied. The "Mussel Watch" concept then spread around the world with the establishment of similar local or regional programmes in places as far afield as Japan, India, South Africa, the USSR and the Mediterranean (Widdows & Donkin, 1992). A number of characteristics of mussels make them ideal candidates for this type of study. The have a wide geographical distribution which eliminates the problems encountered when comparing data for different species. In addition, mussel beds are large which enables repeated sampling and also relatively stable so can be used for long term studies. As mentioned previously, mussels bioaccumulate contaminants. They are also relatively tolerant of, but not insensitive to, a wide range of environmental conditions, including moderately high levels of many types of contaminants (Widdows & Donkin, 1992).

There is considerable evidence to support links between environmental contaminants and non-infectious diseases in marine organisms (Pipe & Coles, 1995), ranging from kidney concretions, mantle recession and proliferative blood cell disorders. There is somewhat more limited knowledge, however, on the links between environmental pollution and infectious diseases. The ecological and evolutionary impact of diseases in the ocean remains unknown, even when these diseases affect economically important species (Harvell et al., 1999). The studies that have been carried out do indicate links between pollution and disease with clams (*Mercenaria mercenaria*) in polluted waters shown to be more susceptible to infestations by polychaetes and bacteria when compared to unstressed clams (Rinkevich, 1999). Pipe and Coles (1995) also demonstrated that mussels are more susceptible to infection by the bacteria, *Vibrio tubiashii,* following pre-exposure to copper or cadmium. Most coastal waters are typically affected by suites of anthropogenic pollutants and inputs, making it difficult to identify any one cause of deteriorating health or disease outbreak (Harvell et al., 1999). A survey of oysters (*Crassostrea virginica*) from sites throughout the Gulf of Mexico by Wilson et al. (1992) linked increased body burdens of metals and petroleum aromatic hydrocarbons (PAH's) with increased *Perkinsus marinus* infection.

Natural environmental variability may also influence immune function. Environmental factors which have the greatest influence on marine bivalve molluscs are salinity and temperature. Hauton et al., (2000) reported significant effects of salinity changes on the proliferation of bacteria and alterations in measured immune parameters. Fisher (1988) also demonstrated that natural environmental variables affect the immune response activities of bivalve haemocytes. He hypothesised that temperature influences the haemocyte membrane by affecting the structure and permeability. Temperature effects on immune function may become of increasing importance if global warming continues as predicted. Growth rates of marine bacteria are also positively correlated with temperature and increased temperatures cause pathogen range expansions (Harvell et al., 2002).

1.4 Aims and objectives of the thesis

A better understanding of the origins of emergent disease and invertebrate immunity is needed before we can evaluate the role of changing environments in host-pathogen interactions (Harvell et al., 1999). The aims of this thesis are

1. To assess immune function in developmental stages of the marine mussel, *Mytilus edulis*. D-shell larvae were examined for presence of haemocytes using electron microscopy. The haemocytes of postlarval stage mussels were compared with adults using light and electron microscopy. The postlarval haemocytes were examined using electron microscopy of sections of whole individuals. Additionally samples of postlarval haemolymph were embedded in BSA and processed for electron microscopy, a methodology previously used to examine adult haemolymph samples (Pipe, 1990a).
2. To examine the susceptibility of both larval and postlarval mussels to several common marine bacteria. Three species of bacteria that have been linked with disease outbreaks were studied; these were *Vibrio tubiashii*, *Vibrio alginolyticus* and *Alteromonas haloplanktis*. Novel methodologies were developed to expose the mussels, and assess the viability of those individuals exposed to bacteria.
3. To consider the interactive effects of pollutants (copper and phenanthrene) and food availability on the susceptibility of postlarvae to these bacteria. This was achieved by co-exposure of the postlarvae to the *V. tubiashii* and the stressors previously listed using the methodologies developed in house.
4. To assess any effects that may occur due to the time of year at which the experiments were carried out. Experiments were repeated at different times of to ascertain whether there were any effects seasonal effects that may cause differences in immune response and disease susceptibility. In this sense "seasonal effects" does not represent any external manipulation of the environment in which the mussels were kept.

Chapter 2. Microscopy of postlarval haemocytes.

2.1 Introduction

In adult molluscs the immune system can be split into cell-mediated and humoral mechanisms; although it has become increasingly apparent that both are interrelated and closely associated with haemocytes (Tirapé et al., 2007). Immune function of bivalve molluscs is largely mediated by phagocytic haemocytes and is complemented by an array of killing mechanisms, which may include the release of degradative and oxidative enzymes and the generation of highly reactive oxygen metabolites (Wootton & Pipe, 2003). The origins and development of bivalve haemocytes has long been debated, as detailed in the introduction to this thesis (Chapter 1). A thorough description of the immune system of adult bivalves is also given in Chapter 1; however brief summaries of haemocyte characterisation are given below.

The study and characterisation of bivalve haemocytes is still an active area of research, although confusion still arises regarding the nomenclature of the blood cells, largely as a result of the use of vertebrate terms such as granulocyte and lymphocyte. The classification of circulating haemocytes from the mussel, *M. edulis*, has received considerable attention (Pipe et al., 1997). Moore and Lowe (1977) and Pipe (1990) recognised 3 cell types: small agranular cells (hyalinocytes) and two types of granular cells, differentiated by the size of the granules into those with comprising mostly of large granules and those with small granules. These cell types have different staining and functional characteristics. The haemocytes containing large granules are eosinophilic and are particularly active during phagocytosis. The other 2 cell types are basophilic but specific functions for these haemocytes are less clear (Pipe et al., 1997). Briefly, there are 2 main schools of thought on the development of haemocytes: Cheng (1981) proposes that there are 2 distinct cell lines that originate from a unique stem cell. Mix (1976) on the other hand proposes that haemocytes originate from one common cell producing a single cell line.

The origins of invertebrate blood cells are highly variable, and in some cases the site of blood cell formation is unknown. In annelids and echinoderms, haemopoiesis is

thought to occur in coelomic lining cells (Sminia & van der Knaap, 1986). In the polychaete worm, *Nicolea zostericola*, agranular phagocytes appear to originate from highly specific sites in the coelomic cavity of the thoracic, and a few of the abdominal, segements (Ecklebarger, 1976). The crustaceans have a very discreet haemopoietic organ made up of a series of nodules surrounded by a thin sheath of connective tissue (Ratcliffe et al, 1985). There do not appear to be any well-defined haemopoietic organs in the molluscs with the exception of the cephalopods, where blood cell formation occurs in the white bodies situated in the orbital pits behind the eyes (Fletcher & Cooper-Willis, 1982). In the case of the gastropods, it has been suggested that in *Biomphalaria glabrata* haemocytes are formed from fibroblasts in the trabeculae of the haemolymph sinuses, similar cells in the wall of the saccular portion of the renal organ and in the loose connective tissue (Cheng, 1981). However, more recently, Jeong et al (1983) have described the ultrastructure of an ameobocyte-producing organ (APO) in the same species which is located between the epithelial cells of the posterior mantle and the epithelial lining of the pericardium. This has been shown to contain both primary and secondary ameoboblasts as well as mature ameobocytes, it has also been stimulated by immunisation. The site of haemopoiesis in bivalves has not yet been identified, Prytherch (1934) believed that the larval eyespots acted as haemopoietic tissue, breaking down and liberating haemocytes at metamorphosis. Although no satisfactory account has yet been published as to where haemopoiesis occurs in bivalves, but the generally accepted belief is that haemocytes arise from differentiation of connective tissue cells (Cheng, 1981; Bachère et al., 2004). However, a recent study on *C. gigas* by Tirapé et al. (2007) indicated that haematopoetic cells could derive from blood vessel endothelium. Tirapé et al. (2007) studied the expression levels of two genes known to be markers of haemocyte precursor cells in serial sections of postlarval *C. gigas*. Gene expression was found to be localised only in haemocytes attached to blood vessel epithelium, with no labelled cells detected in the connective tissue.

While the immune response has be been characterised for adult bivalves, little is known about the immune capabilities during ontogenesis (Tirapé et al., 2007). Larval mussels were examined for the presence of haemocytes (in Chapter 3). In the present study postlarval haemocytes are examined and compared with those of adult mussels.

2.2 Materials and Methods

2.2.1 Light microscopy of postlarval and adult haemocytes

Postlarvae and adult mussels were collected from Whitsand Bay, Cornwall, on transfer to the laboratory they were sorted for size. Haemolymph samples were extracted from the posterior adductor muscle using a 1ml syringe and a 30G hypodermic needle (0.3mm x 13mm or 30½ GA). Samples were collected for light microscopy from postlarvae and adults over a range of sizes (0.73 to 5.2cm shell length). The haemolymph was withdrawn into Baker's formol calcium containing 2% sodium chloride and a note of the animal's size was made. The haemocytes were prepared for differential staining using a cytocentrifuge (Shandon, UK) with 100μl of haemolymph. Cells were then post-fixed in methanol for 3 min., stained with Wrights stain (diluted 1:4 with 0.05M Tris Buffered Saline, pH 7.6) for 5 min., rinsed in water, air dried and mounted in Canada balsam. Relative numbers of eosinophilic (granular) and basophilic (granular and agranular) staining cells were calculated by counting 200 blood cells from each animal using a light microscope.

2.2.2 In situ electron microscopy of postlarval haemocytes

Postlarvae (0.6 to 1.1cm shell length) were collected from Whitsand Bay, Cornwall, on transfer to the laboratory they were sorted for size and fixed in EM fixative (as described in Chapter 2) for 24 hours. Before processing for electron microscopy it was necessary to decalcify the postlarvae. The technique described by McCafferty and Smolowitz (1995) was used to test for decalcification. This briefly involves the use of 2% L-Ascorbic acid to decalcify the specimen, with the end point being detected using a solution of equal volumes of 5% ammonium hydroxide and 5% ammonium oxalate. Decalcification is complete if no milky precipitate forms when the decalcification solution is added to the ammonium mixture. However, 72 hours after the postlarvae were transferred into the ascorbic acid, decalcification was still not complete. This slow decalcification was thought to pose a risk of damage to the postlarval tissues, consequently alternative methods of decalcification were sought.

A commercial preparation was obtained, RDO rapid decalcifier (Apex Engineering, Illinois, USA), the postlarvae were placed in this solution and gently agitated. This solution decalcified the postlarvae in 90 minutes, thus reducing the risk of damage to

the postlarval morphology. The decalcification endpoint was again determined using the ammonium hydroxide/ammonium oxalate method described fully by McCafferty and Smolowitz (1995). All postlarvae processed for electron microscopy were decalcified using RDO.

The postlarvae were fixed in EM fixative for 24 hours before being washed twice for 15 minutes in 0.2M cacodylate buffer, pH 7.4. Secondary fixation was carried out with 1% OsO_4 for 3 hours at room temperature. The postlarvae were then washed twice in cacodylate buffer (15 minutes), before being decalcified in RDO (90 minutes). They were then washed again in cacodylate buffer and dehydrated through an ethanol series before being embedded, via propylene oxide, into "Taab" epoxy resin. Ultrathin sections were cut on a Reichert Ultracut-E ultramicrotome, stained with uranyl acetate and lead citrate and examined in a Philips 300 electron microscope.

2.2.3 Electron microscopy of haemocytes from postlarval blood samples

Samples of postlarval haemolymph were also collected for examination using electron microscopy. Haemolymph from 100 postlarvae (mean size = 65.7 mm ± 1.1mm (2SE)) was withdrawn into EM fixative and the samples were pooled. These samples were processed using the same methodology as used to process the D shell larvae (section 3.3.1). Samples of adult *Mytilus edulis* haemolymph were also processed in the same manner, to allow direct comparison between adult and postlarval haemocytes. Ultrathin sections were cut on a Reichert Ultracut-E ultramicrotome, stained with uranyl acetate and lead citrate and examined in a JEOL 1200EX electron microscope.

2.3 Results

2.3.1 Light microscopy of postlarval and adult haemocytes

Examination of blood cells extracted from postlarvae and differentially stained showed evidence of a blood cell type not previously described. The haemocytes of adult mussels can be divided into 2 groups on the basis of differential staining, basophilic staining cells (blue) and eosinophilic staining cells (red/purple). The basophilic cells

include agranular and granular cells, the eosinophilic cells are restricted to granular cells. In addition to these staining types seen in adults, an intermediate blood cell type was found in small postlarvae. This blood cell appeared to be similar to the agranular basophil of the adults but contained a few (usually less than 4) eosinophilic granules (Figure 2.1). This cell type was only found in postlarvae smaller than 2.5cm and was most common in the smallest group studied, 0.7-1cm (Figure 2.2).

2.3.2 Electron microscopy of postlarval haemocytes in situ

Analysis of the micrographs of the postlarvae showed blood cells associated with the gills and mantle tissue (Figs. 2.3 and 2.4). The blood cells showed similar morphology to those found in adult mussels. Figure 2.3 shows a typical granular haemocyte containing large electron-dense granules and Figure 2.4 shows an agranular cell.

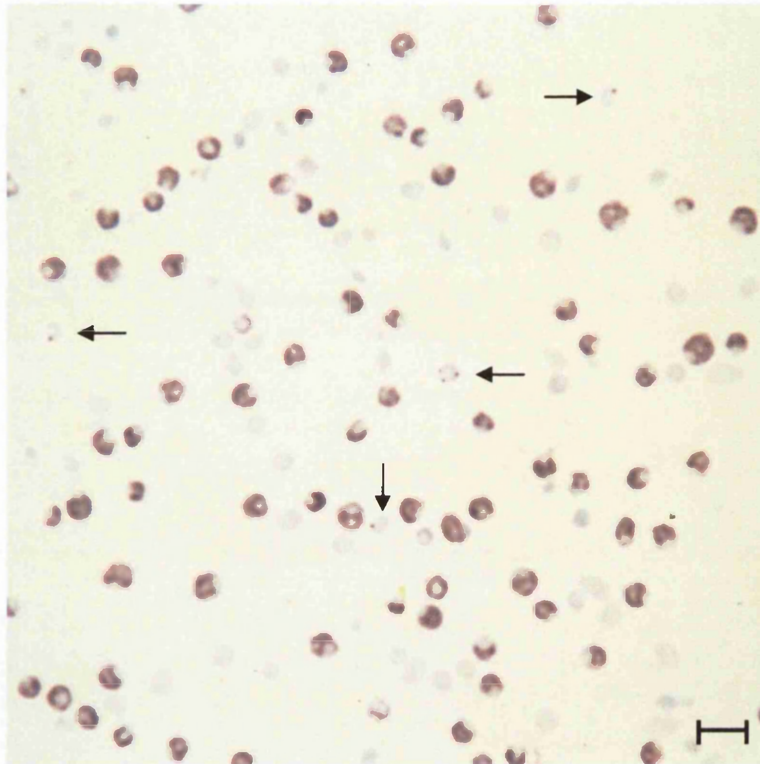


Figure 2.1. Postlarval *M. edulis* haemocytes, differentially stained using Wrights stain. Eosinophilic cells stained purple, basophilic cells stained pale blue Arrows indicate the previously unreported haemocyte type, basophilic in nature with 1-3 eosinophilic granules. Scale bar : 20μm.

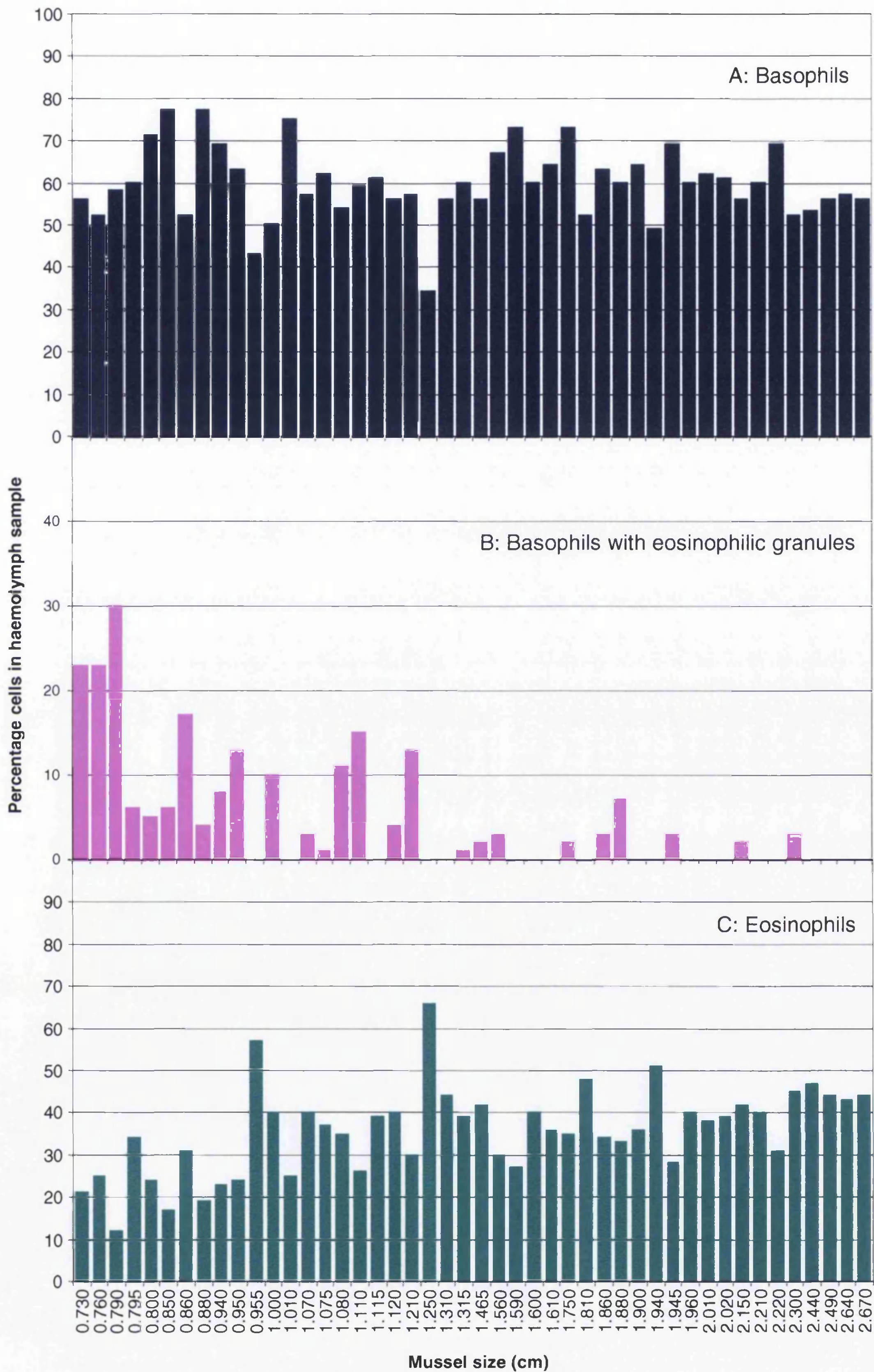


Figure 2.2. The occurrence of blood cell types in differentially-stained haemolymph samples taken from postlarvae and adult *M. edulis* ranging from 0.73 to 2.67 cm in shell length. The previously unreported haemocyte type, basophilic in nature with 1–4 eosinophilic granules, is shown in Fig 2.2B.

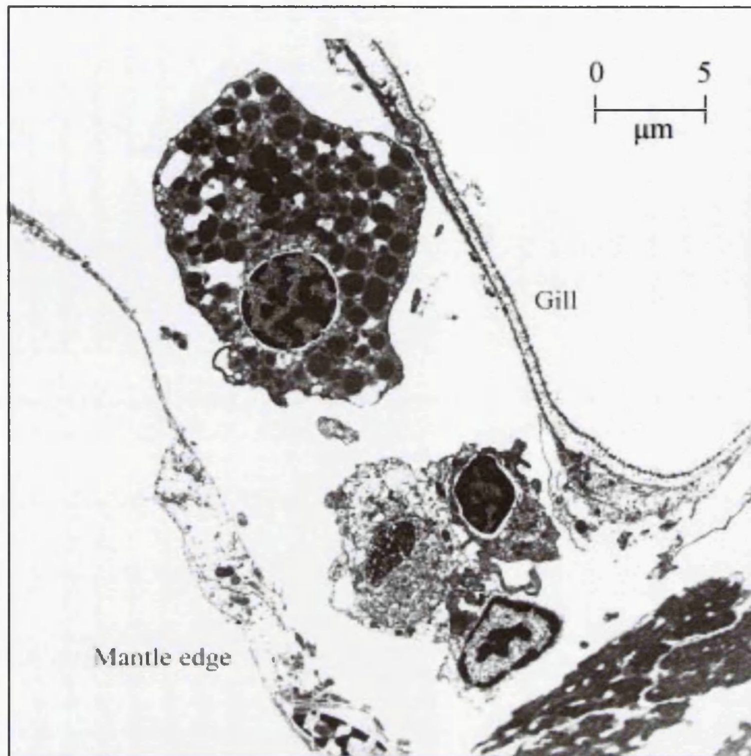


Figure 2.3. TEM of *M. edulis* postlarval haemocytes in situ, 5,000 x magnification. Scale bar = 5μm

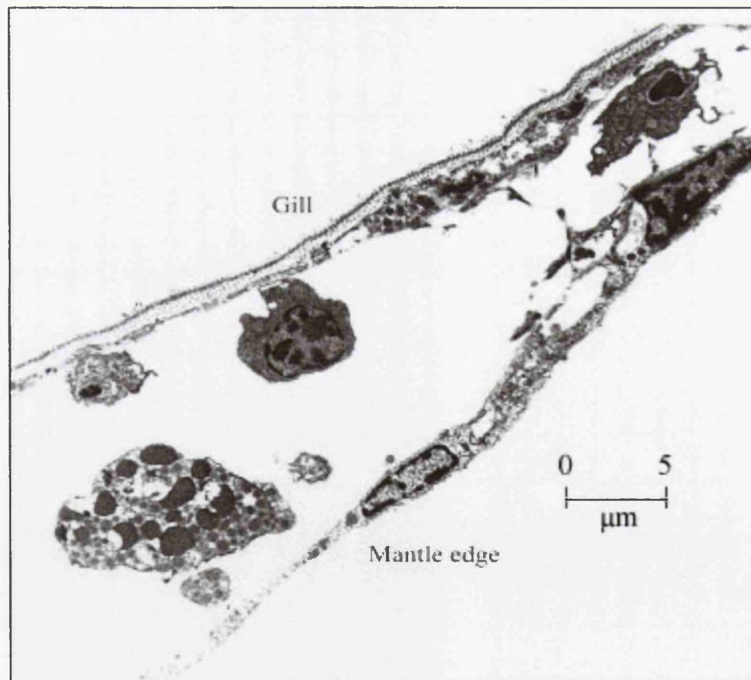


Figure 2.4. TEM of *M. edulis* postlarval tissues showing agranular haemocyte in situ, 5,000 x magnification. Scale Bar = 5μm.

2.3.3 Electron microscopy of haemocytes from postlarval blood samples

Adult haemocytes

Examination of haemolymph samples extracted from adult mussels resulted in the identification of the three blood cell types previously documented, granular cells containing large granules, granular cells containing small granules and agranular cells.

Haemocytes with large electron-dense granules are shown in Figures 2.5A and B, these granules range in size from 0.4-0.8μm. A typical granular cell that contains small electron-dense granules, granule size range of 0.15-0.3.μm, is shown in Figure 2.5C. Possible phagocytic vesicles (arrowed) can be seen in another haemocyte with small granules, Figure 2.5D. A small granule haemocyte that is heavily degranulated (arrow) is shown in Figure 2.5E. Typical agranular haemocytes (hyalinocytes) are shown in Figures 2.5F and G, the hyalinocyte in 2.5F has a filopodium extending from the plasma membrane. Filopodia are characterised by being smaller and narrower than pseudopodia. The hyalinocyte in 2.5G contains an electron-dense particle around which more than 20 mitochondria are clustered. Figure 2.5 H is a haemocyte with small granules showing evidence of phagocytic activity with the formation of a phagocytic vesicle (asterisk).

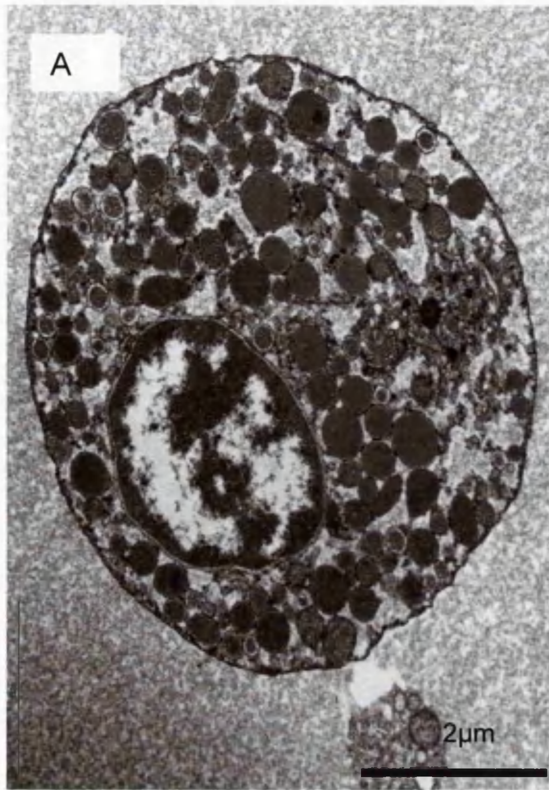
Postlarval haemocytes

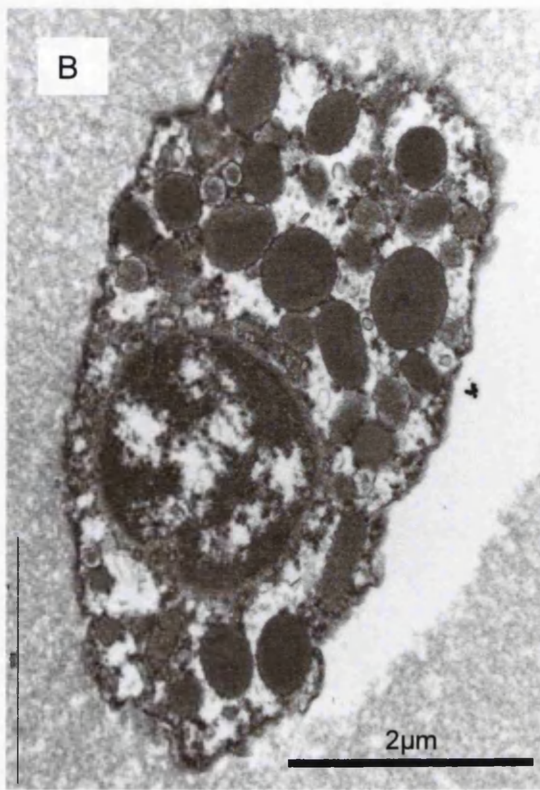
All three blood cell types seen in adult mussels were seen in postlarval haemolymph samples processed for electron microscopy, in addition a blood cell type not previously described, but also seen at light microscope level, was also identified.

Agranular haemocytes which appear to be identical to those are seen in adult mussels were identified in postlarval haemocyte samples, (Figures 2.5I and 2.5J). When seen in the postlarvae these cells often contain numerous mitochondria, as shown in Figures 2.5I and J. The haemocyte shown in Figure 2.5K is an agranular cell containing mitochondria which, in addition, also has prominent Golgi apparatus and filopodia. Figures 2.5L and 2.5M show granular haemocytes with small granules, again as seen in the adults. Vesiculation can be seen in the small-granule haemocyte in Figure 2.5L. Haemocytes containing large granules of the type seen in adult mussels were also seen in the postlarval blood samples (Figure 2.5N).

Fig 2.5A-D

Fig 2.5 E-H





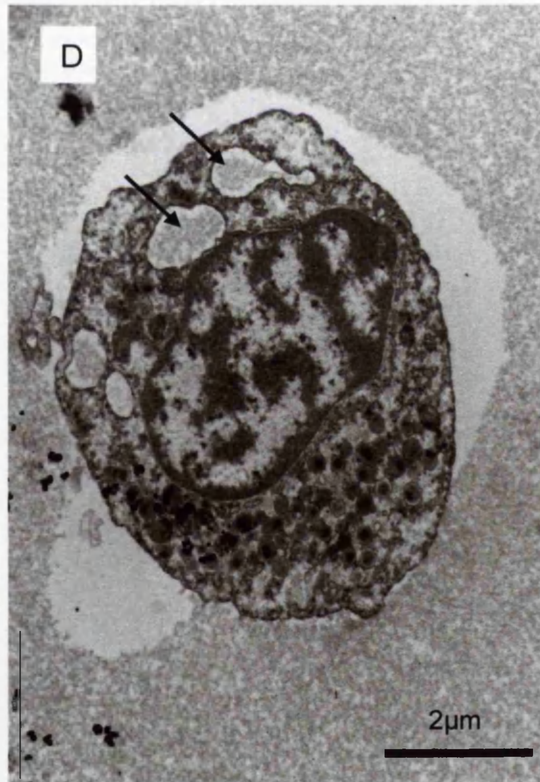
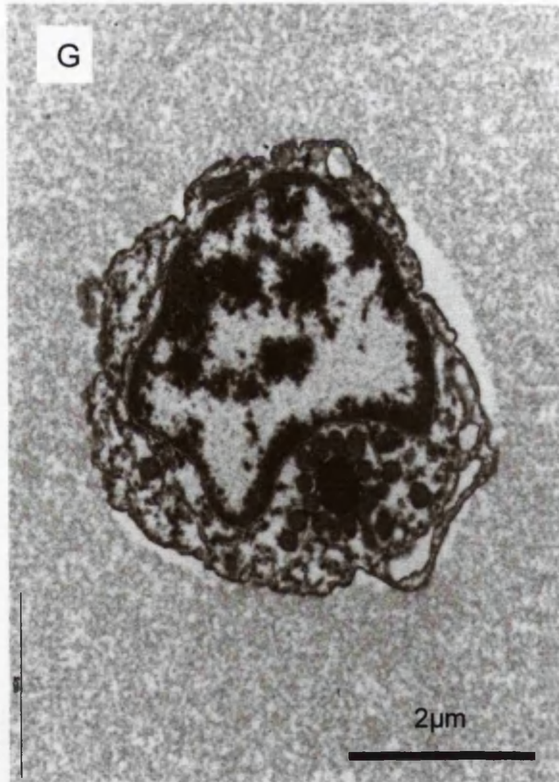


Figure 2.5A-D. Adult *M.edulis* haemocytes, ultrathin sections stained using lead citrate and uranyl acetate. Figure 2.5A and B show granular haemocytes with large granules. Figure 2.5C granular haemocyte with small granules, Figure 2.5D shows granular haemocyte with small granules and phagocytic vesicles (arrows).



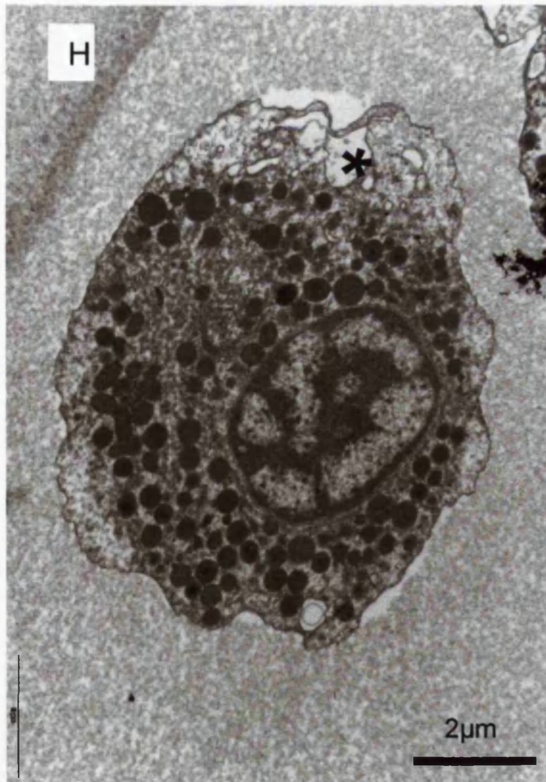
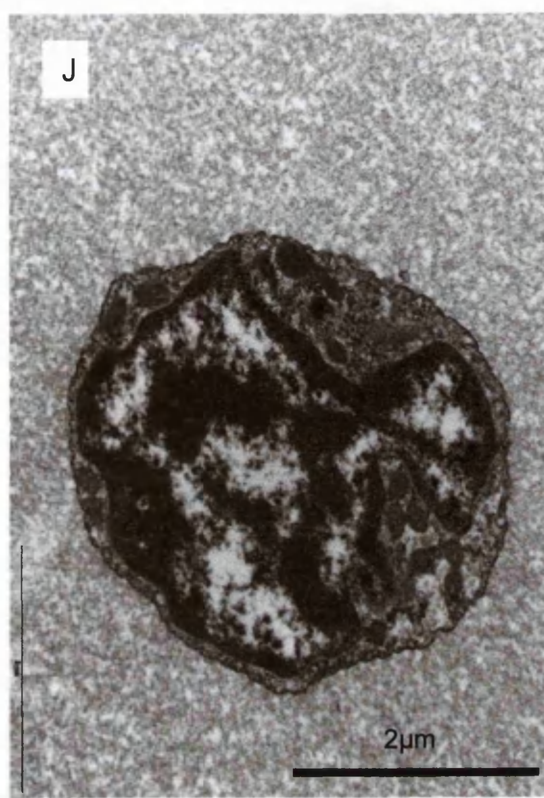
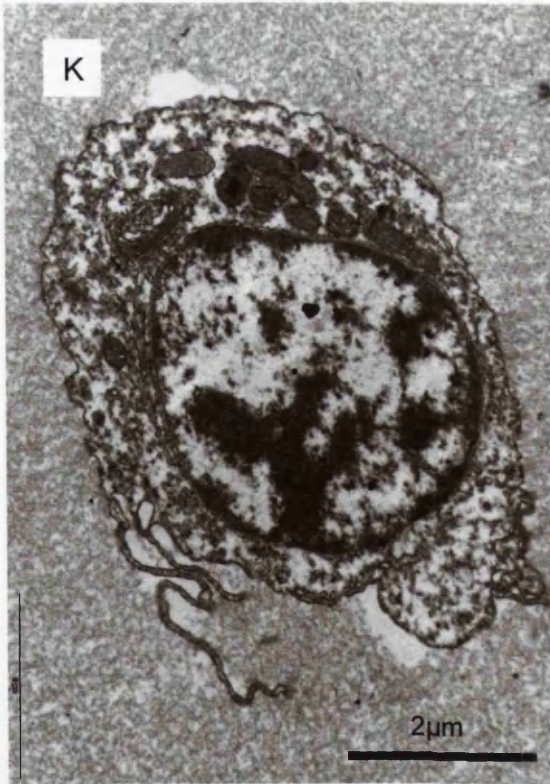


Figure 2.5E-H. Adult *M.edulis* haemocytes, ultrathin sections stained using lead citrate and uranyl acetate. Figure 2.5E shows a largely degranulated small-granule haemocyte. Figure 2.5F and G show agranular haemocytes. Figure 2.5H granular haemocyte with small granules and phagocytic vesicle (asterisk).





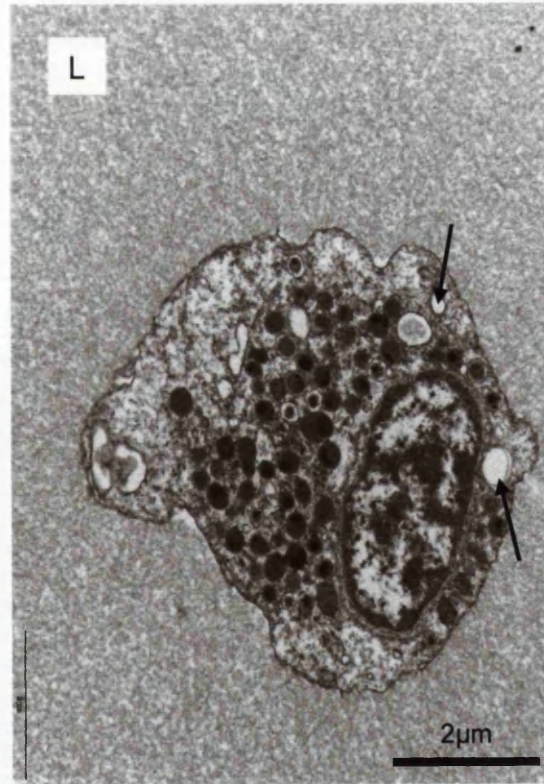
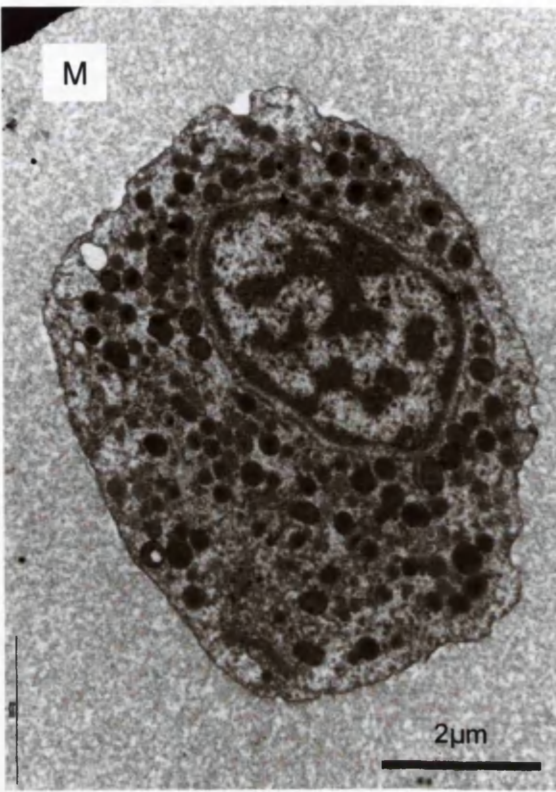


Figure 2.5I-L. Postlarval *M.edulis* haemocytes, ultrathin sections stained using lead citrate and uranyl acetate. Figure 2.5I, J and K show typical agranular haemocytes. Figure 2.5L granular haemocyte containing small granules, showing evidence of vesiculation (arrows).



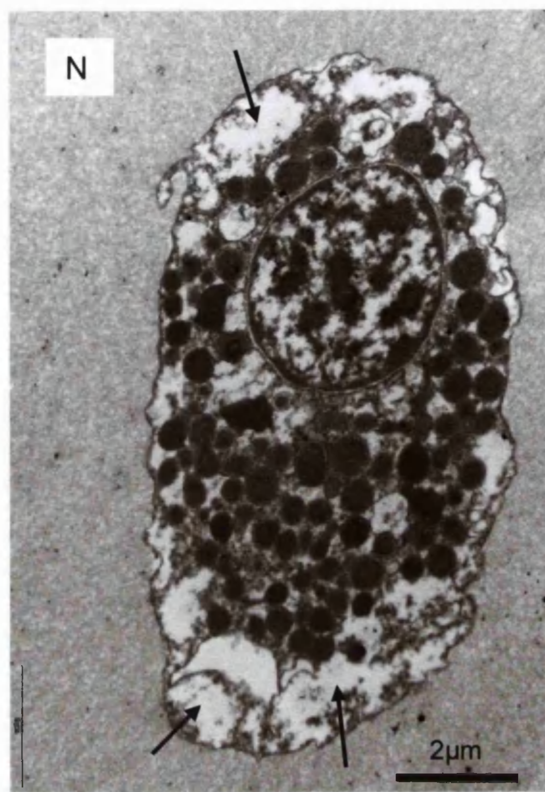


Figure 2.5M-P. Postlarval *M.edulis* haemocytes, ultrathin sections stained using lead citrate and uranyl acetate. Figure 2..5M granulair haemocyte containing small granules. Figure 2.5N, O and P show granular haemocytes containing large granules, showing varying levels of degranulation (arrows).

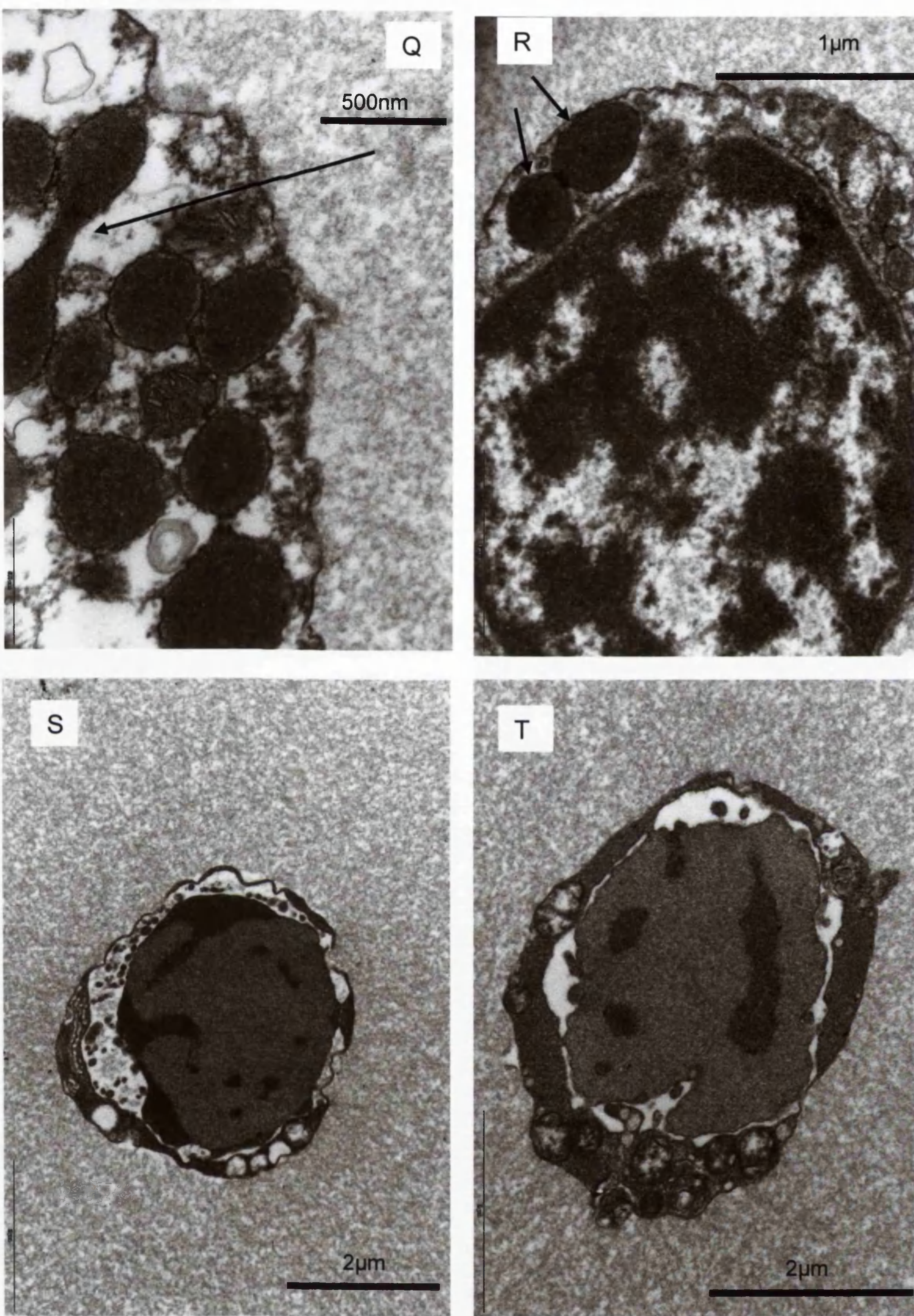


Figure 2.5Q-T. Postlarval *M.edulis* haemocytes, ultrathin sections stained using lead citrate and uranyl acetate. Figure 2.5Q granules within a haemocyte undergoing division (arrow). Figure 2.5R previously unreported haemocyte, granules arrowed. Figure 2.5S and T agranular haemocytes showing condensed nuclei, characteristic of apoptosis.

The large-granule haemocyte, Figure 2.5N., shows some degranulation around the cell margins (arrowed). Figure 2.5O shows a haemocyte containing large granules with further signs of degranulation, a granule that appears to be in the process of breaking down is next to the area of degranulation (arrowed). A large-granule haemocyte with over 50% degranulation is shown in Figure 2.5P. Granules contained within haemocytes are membrane bound, as demonstrated by Figure 2.5Q, the granule arrowed in this figure may be undergoing division as it appears dumbell-like in shape and is also in close proximity to a number of mitochondria. It is possible that granules are able to multiply by division; the proximity of the mitochondria implies that the process of granule division may require energy input. The previously unreported haemocyte type seen at the light microscope level, when it is characterised by being basophilic in nature with the cytoplasm containing a small number (1-4) of strongly eosinophilic granules, is shown in Figure 2.5R.

Some postlarval haemocytes appeared to show morphological changes characteristic of apoptosis, Figures 2.5S and 2.5T show what appear to be apoptotic agranular haemocytes. The chromatin within the nuclei is very condensed, there is very little cytoplasm indicating that cell shrinkage has occurred.

2.4 Discussion

Adult haemocytes fall into three categories: agranular hyalinocytes; haemocytes containing small granules; and haemocytes containing large granules (Pipe, 1990). All three of these haemocyte types were seen in the present study in both the haemolymph samples taken from the adult mussels and the samples taken from mussel postlarvae. In addition to the three haemocyte cell types seen in adult mussels a haemocyte type not previously described was seen in the postlarval samples that were examined. These cells were characterised as being largely agranular but did, however, contain a small number of granules (1-4). The numbers of these previously unseen cells seen decreased as the size of the postlarvae increased (Figure 2.2). When differentially stained the cell was basophilic in nature and contained a few eosinophilic staining granules. The previously unseen cell type was also seen in the postlarval haemocyte samples examined using electron microscopy.

Dyrynda (1995) reported that there was no reactivity with antibodies to eosinophilic haemocytes in cells from disaggregated mussel larvae, suggesting that eosinophilic cells may be last in a cell line and only present at comparatively late stages of the life history. Haemocytes were not identified in the larval sections examined in the present study (Chapter 3). The observations on postlarval haemocytes may support the postulation that haemocytes originate from a single cell line with eosinophilic cells being formed late in the cell line and therefore only present at comparatively late stages of *M. edulis* development. The percentage of eosinophilic cells seen in the smallest postlarvae examined was low, increasing with increasing size of the animals examined (Figure 27.2). The putative new blood cell type described in these small postlarvae may be a precursor to the eosinophilic cells seen in adults. As discussed previously there are two main schools of thought on the ontogeny of mussel blood cells. Cheng's (1981) theory proposes that there are two distinct cell lines one for basophilic haemocytes and another for eosinophilic haemocytes whilst Mix (1976) suggests that haemocytes originate from one common stem cell which develops into the hyalinocyte (or agranular) cell. Moore and Lowe (1977) also suggest that haemocytes originate from two separate lines. Small basophilic cells (agranular hyalinocytes) are thought to be part of a developmental series of basophilic cells that develop into larger macrophages whilst the eosinophilic granular haemocytes represent a separate cell line (Moore & Lowe, 1977). The new putative blood cell type recorded here appears to be a largely agranular cell with eosinophilic granules and may represent a precursor to the mature eosinophilic cell. This supports Mix's (1976) model of haemocyte development, in that it is possible that the agranular hyalinocytes are the precursor cells to all three mature haemocyte cell types seen in adult mussels. Bachère et al. (2004) feel that according to their maturation and activity, agranular cells may evolve from young cells with a high nucleocytoplasmic ratio to large haemocytes with a small nucleus and cytoplasm rich in vesicles.

Apoptotic agranular haemocytes were noted when examining samples of postlarval haemolymph using electron microscopy. Apoptosis is the process of programmed cell death; cells essentially commit suicide by activating an intracellular death programme. It is characterised by cytoplasm shrinkage, condensation and degradation of the chromatin in the nucleus, development of bubble-like blebs on the cell surface and break down into small membrane-bound fragments. In contrast cell death as a result of injury or damage (necrosis) is characterised by swelling due to the loss of homeostatic

control, the cells and their organelles swell and the contents of the cell leak out leading to inflammation of the surrounding tissues. Studies of the role of apoptosis as a defence mechanism have been undertaken in organisms ranging from nematodes to mammals (Goedken et al., 2005). Host cells infected by protozoa, bacteria, or viruses may counteract these intracellular infections by initiating apoptosis and their subsequent removal by phagocytosis (Luder et al., 2001). Until recently little had been documented regarding apoptosis of bivalve haemocytes, despite there being many studies that show increased hemocytes numbers in circulation as a result of exposure to pollutants. However, haemocyte apoptosis has recently been reported in *Crassostrea gigas* (Terahara et al., 2003; 2004) and *Crassostrea virginica* (Goedken, 2005). Terahara et al. (2004) demonstrated that both adherent and non adherent haemocytes extracted from *C. gigas* and treated with the fibronectin peptide containing the Arg-Gly-Asp (RGD) motif simultaneously undergo apoptosis. Goedken et al., (2005) have quantified *C. virginica* haemocyte apoptosis, finding that apoptosis was higher in granulocytes compared with hyalinocytes. This is postulated to be a result of granulocytes having higher phagocytic and respiratory burst activity. Sunila and LaBanca (2003) also reported increased haemocyte apoptosis in *C. virginica* mucosa and submucosa. In contrast to both of these findings, in postlarval mussels, apoptosis appeared to be higher in agranular (hyalinocytes) haemocytes compared with granular cells. This may be due to differences in the cause of apoptosis between developmental stages, the apoptotic haemocytes noted in the present study were from postlarvae whereas Goedken et al. (2005) and Sunila and LaBanca (2003) studied adult individuals. It is possible that the apoptosis seen by Goedken et al. (2005) and Sunila and LaBanca (2003) in adult oyster haemocytes was a result of cells that had phagocytosed foreign materials, indeed both groups were studying apoptosis and its role in defence against the protozoan parasite, *Perkinsus marinus*. The apoptosis seen in postlarval haemocytes in the present study may be due to a rapid rate of cell turnover. Many of the postlarval haemocytes contain large numbers of mitochondria, or prominent Golgi apparatus, both of which can also be associated with cell turnover, tissue development and morphological changes in organisms. The Golgi apparatus modify and process proteins and fats from the endoplasmic reticulum, preparing them for export outside the cell, prominent Golgi apparatus often indicate that the tissue is active. Mitochondria are the main power generators of the animal cell, the number of mitochondria is based upon the cell's level of metabolic activity, demonstrating that these haemocytes have high energy

requirements and are possible still developing. Moore and Lowe (1977) reported that agranular cells were characterised by small amounts of surrounding cytoplasm with a few mitochondria. However, in the postlarvae examined in the present study agranular haemocytes were often found to contain numerous mitochondria, as shown in Figures 2.5I, J and K. The haemocyte pictured in 2.5K also contains prominent Golgi apparatus. These observations lend further support to the theory that agranular cells are part of a continuous series of related cells and are the precursor cells to all three mature *M. edulis* haemocyte cell types.

In conclusion, haemocytes from postlarval mussels were examined at both the light and electron microscope level. All three haemocyte types seen in adult *M. edulis* were identified in the postlarvae and in addition a novel haemocyte was found. This novel haemocyte was basophilic in nature when differentially stained but was also seen to contain a few eosinophilic granules. It is possible that this cell type is a precursor to the eosinophilic haemocyte seen in postlarvae and adults, and its existence lends support to the theory that all mussel haemocytes originate from one common stem cell, as originally proposed by Mix (1976).

Chapter 3. Susceptibility of larvae to bacteria.

3.1 Introduction

3.1.1 Developmental stages of mussels

Bivalves undergo an annual reproductive cycle which involves a period of gametogenesis followed by a single, an extended or even several spawning events, which in turn is followed by a period of gonad reconstitution (Gosling, 2003). Mussels are gonochoristic i.e. they have separate sexes (apart from the occasional hermaphrodite) and shed gametes into the sea, demonstrating external fertilisation and pelagic planktotrophic larvae. Many populations of *Mytilus edulis* exhibit some spawning all year round, with major peaks of spawning in spring. Often, after a further period of gametogenesis, a number of "opportunist" spawnings occur later in the summer/autumn. More restricted spawning periods occur when adult feeding conditions have been poor over the preceding winter and continue to be so into the spring. The pattern of two spawning periods per year is generally seen in Southern Britain, with populations living in the north only spawning once a year; in the late spring (Seed, 1976). Lowe et al. (1982) recorded mussels from Anglesey having only one spawning event in the spring, whereas mussels from Plymouth had 2 distinct spawning events, one in the spring and a second in the summer/autumn. Lebour (1938) records the spawning period of *M. edulis* in Plymouth as between April and June with the resultant veligers dominant in late spring-early summer. The reproductive cycle of *Mytilus* also varies geographically from east to west; spawning on the west coast of Britain occurs a few weeks earlier than on the colder east coast (Seed & Suchanek, 1992). Temperature is the principal factor controlling the reproductive cycle (Seed, 1976), however, it is also affected by food supply and the nutrient reserves of each individual animal. Food supply, in the form of phytoplankton, varies in a cyclical manner throughout the year, being lower in the winter than in the summer. The variation in food supply and temperature changes result in the existence of "winter" and "summer" physiological states in ostreid bivalves (Hawkins et al., 2005). The different metabolic responses resulting from this bi-stable physiology can also effect immunocompetence (Hauton et al., 2001).

Gametes of *M. edulis* are generated within the gonadal tissue of the mantle (White, 1937; Seed & Suchanek, 1992) and are discharged via paired gonoducts from each gonad. These unite into a common duct that opens on the genital papilla, situated anterior to the posterior adductor muscle (Seed, 1976). The gametes are shed directly from this duct into the water column where fertilisation takes place. The acrosome reaction facilitates the penetration of sperm into the egg (Niijima & Dan, 1965; Pipe & DaSilveira, 1989). The initial cleavage division typically occurs within one hour of fertlisation (Lutz et al., 1991). The fertilised egg then rapidly divides to become a ball of cells, the first cilia appearing after 4-5 hours (at 18°C), after which the embryo begins to swim (Bayne, 1976). In ideal conditions, the trochophore stage will be reached 12 hours after fertilisation, however, commonly, it is reached approximately 24-48 hours after fertilisation (Loosanoff & Davis, 1963). The trochophore possesses a ring of cilia at its anterior end as well as flagella and as a consequence they are strong swimmers. This stage is non-feeding and nourishment is provided by the yolk from the egg.

The trochophore stage is relatively brief, and rarely seen outside of the laboratory. The shell gland is present at the dorsal end and is responsible for secreting the first larval shell. The first larval shell, the prodissoconch I, is secreted around 24-48 hours after the larva reaches the trochophore stage. The shell is D-shaped in outline (larvae at this developmental stage are sometimes referred to as D-shells) with a straight-hinge and a shell length of 100 to 120μm (Sprung, 1984). This planktotrophic (plankton-feeding) stage is more commonly termed the veliger stage (Lutz & Kennish, 1992). The larvae are relatively simple, with a ciliated velum that functions as both a feeding and a swimming organ. The veligers have a functioning gut. The shell is thin and transparent with the internal organs clearly visible through it. The 2 prodissoconch stages (I and II) can last up to 4 weeks and are characterised by rapid larval growth, increasing from approximately 120 to 250μm in length, but with little increase in morphological complexity (Bayne, 1976). The velum is a circular lobe of tissue bearing a ring of cilia that develops from the apical plate of the trochophore. Small particles (1-2μm diameter) caught by the cilia are swept towards the mouth and onwards in a simple gut (Gosling, 2003). This gut lacks muscles, the movement of food is accomplished by cilia alone. Flagellate algae, such as *Monochrysis* and *Isochrysis*, devoid of a cell wall and forming no toxic substances, constitute the best food for larvae (Ukeles, 1969).

As soon as the larva develops into prodissoconch I it immediately starts to develop into the next stage, it secretes a second shell, or prodissoconch II (Gosling, 2003). The mantle, rather than the shell gland, secretes the prodissoconch II, which has concentric growth lines (Millar, 1968). This shell has a more pronounced umbonal region near the hinges and is rounded in profile. This transition from the "straight hinge" to the "umbo" stage of development usually occurs at a length of around 140 to 150µm (Lutz & Kennish, 1992). At a shell length of between 220 and 260µm, the veliger acquires a pair of pigmented "eye spots" followed shortly after by the development of a foot (Bayne, 1976). The larva is now known as a pediveliger, this is the stage that immediately precedes settlement and metamorphosis. The larval life of *Mytilus* lasts for a minimum of 3 weeks at 18°C with the larvae showing a maximum growth rate of ca. 12µm per day at this temperature, and metamorphosis occurring at a shell length of ca. 300µm (Widdows, 1991).

The pedal organ, or foot, of the pediveliger rapidly becomes functional (Lutz & Kennish, 1992) with individuals having the ability to actively extend the organ and creep along. The pediveliger also retains the ability to swim by means of its developed velum. The ultimate objective of the pediveliger is to select and colonise a substrate and metamorphose into its adult form (Kasyanov et al., 1998). As such, it is morphologically more complex than the D-shell stage with the general morphological features of pediveliger larvae having been described by Bayne (1971). These morphological features can be summarised as follows (after Bayne (1971) and Lutz & Kentish (1992)):

- A large velum that is used for swimming and feeding
- A foot that is used for feeding and has a byssus system capable of secreting byssal threads
- A thin mantle that secretes the shell
- A mouth, oesophagus, stomach (with style sac and large digestive gland), and a simple intestine
- A ciliated palp that sorts food particles
- 2 or 3 pairs of gill filaments

- A nervous system comprising cerebral, pedal, and visceral ganglia, together with a sensory system of statocysts (gravity sensors), apical plate, and pigment spots also referred to as "eye spots"

Once the pediveliger comes into contact with a suitable substrate, it will settle and metamorphose into a postlarva which is the earliest stage of the adult form.

3.1.2 Bacteria associated with mussels

Since bivalves live in an environment rich in bacteria, their filter feeding habit results in ingestion of many kinds of microorganism which may be potentially pathogenic to either the animal itself or consumers of the animal. As a result of the commercial interest in several species of bivalve (oysters, mussels, clams and scallops), bacteria associated with their tissues were first investigated in regard to their effect on food quality (Prieur, 1987). The general public on the whole consider mussels and oysters as relatively unsafe to eat; fearing consumption will result in food poisoning. It is the case that edible bivalves can accumulate large numbers of bacteria in their tissues and may act as passive carriers of human pathogens (Zampini et al., 2003). The accumulation of microorganisms by bivalves, coupled with the tradition of consuming them raw or lightly cooked, means that they do act as potential vectors for human infection from a number of water-bourne agents such as bacteria, viruses and algal toxins (Gosling, 2003). The diseases associated with shellfish range from serious infectious diseases including typhoid, paratyphoid, cholera, and dysentery, to miscellaneous cases of more or less serious gastroenteritis (Prieur et al., 1990).

We have come a long way since the 1900's, when Asiatic cholera and typhoid fever were commonly associated with the consumption of polluted shellfish (Shumway, 1992). Under the EC Shellfish Hygiene Directive (91/492/EEC) areas from which shellfish are to be harvested are now classified, and treatments to be taken before the bivalves are consumed have been laid down. Classification of these areas in England and Wales is based on the *Escherichi coli* content of the flesh; there is no equivalent classification for viral contamination. Shellfish are also regularly sampled for the presence of toxins produced by microalgae (Laing et al., 2004). Bivalve cultivation is frequently carried out in areas affected by blooms of toxic microalgae, as the bivalves themselves appear not to be harmed by the algae (Laing & Spencer, 1997). The toxins

from the algae accumulate in the flesh of the bivalves and people eating contaminated meat can become ill from paralytic, amnesiac or diarrhoetic shellfish poisoning. These toxins are not denatured by cooking. As a consequence, if the toxin level exceeds a certain threshold in a sampled population, the collection and sale of shellfish is prohibited from that area until the level falls back below the threshold (Laing et al., 2004). The mussels used in the present study were collected from Whitsand Bay, Cornwall. These mussels have been allocated to Class B in the 1992 Provisional Harvesting Classification List (MAFF, 1995). Table 3.1 shows the classification criteria for harvesting mussels, note that this classification relates purely to microorganisms that are detrimental to human health. The Whitsand Bay mussels can therefore be considered to be exposed to relatively low levels of microbial pollution in their natural environment, and are consequently relatively "clean". However, as will be shown later (Chapter 4), they are still heavily infected with *Vibrio* species.

Class	Microbiological standard	Treatment Level
A	All samples contain <300 faecal coliforms or <230 E.coli $100g^{-1}$ of mollusc flesh	None; suitable for consumption, can be marketed
B	90% of samples must not exceed 6,000 faecal coliforms or 4,600 E coli $100g^{-1}$ of mollusc flesh	Depuration needed (either by relaying to Category A area or heat treat by an approved method).
C	All samples must not exceed 60,000 faecal coliforms $100g^{-1}$ mollusc flesh	Relay for minimum of 2 months in an approved relaying area, or heat treat by an approved method
D	Do not conform to at least Class C	Prohibited

Table 3.1. Criteria for the classifying shellfish harvesting areas (Laing et al., 2004).

In addition to human pathogens and algal toxins, bivalves accumulate pathogenic bacteria that they can be sensitive to. Whilst the majority of bacteria in the environment are not harmful, some can be facultatively pathogenic to weakened animals (Gosling, 2003). However, bacterial diseases of molluscs are relatively insignificant in populations of adult animals, commonly only affecting the early life history stages.

This is in part due to the primary defence mechanisms of molluscs, including phagocytosis and encapsulation, which are very efficient at dealing with pathogens (Feng, 1988).

Not only are bivalve larvae exposed to bacteria in their surrounding environment but direct transfer of bacteria from "parent" or "adult" to larvae has also been reported. For example, Riquelme et al. (1994) reported evidence for the vertical transfer of bacteria from scallop (*Argopecten purpuratus*) gametes to larvae. The genus *Vibrio* is one of the most frequently detected in samples of shellfish, dominating the bacteria found in tissue samples although it does not dominate the microbiota of their surrounding water (Pujalte et al., 1999). *Vibrio* species cause vibriosis, or bacillary necrosis, in bivalve larvae in culture environments. Bacterial disease is commonly seen in hatcheries due to the artificial culturing conditions, with larvae and juveniles being stocked at high densities. Disease outbreaks can wipe-out entire cohorts, thus having a major impact on the aquaculture of oysters and mussels. The effects of *Vibrio* species on wild populations of bivalve molluscs are less well known. Disease outbreaks have been linked to a number of bacteria including *Vibrio tubiashii* (Hada et al., 1984) and *Vibrio alginolyticus* (Gosling, 2003). Virulence of these *Vibrio* infections is reported to be so great as to cause almost 100% mortality in 24 hours (Perkins, 1993). Vibriosis can occur in any marine hatchery since the causative bacteria are ubiquitous, however, they are opportunistic pathogens and disease is normally associated with suboptimal conditions including warm temperatures (Elston, 1993). Studies have shown that high temperature stress accompanied by *Vibrio* infections have caused mortalities of *Crassostrea gigas* (Sinderman, 1988). This may have been due to increased pathogen populations caused by the higher temperatures or due to the raised temperature compromising the oysters' immune system.

The bacteria used in the present study were obtained from The National Collections of Industrial and Marine Bacteria ltd, Aberdeen (NCIMB) and had previously all been isolated from molluscs. The species selected for larval exposure experiments were: *Vibrio tubiashii* and *Vibrio alginolyticus*. *Vibrio tubiashii* was originally characterised by Hada et al., (1984) who demonstrated that six strains of bacteria isolated during unrelated incidents of disease outbreaks were distinct from other species of *Vibrio*. These strains were then aggregated into a new species, *Vibrio tubiashii*. This species

was also shown to be pathogenic to *Crassostrea gigas* and *Ostrea edulis* larvae (Jeffries, 1982) and was consequently chosen for the present study. *Vibrio alginolyticus* is pathogenic to humans causing food poisoning, evidenced by chronic diarrhoea, as well as causing wound infections in individuals with occupational activities around seawater (Hlady & Klontz, 1996). *Vibrio alginolyticus* has also been demonstrated to be pathogenic to white shrimp (*Litopenaeus vannamei*) under nitrite stress (Tseng & Chen, 2004), and it has also been isolated from cultured tiger prawns (*Penaeus monodon*) during outbreaks of mass mortality (Lee et al., 1996).

3.2 Development of exposure method

3.2.1 Bacterial viability in exposure vessels

The *Vibrio alginolyticus* used was NCIMB culture number 1339: isolated by Tubiash (1965) from moribund larvae of the hard clam (*Mercenaria mercenaria*). The *Vibrio tubiashii* was NCIMB culture number 1336: this was also isolated by Tubiash (1965) from moribund larvae of *M. mercenaria*. Both species of bacteria are Gram negative.

The bacteria were supplied in ampoules containing freeze-dried cultures. The cultures were revived as instructed by NCIMB, briefly, the bacteria were suspended in 0.5ml of liquid media recommended for the strain. Sea water agar is recommended for both species: 10.0g Beef extract (Lab-Lemco), 10.0g neutralized bacteriological peptone, 750ml filtered, aged seawater and 250ml distilled water. The pH was adjusted to 7.8 and the agar then boiled for 3 to 5 minutes before cooling and filtering. The pH was then readjusted to 7.3 and the agar autoclaved at 121°C for 15 minutes. Solid seawater agar was made by adding 15g agar L^{-1} after readjusting the pH, prior to autoclaving. Liquid media were inoculated with the revived bacteria, as were solid agar slopes to serve as stock cultures.

In accordance with NCIMB recommendations, the bacteria were sub-cultured at least twice before use in experiments. A commercial agar, Marine Broth (BD, Oxford, U.K) was tested for suitability as a culture medium. It was found to support growth of all 3 bacterial species; subsequently the bacteria were maintained in this medium due to its ease of use (heat 37.4 g of Marine Broth in 1 litre of distilled water, boil for 1 to 2

minutes, then autoclave at 121°C for 15 minutes). Bacterial concentrations were determined using turbidometric methodology, samples were loaded into a 96 well microplate and absorbance measurements were read at 550nm (Smith et al., 1995).

Initial experiments inoculating cultures of larvae with *Vibrio tubiashii* demonstrated that the bacterial levels in the seawater declined rapidly, with live bacteria being undetectable within 24 hours. It was therefore necessary to add nutrients to the larval cultures to maintain the bacteria for the duration of the experiments.

Two different bacterial culture media were examined for their ability to maintain bacteria abundance, in the absence of larvae, around the initial dosing concentration for the 24 hours between water changes.

- Marine Broth (Difco)

Range finding experiments were performed to determine the lowest concentration of Marine Broth at which bacteria remained viable for 24 hours. Solutions of 100%, 10%, 5%, 2% and 1% Marine Broth in filtered (0.2µm membrane) seawater were autoclaved and inoculated with 6×10^5 *Vibrio*/ml of culture media.

- Thiosulfite citrate bile sucrose (TCBS) agar (Merck)

TCBS agar is used for the isolation and selective cultivation of *Vibrio cholerae* and other enteropathogenic *Vibrios*. Larvae were exposed to a 5% solution of TCBS agar in filtered (0.2µm) seawater. The agar was present in the larval cultures in one of two forms, a solidified layer on the base of the vessel or in solution. The larvae were also exposed to both forms of TCBS agar and *V. tubiashii* at a concentration of 1×10^4 bacteria/ml.

After 24 hours the abundance of *V. tubiashii* in vessels containing Marine Broth were as follows:

Percentage Marine Broth	Bacterial abundance after 24 hours
100%	1.01×10^8 cells ml^{-1}
10%	2.1×10^7 cells ml^{-1}
5%	1.8×10^6 cells ml^{-1}
2%	4.1×10^4 cells ml^{-1}
1%	undetectable

Table 3.2: Results of bacterial viability in exposure vessels containing Marine Broth after 24 hours.

It was therefore shown that cultures of 100%, 10% and 5% Marine Broth maintained bacterial growth, but bacterial abundance in cultures of 2% and 1% Marine Broth declined over 24 hours. A solution of 5% Marine Broth was used in all subsequent exposure experiments, since it was the lowest concentration that maintained bacteria growth. Exposure of larvae to a solution of 5% Marine Broth was shown not to effect the survival of larvae. A sub-sample of 100 D-shell larvae were removed from each of 3 exposure vessels containing 5% Marine Broth for assessment. From these subsamples, 300 larvae in total, 287 were found to be viable (using morphological assessment criteria detailed in 3.2.2). This process was repeated with the "control" vessels containing filtered seawater alone; of the sub-sample of 300 larvae 293 larvae were found to be viable. Thus demonstrating that the presence of Marine Broth alone, at a concentration of 5%, did not affect the viability of larval *M. edulis*.

Larvae exposed to TCBS agar were found to die within 24 hours regardless of bacterial treatment. When larvae were placed on a TCBS agar plate containing 1% NaCl they were found to lyse within 5 minutes. TCBS agar has a much higher enzymatic content than Marine Broth. Marine Broth contains 5g peptone per litre, whereas TCBS contains 10g of peptone per litre as well as 5g of "Bacto oxgall" (ox bile). Bile aids digestion of fats and peptones act to break proteins down into their constituent amino acids. High levels of these compounds may lead to "digestion" of the larvae, consequently it deemed unsuitable for use in exposure experiments.

3.2.2 Quantification of larval viability

Methodology for assessing larval viability was developed so that the effects of exposure to bacteria could be evaluated. Various measures of viability could be used, such as vital staining, morphological/behavioural criteria or a combination of both.

Ripe mussels, *Mytilus edulis*, were collected from the field (Whitsand Bay, Cornwall) and were placed in holding tanks at 15°C. Encrusting organisms removed and external byssus threads cut off before spawning was induced. The posterior adductor muscle was injected with 0.5ml of 0.5M potassium chloride solution and the mussels placed into a tank of filtered seawater at 15°C. After 30 minutes the temperature of the seawater was raised to approximately 25°C and the mussels were then immediately transferred to fresh filtered seawater (15°C). The thermal shock treatment was repeated, up to a maximum of 4 times, until mussels began spawning. Mussels not producing significant quantities of gametes within 3 hours were rejected.

Spawning mussels were transferred to individual containers with filtered seawater. On cessation of spawning, mussels were removed from the containers and discarded. Eggs were then filtered through a 200μm mesh into a 500ml flask containing filtered seawater (concentration 25 eggs ml^{-1}). A small volume of sperm was added to the cylinders and after 30 minutes the eggs were examined for fertilisation success rate, more sperm could then be added if necessary. A 30μm mesh was used to concentrate eggs and larvae.

The fertilised eggs were collected and resuspended in fresh filtered seawater. All cultures were kept at 15°C, at which temperature trochophore larvae were present approximately 24 hours post fertilization. After a further 24 to 48 hours, the larvae reached the D-shell stage. On reaching this stage, the larvae were fed *Dunaliella tertiolecta* daily, at a concentration of 150 cells μl^{-1} of larval suspension, and the cultures were aerated (seawater was changed daily). Concentrations of *Dunaliella tertiolecta* were determined using an improved Neubauer haemacytometer. All experiments were carried out in 500ml vessels at 15°C and the larvae were kept at maximum concentration of 25,000 larvae $litre^{-1}$ of seawater (25 larvae ml^{-1} of seawater).

Staining criteria

Vital stains provide a method for distinguishing between viable (i.e., capable of growth) and nonviable cells. They are generally based on "dye exclusion": cells with intact membranes exclude the dye and are therefore considered viable. The dyes are cytotoxic, hence over a period of time (this varies with the stain used) all cells will eventually take up the stain. A sub-sample of larvae were taken from each exposure vessel for assessment, 100 from each vessel.

- *Eosin Y exclusion*

To one part of 0.2% eosin Y (w/v in phosphate buffered saline (PBS) containing 2% NaCl), one part of larval suspension was added. Eosin Y solution is stable at room temperature, if a precipitate formed the solution was passed through a Whatman #1 filter paper before use. The percentage of viable cells remains constant up to 10 minutes in the eosin Y solution. The number of viable larvae per ml was determined by pipetting 100µl of the suspension onto a glass slide, the percentage of viable larvae was then calculated.

- *Trypan Blue exclusion*

A 0.2% trypan blue saline solution (w/v in PBS containing 2% NaCl) was made up and filtered through a Whatman #1 paper. One part of larval suspension was added to one part of the 0.2% trypan blue solution. The larvae were then counted within 3 minutes, after which time viable larvae begin to take up the dye. The number of viable larvae/ml and the percentage of viable larvae were determined using the procedure described for eosin Y.

- *Nigrosin exclusion*

A 1% (w/v) nigrosin solution in PBS (containing 2% NaCl) was made up; this stock solution was filtered through a Whatman #1 paper. The 1% nigrosin stock was then diluted 1:10 in PBS, just prior to use and mixed with the larval suspension such that the larval suspension: nigrosin solution ratio was between 1:2 and 1:10. This mixture was then left for 5-10 minutes; before the percentage of viable larvae and number per ml were determined (as described for eosin Y).

- *Fluorescein Diacetate*

The fluorescein diacetate was added to acetone (5mg per ml of acetone), and stored in tightly capped containers at –20°C. The fluorescein diacetate solution was then diluted

for use (1:50) in PBS (with 2% NaCl) at room temperature, on dilution a fine suspension forms. One volume of this fluorescein diacetate suspension was then added to 9 volumes of the larval suspension (10^6 larvae/ml). The mixture was then left to stand at room temperature for 15 minutes. The larvae were examined using a (Zeiss Axiovert 100) fluorescence microscope, the viable larvae appeared green. The percentage of viable larvae was determined as described for eosin Y exclusion.

Behavioural and morphological criteria

Behavioural analysis involved assessing the swimming behaviour and general motility of the larvae. The morphology of the larvae was also examined, specifically looking for any gross abnormalities such as altered hinges and shells. Figure 3.4 shows live D-stage larvae. The use of vital stains to assess larval viability precludes the use of fixatives. However, assessment of larval viability using morphological criteria allows the use of fixatives such as Bakers formol calcium (4% formaldehyde, 2% NaCl, 1% calcium acetate) or EM fixative (2% formaldehyde, 2.5% glutaraldehyde, 2%NaCl, 2mM $CaCl_2$ in 0.2M cacodylate buffer, pH7.4). The addition of a small amount of fixative leads to the retraction of the velum (in normal larvae) after which more is added to complete the fixation.

The use of staining criteria to assess larval viability produced mixed results, each stain is considered individually below:

- Eosin Y exclusion

Trochophore larvae that were non-motile, and hence appeared to be non-viable, did not take up this stain, nor did D-shell larvae that had gross abnormalities of the velum and shell (Figure 3.1). The stain was only seen in a few D-shell larvae in which the internal organs could be seen to be degenerating and was not noted at all in the trochophore larvae. Figure 3.2 shows a viable trochophore larva in eosin Y solution.

- Trypan Blue exclusion

Trypan blue solution proved to be an unstable stain that often crystallised out of solution. As a consequence it frequently had to be filtered, however, this did not always prevent the stain aggregating when added to the larval culture. Few larvae took up the stain even when they appeared morphologically abnormal.

- Nigrosin exclusion

As for the 2 previous stains this dye only appeared to be taken up when the larvae were grossly abnormal or dead and decaying.

- Fluorescein diacetate

Clear differences in the intensity of fluorescence were visible in both trochophore and D-shell larval cultures (Figure 3.3). Further work to develop a method for grading the intensity of fluorescence would be needed if this stain were to be chosen as the sole method for assessing larval viability.

The alteration of larval morphology by pollutants is well documented; an American Society for Testing and Materials (ASTM) method exists for conducting acute toxicity test on embryos of four species of saltwater bivalve molluscs (ASTM, 1989). The assessment is based morphological criteria, the percentage of abnormal D-larvae is assessed after the fertilized eggs have been incubated for 1 or 2 days in water containing the pollutant under investigation (His et al., 1997). In the present study, once the methodology has been developed, the "pollutant" will actually be a potentially pathogenic bacterium. The chosen method for assessment of the effects of the bacterium should be sensitive enough to pick up sublethal effects.

Fixed larvae proved easier to examine, more detailed analysis of the morphology was then possible (Figure 3.5). Larval abnormalities have been used by many authors as a method for assessing larval "health", although the criteria used often differ (His et al., 2000). The ASTM (1980) consider that larvae possessing misshapen or otherwise malformed shells are normal, provided that shell development has been completed. Klöckner et al. (1985), however, consider that any retardation resulting in incomplete soft parts or deformed shells (asymmetrical shape, concave D-line, carved shells) to be abnormal.

Many different abnormalities in the larval cultures incubated with bacteria were noted. In trochophore cultures, these abnormalities included segmented eggs that have ceased development, malformed trochophores that appear blistered and larvae that are partially developed with retarded misshapen shell plates. In cultures of D-shell larvae, segmented eggs and larvae that had not reached the D-shell stage were considered abnormal. Abnormalities of the shell of D-stage larvae were also seen and recorded; these included those documented by His et al. (1999) such as incomplete shell

formation, indentations in the shell margins, convex hinges, and protrusion of the mantle beyond the shell margins.

It was concluded that, of the staining techniques examined, only fluorescein diacetate was suitable for use in assessing larval viability. All the other dyes examined proved too insensitive, staining was only observed in grossly abnormal and dying larvae. Further work would be needed to develop a method for grading the intensity of fluorescence if fluorescein diacetate were to be chosen as a method for assessing larval viability. Unfortunately the time needed to further develop this methodology was not available. The staining techniques used relied on differentiation between viable and non-viable tissue, as a result it was not possible to fix the larvae for examination. Larvae that were not fixed proved considerably more difficult to examine closely, and hence evaluate, than those that were fixed. In conclusion, it was found that morphological criteria were more sensitive and easier to use, allowing fixation and consequently more accurate detection of abnormalities. As a result, morphological criteria alone were used to assess larval viability in all subsequent exposure experiments.

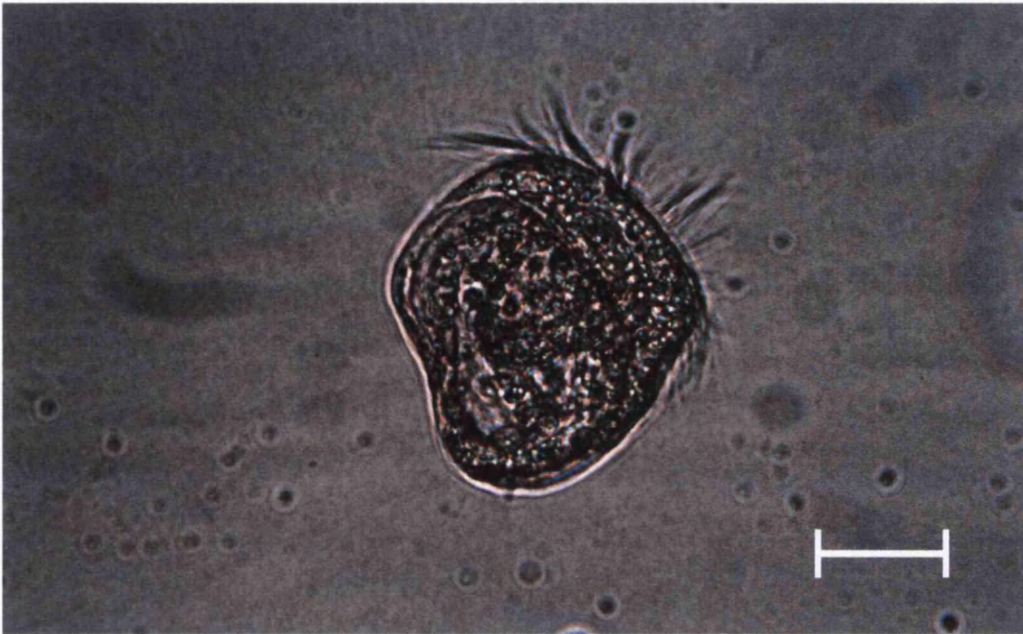


Figure 3.1. Phase contrast light micrograph of D-shell larva exposed to Eosin Y solution, larva has a pinched hinge and enlarged velum but has not taken up the dye. Scale bar = 50μm.

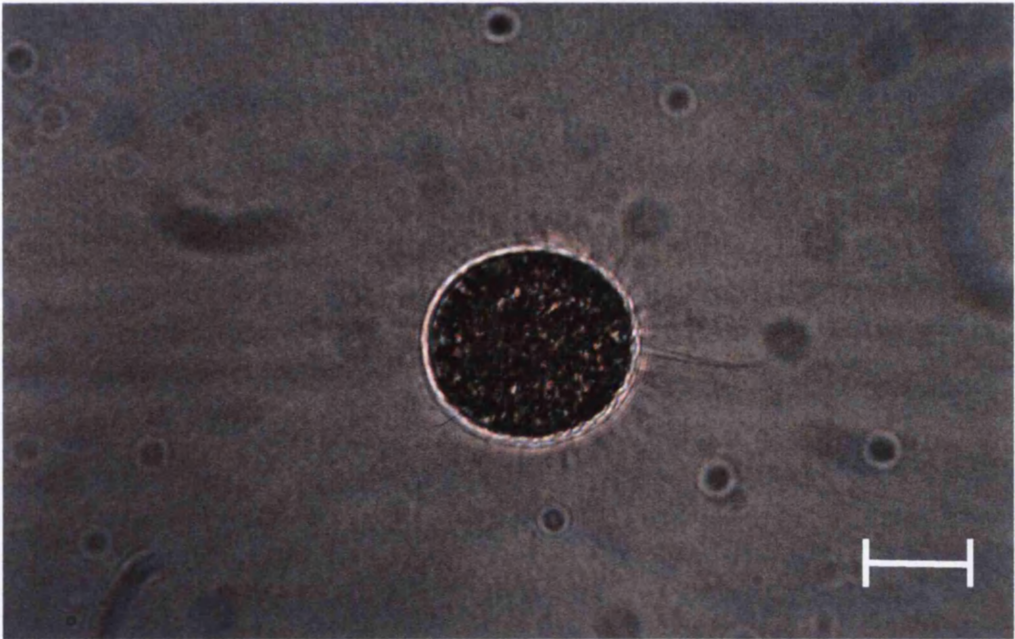


Figure 3.2. Phase contrast light micrograph of a trochophore larva exposed to Eosin Y solution. Scale bar = 50μm.

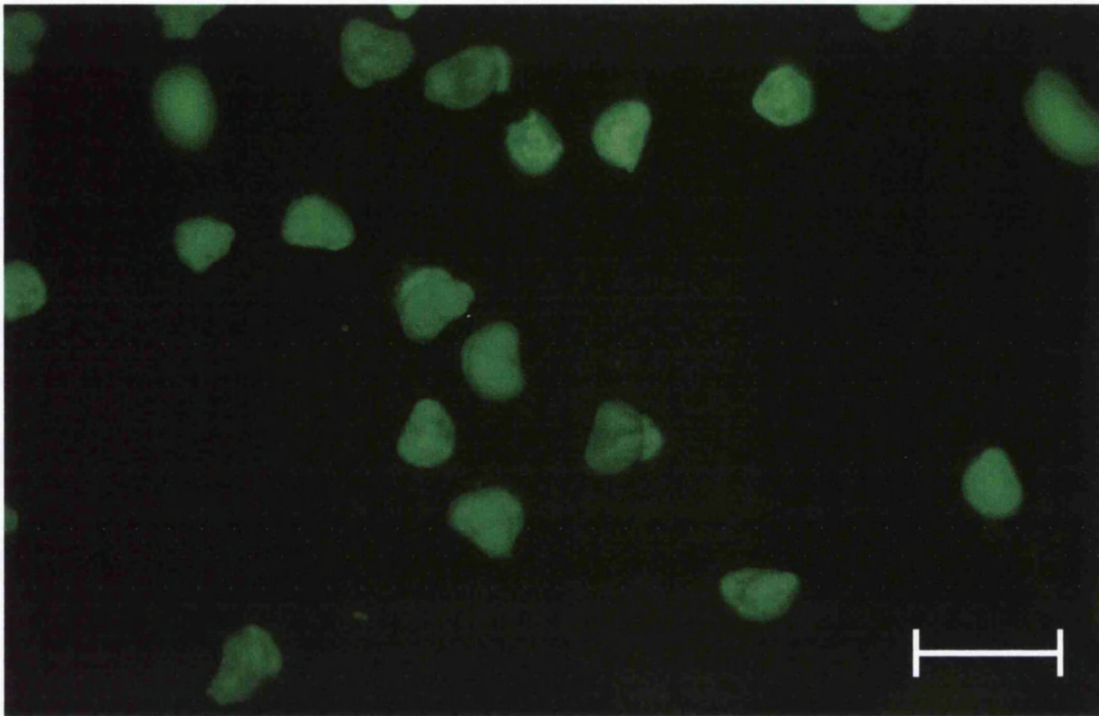


Figure 3.3. Fluorescent light micrograph of D-shell larvae exposed to fluorescein diacetate. Scale bar = 200μm..

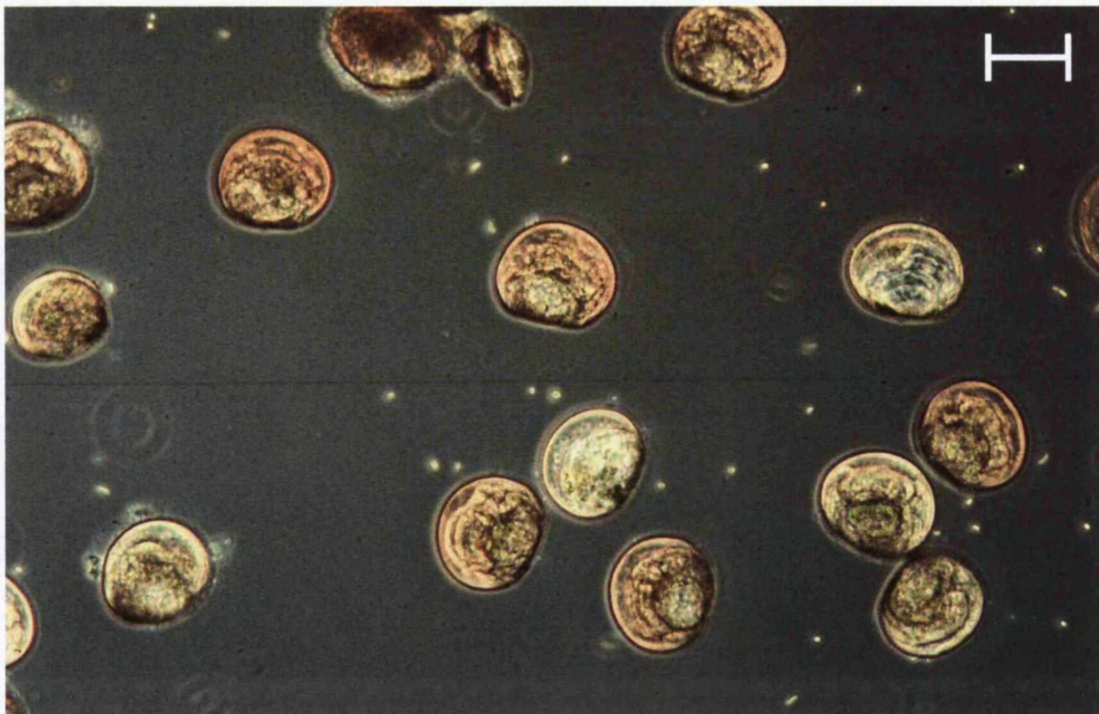


Figure 3.4. Phase contrast light micrograph of live D-shell larvae. Scale bar = 50μm.

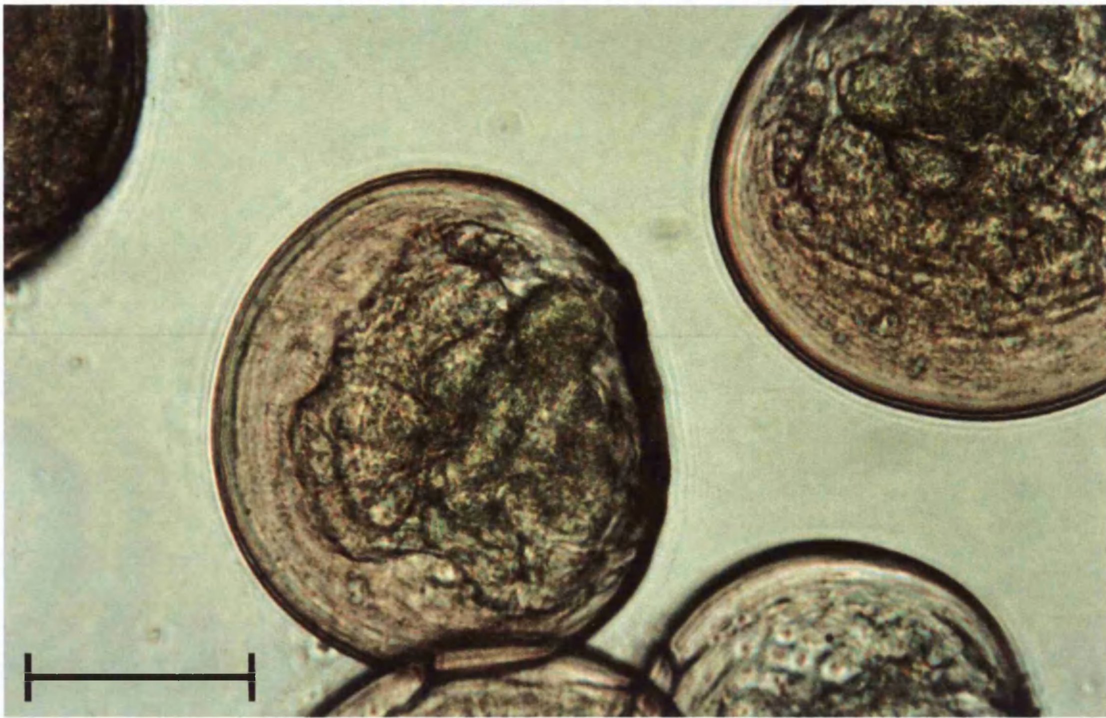


Figure 3.5. Light micrograph of fixed D-shell larvae. Scale bar = 50μm.

3.3 Electron microscopy of D-shell larvae

3.3.1 Materials and methods

D-stage larvae were fixed in EM fixative (2% formaldehyde, 2.5% glutaraldehyde, 2% NaCl, 2mM $CaCl_2$ in 0.2M cacodylate buffer, pH 7.4). The fixed samples were spun very gently, at only 25g, for 5 minutes and the fixative removed. The larval pellets were then resuspended in 15% bovine serum albumin (BSA), mixed with an equal volume of fresh EM fixative and drops pipetted onto dental wax where they were allowed to gel. The BSA-embedded larvae were then washed in 0.2M cacodylate buffer, pH 7.4 for 5 minutes (Pipe, 1990a). Secondary fixation was carried out with 1% OsO_4 (Osmium tetroxide) for 3 hours at room temperature. The larvae were then washed twice in cacodylate buffer (15 minutes), dehydrated through an ethanol series before being embedded via propylene oxide into TAAB epoxy resin (TAAB Laboratories Equipment Ltd, Berkshire, U.K). Ultrathin sections were cut on a Reichert Ultracut-E ultramicrotome, stained with uranyl acetate and lead citrate and examined in a Philips 300 electron microscope.

3.3.2 Results

The results obtained demonstrate that at this early developmental stage the larvae consist mainly of undifferentiated cells which are densely packed. No putative blood cells were identified. Figure 3.6, shows a composite TEM of a typical 48 hour D-shell larva with a retracted velum. The undifferentiated cells typically have a large nucleus and dense cytoplasm. The developing digestive gland has been identified. The gut is also clearly visible, lined with microvilli. Large developing muscles can also clearly be seen running along the base of the larvae and the right hand margin of the larva close to the shell. Many of the larval cells contain large numbers of mitochondria indicating that they require substantial amounts of energy, possibly because they are undergoing differentiation and development.

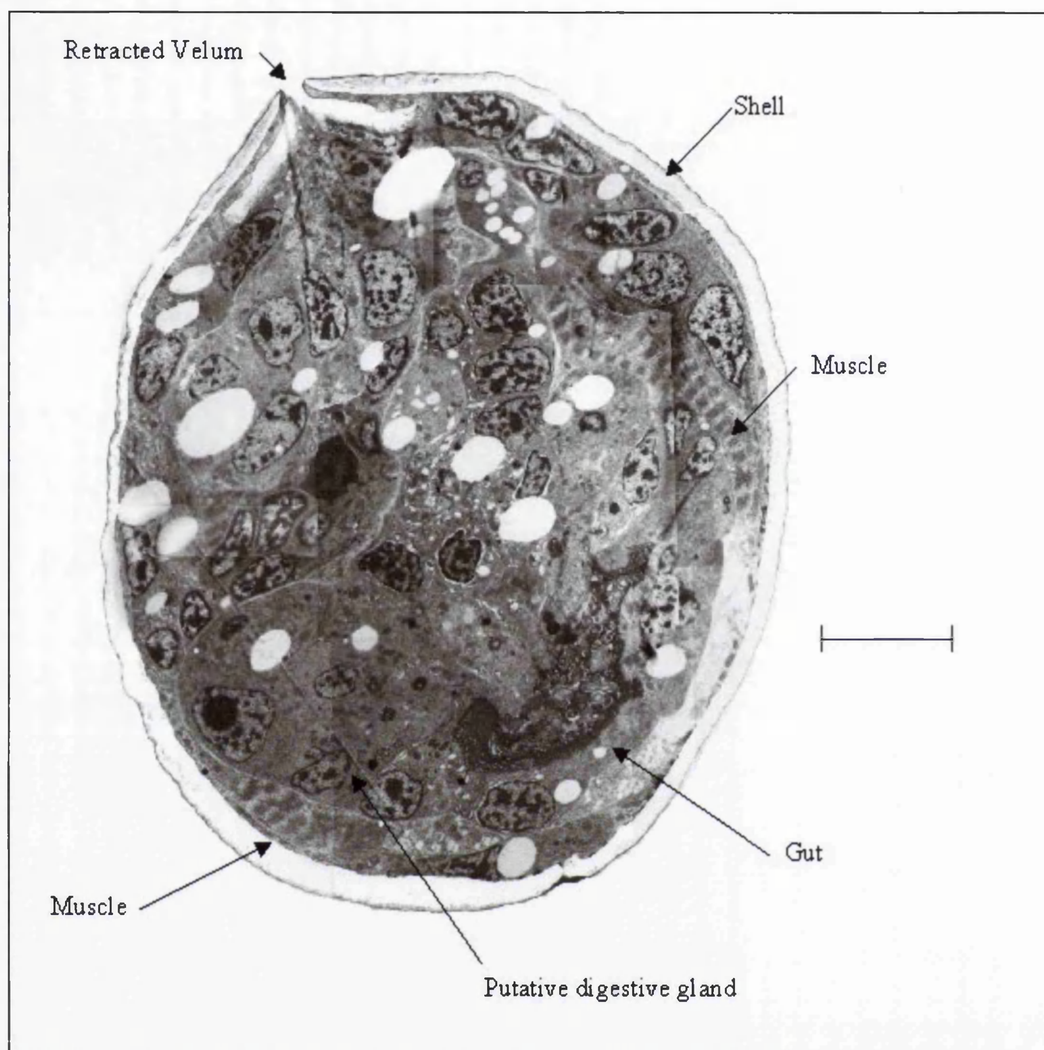


Figure 3.6. Composite TEM of 48h D-shell larva. Scale bar = 10μm.

3.3.3 Discussion

Elston stated (1980b) that the larval bivalve is an animal structurally and functionally distinct from the adult. This is not entirely surprising as the larvae and adults have very different modes of life, with the larvae being free-swimming and pelagic until they settle on a suitable substrate and metamorphose into the sedentary adult form.

Few morphological studies of larval mussels have been made, with the exception of work by Dyrynda (1995) and Bayne (1971; 1976), most work has concentrated on oysters of various species. Bayne (1976) makes little detailed reference to the morphology of larvae until they reach the pediveliger stage. His 1971 work also examines the pediveliger stage and changes that occur at metamorphosis. Dyrynda (1995) gives more detailed morphological details of 7 day old mussel larvae. Elston (1980a, 1999) gives detailed descriptions of the larval morphology of the oyster *Crassostrea virginica*, and it is with this work that we can draw the most comparisons between larval oysters and mussels.

Elston (1980a, 1999) notes the presence of a striking enclosed fluid filled visceral cavity in larval *Crassostrea virginica*, the larva is also reported to contain digestive organs, musculature and free cells. No evidence of this cavity was found in D-shell larvae of *Mytilus edulis* examined in the present study as they full of densely packed mostly undifferentiated cells. Many sections of numerous larvae were examined so it is unlikely that the areas of mantle and visceral cavity were missed due to sectioning in a different plane to that of the cavity.

It is possible that the differences in between Elston's observations and those noted in the present study are due to the age of the larvae studied. The *Mytilus* larvae examined in the present study were 48 hours old, whereas the *Crassostrea* larvae Elston (1980a) studied were between 24 hours and 21 days old. Prytherch (1934) studied the same species as Elston but does not report the presence of a visceral cavity in the American oyster, *Crassostrea (Ostrea) virginica*. No age is given for the oysters studied by Prytherch (1934), however, all the monographs and diagrams show larvae with feet, implying that these were pediveliger larvae that were ready to settle and metamorphose. This supports the theory that the presence of a visceral cavity is dependant on the age of

the larvae studied. Dyrynda (1995) studied 7 day old *Mytilus edulis* larvae and also reported the lack of a visceral cavity, concluding that there was little differentiation of cell types at this stage. It may be that the visceral cavity appears later in the development of the mussel larvae. However, no mention is given of a visceral cavity in the Sydney rock oyster, *Ostrea commercialis*, at any developmental stage (Roughley, 1933).

Alternatively, it may be that the visceral cavity was not visible due to the fact that the micrographs were taken on mussel larvae that all had their velum retracted since larvae were treated with the addition of a small amount of fixative (as detailed in section 3.2.2) to retract the velum. Elston (1980a) cites this as a possible reason that several other authors having failed to report a visceral cavity.

A gut was evident in the 48 hour mussel D-shell larvae, although again it was not as developed as the digestive system seen in 21 day old oysters (Elston, 1980a). There were also 2 prominent muscles visible in the mussel larvae possibly the anterior and posterior adductors. The anterior adductor muscle develops first in early oyster larvae but, by the end of the D-shell stage, before the larvae have completely formed a new shell and become umbo veligers, both the adductor muscles have developed nearly equal size (Elston, 1980a). Elston (1980a) noted that in oyster larvae of 102 μm in shell height (approximately the same size as the larvae in Figure 3.6) each adductor muscle consists of 8 muscle fibres. However, none of the sections examined in the present study showed the muscle in transverse section, so it was not possible to confirm this finding in mussels.

Elston (1980a) states cells with phagocytic capacity appear in the oyster prodissoconch I (D-shell) stage or earlier. In a later paper, Elston (1980b) used electron and contrast microscopy to further examine the phagocytic cells of both *Crassostrea virginica* and *C. gigas* larvae. He found that the phagocytes were easily identified after several hours exposure of the larvae to India ink suspensions, although neither the age nor the developmental stage of the larvae are given. He observed various stages of the engulfment and digestion process associated with phagocytosis and suggested that the apparent movement of these cells along the visceral cavity surface suggests that they serve to remove foreign materials as well as tissue debris.

Although Dyrynda (1995) reported that there was little differentiation of cell types in 7 day old veliger larvae, disaggregation of the larvae revealed the presence of haemocytes. The disaggregated cells of both trochophore and veliger larvae were shown to contain some of the common enzymes associated with adult mussel haemocytes (Dyrynda et al., 1995). The average size of cells was not significantly different between the two stages examined, at around 6μm, with the diameters ranging between 3-13μm. This size range is slightly smaller than that of adult *M. edulis* haemocytes which range from 4-24μm (Renwrantz, 1990). Cells of veliger larvae at the 7 day old stage stained uniformly basophilic with no eosinophilic cells seen (Dyrynda, 1995). This is in contrast with observations of larval oysters which have been shown to possess some eosinophilic cells including phagocytic coelomocytes (Cole, 1938; Elston, 1980a, 1980b). The phagocytic cells were seen as early as prodissoconch I (D-shell veliger) of *Crassostrea virginica* by Elston (1980a) associated with the surfaces of the visceral cavity and in the 1980b paper were shown to phagocytose India ink particles. Cole (1938) reported the presence of eosinophilic phagocytic cells in pediveliger *Ostrea edulis* larvae. There were no haemocytes seen in the sections examined in the present study, but Dyrynda (1995) found that the undifferentiated larval cells did stain using monoclonal antibodies raised against adult haemocytes of *Mytilus edulis*.

The work by Dyrynda (1995) appears to be the only other detailed study of mussel early larval morphology (larvae less than 2 weeks old). Dyrynda studied 7 day old larvae and noted that the undifferentiated cells had large nuclei and dense cytoplasm that was characterised by large quantities of rough endoplasmic reticulum indicating that they were likely to be actively involved in protein synthesis. The 48 hour old larvae examined in the present study were also found to comprise of mostly undifferentiated cells that had large nuclei and dense cytoplasm. They were not noted, however, to contain large quantities of rough endoplasmic reticulum. The only differentiated cells observed in electron micrographs were the heavily ciliated cells of the velum and alimentary tract.

3.4 Exposure of larvae to bacteria

3.4.1 Materials and methods

Mussels were spawned as detailed earlier (section 3.2.2). The cultures were checked for fertilisation success 1 hour after the eggs and sperm were mixed, and the larvae collected on a 30μm mesh. All experiments were carried out at 15°C and the larvae were kept at a concentration of 25,000 larvae L^{-1}. Exposure of the trochophore larvae was carried out immediately, with larvae being transferred into 500ml volumetric flasks containing 5% Marine Broth in autoclaved filtered (0.2μm) seawater. These flasks were aerated and dosed with bacteria. The bacteria used were *Vibrio tubiashii* and *Vibrio alginolyticus*. For each bacterium, two concentrations were used, 1×10^4 bacteria ml^{-1} and 1×10^6 bacteria ml^{-1}, controls were run exposing larvae to 5% Marine Broth alone. The exposures were set up with each treatment (Control, low and high bacterial concentrations) being run in triplicate so as to determine whether there were any vessel effects. The larval cultures were checked and culture media changed daily. After 24 hours, the larvae were fed the unicellular chlorophyte alga, *Dunaliella tertiolecta,* at a concentration of 100 cells μl^{-1} of larval culture (concentrations determined using an improved Neubauer haemacytometer). The algae were added daily, 1 hour after the culture medium was changed. A sub-sample of 200 larvae from each vessel was removed from each vessel, when the culture medium was changed, to assess larval viability.

Exposure experiments were also carried out on D-shell larvae following the same protocol, although they did not commence until 80% of the larvae had reached the D-shell stage (within 48 hours post fertilisation). Each experiment was run for a minimum of 48 hours, longer if the larvae remained viable. As a result, the length of exposure varied according to the bacterium used and the developmental stage of larvae being studied. A sub-sample of 100 larvae from each vessel was removed from each vessel, when the culture medium was changed, to assess larval viability.

Results were analysed using z-tests (Excel, Microsoft Office 2003), the data were log transformed before analysis.

3.4.2 Results

3.4.2.1 Trochophore exposures

Exposure of the trochophore larvae to *Vibrio tubiashii* for 48 hours, resulted in 100% mortality at the higher dose (1 x 10^6 *Vibrio*/ml). Figure 3.7 shows that, although larvae exposed to the lower *V. tubiashii* dose (1 x 10^4 *Vibrio* ml^{-1}) had a much lower mortality rate, very few (41 larvae, from a sub sample of 600) of these larvae were viable after 48 hours. Larval cultures were examined and, using morphological criteria, the larvae categorised as either live, dead or abnormal. The experiment was stopped after only 48 hours and most of the larvae were still trochophores, no differentiation was made between live, normal trochophores and D-shells, they were all categorised as "live". Larvae exposed to the lower dose of *Vibrio* showed gross abnormalities, and did not appear to be developing into D-shells. The outlines of the trochophores appeared blistered as if they were about to break down. There was a low level of mortality in the controls, the larvae appeared normal with a few D-shells present. A z-test pooling the results of exposure to the 2 different levels of bacteria and contrasting them to the control vessels demonstrated that exposure to *V. tubaishii* resulted in increased mortality (p=0.00). However this response was not dose dependant, a z test revealed that there was no significant difference (p=0.109) between larvae exposed to the high dose (1 x 10^6 *V. tubaishii* ml^{-1}) compared to those exposed to the lower dose (1 x 10^4 *V. tubaishii* ml^{-1}).

Exposure of the trochophore larvae to *Vibrio alginolyticus* for 96 hours, resulted in no detectable mortality in any treatment. This experimental treatment was stopped due to the high percentage of abnormal D-shells present in the high (1 x10^6 *Vibrio*/ml) dose vessels (Figure 3.8). The results imply that this bacterium retards development, with 6 times more larvae remaining as trochophores in the high bacterial dose (368 ± 1.33, 2SE) compared with the control vessels (60 ± 4.62, 2SE). In addition, those larvae exposed to the bacteria that did develop to the D-shell stage were mostly abnormal. Approximately equal numbers of abnormal (216 ± 9.24, 2SE) and normal (236 ± 3.53, 2SE) D-shells were seen in larvae exposed to 1 x 10^4 *Vibrio*/ml. However, even this lower level of *Vibrio alginolyticus* seemed to retard development with over twice as many trochophores present in cultures exposed to the bacterium (148 ± 10.41, 2SE) compared with the controls (60 ± 4.62, 2SE). For the purpose of statistical analysis the

abnormal D-shell and trochophore categories were pooled and considered "abnormal", as, under the environmental conditions used for the exposure experiments, trochophores should develop into D-shell larvae within 96 hours. A z-test to assess the effects of exposing trochophore larvae to *V. alginolyticus*, pooling the results of exposure to the two different levels of bacteria and contrasting them to the control vessels demonstrated that bacterial exposure resulted in an increased level of abnormal larvae ($p=0.00$). This response was shown to be dose dependant, a z test revealed that there was significantly more ($p<0.05$) abnormal larvae in the high dose (1×10^6 *V. alginolyticus* ml^{-1}) compared to those exposed to the lower dose exposure (1×10^4 .*V alginolyticus* ml^{-1}).

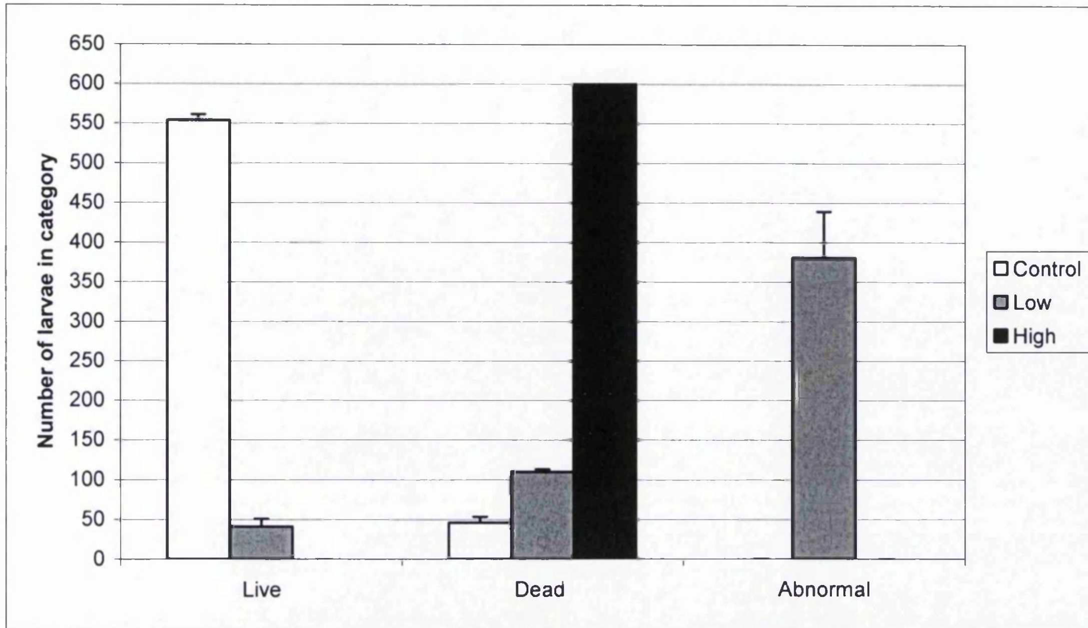


Figure 3.7. Trochophore larval viability after 48 hour exposure to *Vibrio tubiashii*. (Control: No *Vibrio*. Low: 10^4 *Vibrio* ml^{-1}. High: 10^6 *Vibrio* ml^{-1}.). Actual values are given ± 2SE, n=600 larvae from each treatment.

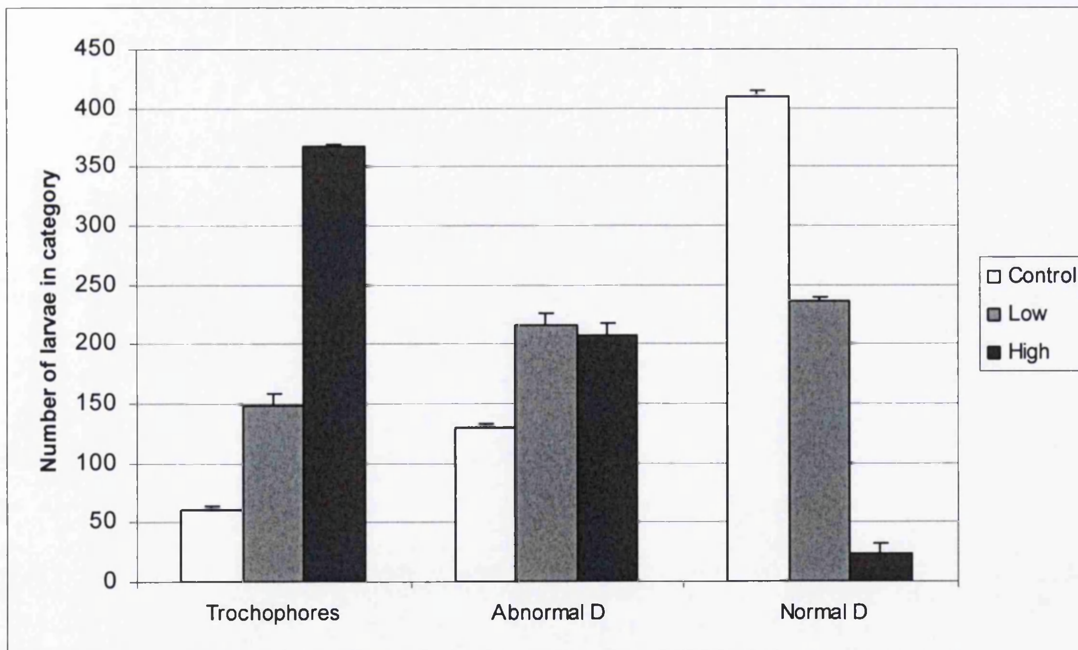


Figure 3.8. Trochophore larval viability after 96 hour exposure to *Vibrio alginolyticus*. (Control: No *Vibrio*. Low: 10^4 *Vibrio* ml^{-1}. High: 10^6 *Vibrio* ml^{-1}.). Actual values are given ± 2SE, n=600 larvae from each treatment.

3.4.2.2 D-shell exposures

An initial 48 hour experiment was run to allow direct comparison of the effects of exposure to *V. tubiashii* at the different developmental stages (D-shell and trochophore). In contrast to the trochophore exposures, a dose dependant response can be seen in the D-shell exposures, a z-test showed that a high dose (1×10^6 *V. tubaishii* ml^{-1}) caused significantly ($p<0.01$) higher mortality of D-shell larvae than the lower dose exposure (1×10^4 *V. tubaishii* ml^{-1}). Larvae that were exposed to the higher dose of *Vibrio tubiashii* for 48 hours as D-shells showed a lower mortality rate than those exposed as trochophores (Figure 3.9 c.f. Figure 3.7). However, exposure to 1×10^6 *Vibrio* ml^{-1} still resulted in high (253± 7.42, 2SE) mortality, while exposure to the lower dose of 1×10^4 *Vibrio* ml^{-1} lowered the mortality to 144 (±10.07, 2SE) (Figure 3.9). Unfortunately, for this experiment, problems with fixation resulted in morphological changes to the larvae and so it was not possible to determine whether the bacteria had caused any abnormal development. As abnormal larvae seen in the trochophore experiment were unlikely to develop into normal live D-shells, the abnormal and dead categories were pooled for the purpose of statistical analysis. A z-test was run to assess whether there was a statistically significant difference in the virulence of *Vibrio tubaishii* between the two developmental stages of *M. edulis* larvae exposed to this potential pathogen. The low and high doses of bacteria were combined and the results of exposure to *Vibrio tubaishii* at the trochophore stage were contrasted to the results obtained when D-shell larvae were exposed to the same species of bacteria. The developmental stage did significantly alter the response of *M. edulis* larvae to *V. tubaishii*, trochophores show a significantly ($p=0.00$) higher level of mortality than D-shells.

This initial 48 hour exposure therefore showed that D-shell larvae were less susceptible to *V. tubiashii* than trochophores. Exposure to *V. tubiashii*, was subsequently run over a longer period of 5 days. Unfortunately, fungal contamination was found in 2 of the 3 culture vessels, therefore these were not used in subsequent analysis nor was any statistical analysis carried out. A sub sample of 200 larvae from the remaining vessel were removed and assessed for viability. The results for the remaining vessel (Figure 3.10) showed that the presence of *V. tubiashii* led to a decrease in normal D-shells and an increase in the number of abnormal or empty D-shells. A small number of trochophores (2 from a sub sample of 200 larvae) in the control vessel did not develop

into D-shells. In order to visually compare the results of this experiment with the results from the 48 hour exposure to *V. tubiashii,* the D-shell and abnormal D-shell categories have been pooled to form a "live" category (Figure 3.11). Comparison of figures 3.9 and 3.11 shows that the overall trend appears to be the same, in that there is a dose dependant response to *V. tubiashii* (increased levels of bacteria lead to increased mortality). However, surprisingly the level of mortality in those D-shells exposed for 5 days is lower (59, from a sub sample of 200 larvae) than those exposed for 48 hours (253 ± 7.42, 2SE, from a sub sample of 300 larvae), possibly due to gamete quality (see discussion, section 3.4.3).

Figure 3.12 shows the results of exposing D-shell larvae to *Vibrio alginolyticus* for 72 hours. This bacterium appears to have a different effect on the D-shells compared with the trochophores; dead animals were seen in the latter experiment but not in the former. However; it is possible that there were mortalities in the trochophore experiments but that the dead trochophore larvae decomposed by the time the cultures were examined at 96 hours. Unlike the empty shells seen in D-shell larval exposures, decomposed trochophores would leave no lasting evidence. A z-test was run to examine the effects of exposure of D-shell larvae to *V. alginolyticus*, the results of exposure to high and low levels of the bacterium were pooled and contrasted against the results obtained for the controls, showing that exposure to *V. alginolyticus* had a significant ($p=0.00$) effect on the level of mortality of D-shells. A further z-test was then run to assess the effects of the concentration of *V. alginolyticus* on D-shell mortality; a dose dependant response was seen with the high concentration (1×10^6 *V. alginolyticus* ml^{-1}) of V. *alginolyticus* causing significantly ($p<0.05$) more mortalities than the lower concentration (1×10^4 *V. alginolyticus* ml^{-1}).

There is an indication that *V. alginolyticus* is less virulent to D-shells as 194 (from a sub sample of 300 animals) of D-shells exposed to the high dose remained live and normal compared with only 24 (from sub sample of 600 animals) reaching normal D-shell stage in the trochophore exposure. Care, however, must be taken when comparing these 2 experiments as the trochophore larvae were exposed to the bacterium for 24 hours longer than the D-shells were, consequently no statistical analysis was undertaken to compare the results of the two experiments. No abnormal D-shells were recorded in the 72 hour D-shell exposure experiment.

No stastical analysis was undertaken to assess the relative virulence of the two species of *Vibrio* used in the exposure experiments as the periods of exposure to the two potential pathogens differed. However, the exposure times differed due to the fact that larvae, at both developmental stages, remained viable for longer when exposed to *V. alginolyticus* compared with *V. tubaishii* which indicates that the former species is less pathogenic than the latter.

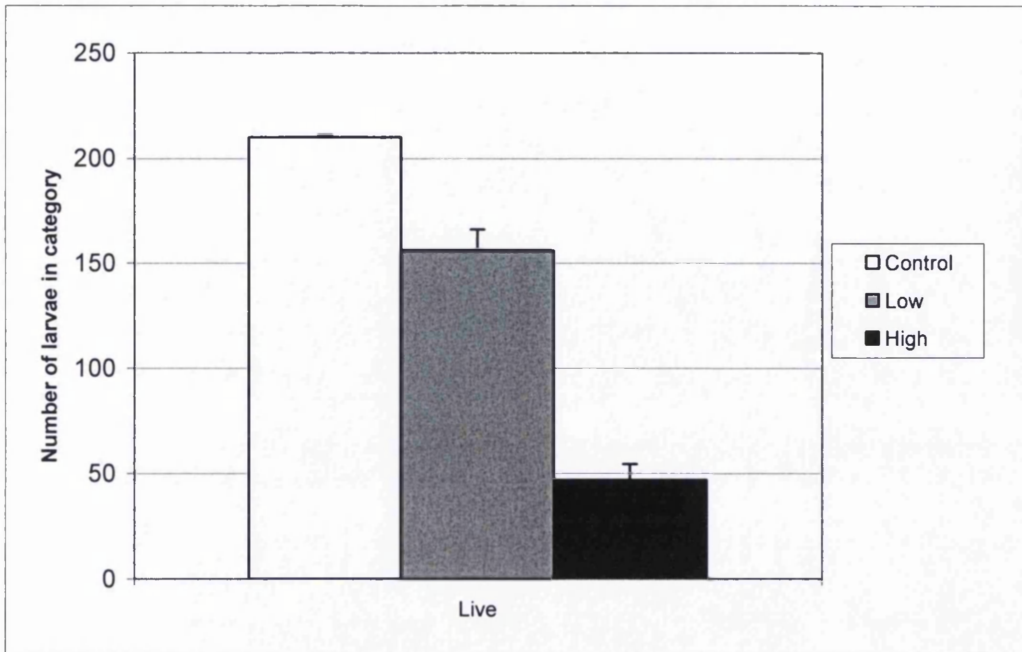


Figure 3.9. D-shell larval viability after 48 hour exposure to *Vibrio tubiashii.* (Control: No *Vibrio.* Low: 10^4 *Vibrio* ml^{-1}. High: 10^6 *Vibrio* ml^{-1}.). Total larval counts are given ± 2SE, n=300 larvae from each treatment.

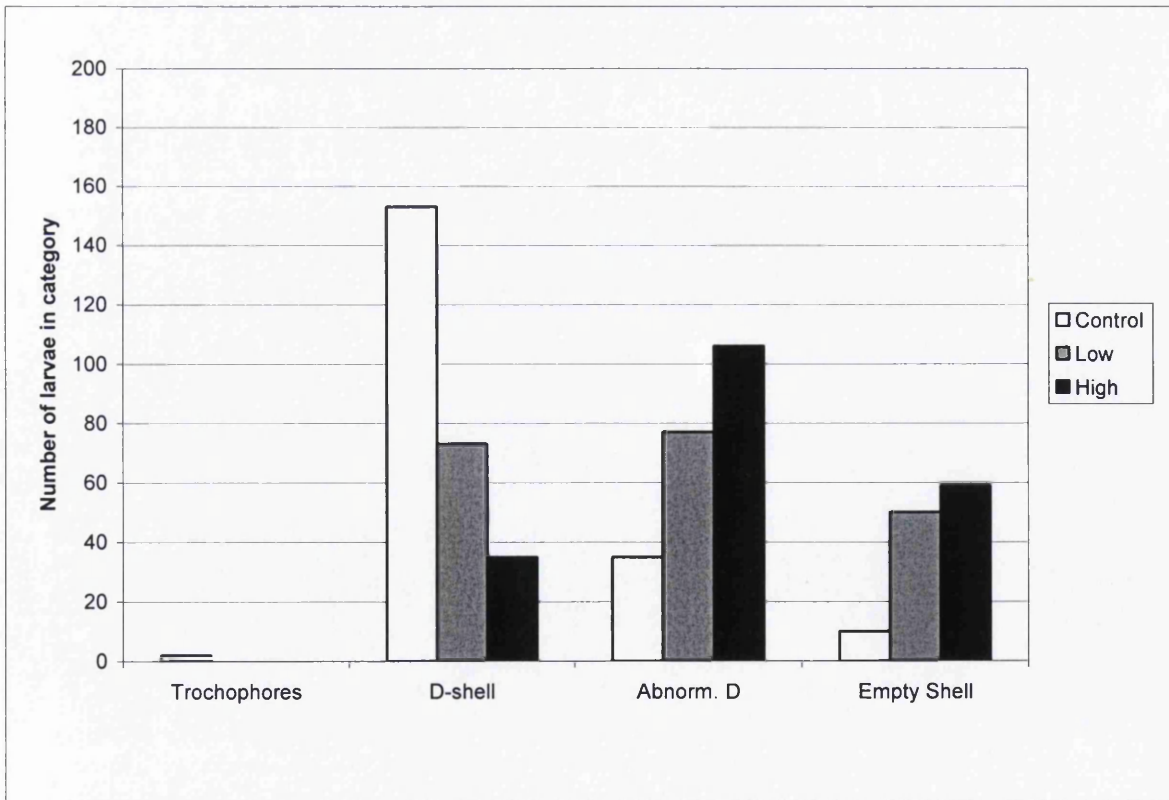


Figure 3.10. D-shell larval viability after 5 day exposure to *Vibrio tubiashii.* (Control: No *Vibrio.* Low: 10^4 *Vibrio* ml^{-1}. High: 10^6 *Vibrio* ml^{-1}.). Due to fungal contamination only 1 vessel was analysed, values are total counts, n = 200 larvae from each treatment.

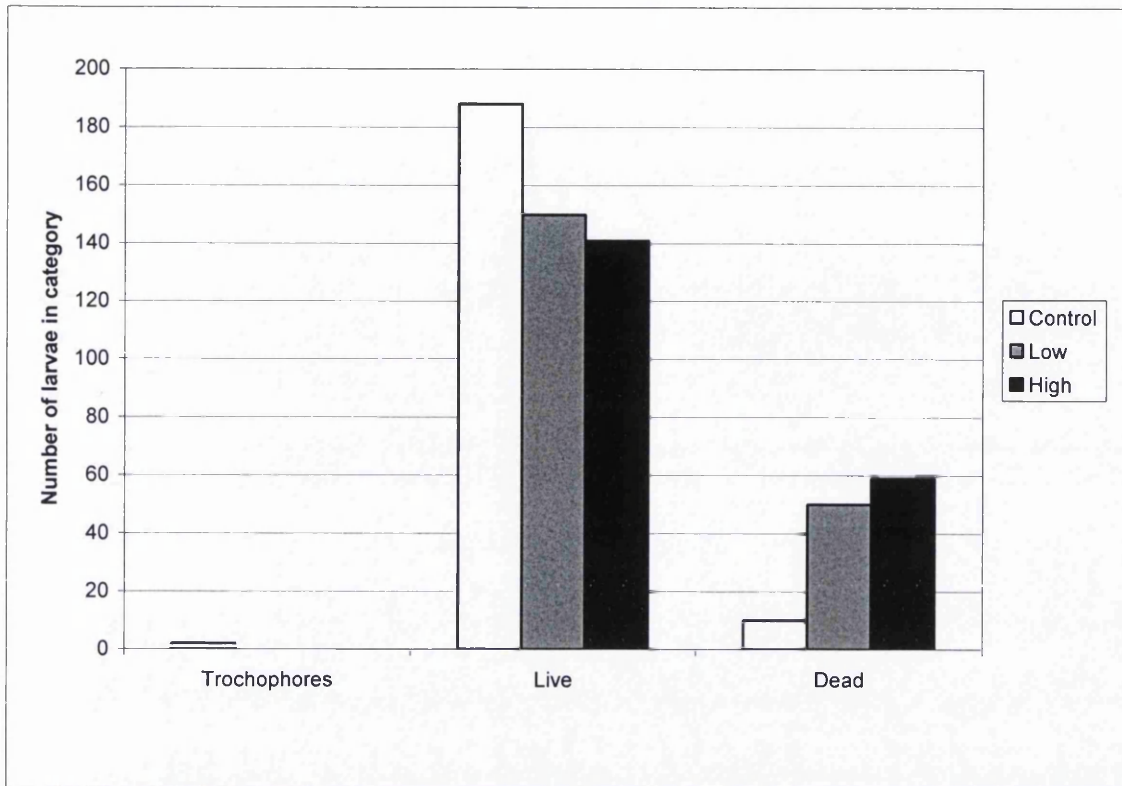


Figure 3.11. D-shell larval viability after 5 day exposure to *Vibrio tubiashii*. (Control: No *Vibrio*. Low: 10^4 *Vibrio* ml^{-1}. High: 10^6 *Vibrio* ml^{-1}.). Due to fungal contamination only 1 vessel was analysed, values are total counts, n = 200 larvae from each treatment.

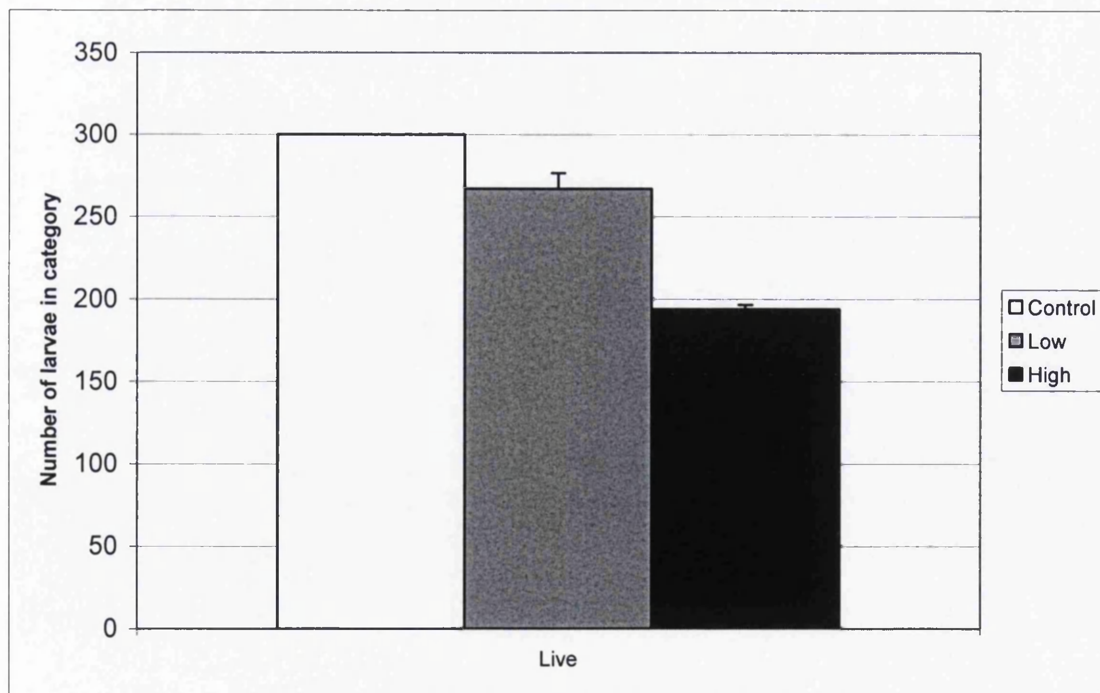


Figure 3.12. D-shell larval viability after 72 hour exposure to *Vibrio alginolyticus*. (Control: No *Vibrio*. Low: 10^4 *Vibrio* ml^{-1}. High: 10^6 *Vibrio* ml^{-1}.) Total larval counts are given ± 2SE, n=300 larvae from each treatment.

3.4.3 Discussion

The *Vibrio* species studied appear to be more virulent to trochophores than D-shells. A direct comparison made between trochophore and D-shell larvae exposed to *V. tubaishii* for 48 hours demonstrated that this species was significantly (p=0.00) more pathogenic to trochophores compared with D-shell larvae. This is consistent with previous studies which report that pathogenicity decreases as age increases, with *Vibrios* being more pathogenic to larvae than adults. Susceptibility also alters with age within the larval stages of mussels, the older the larvae are the less sensitive they are (Nottage & Birkbeck, 1986). This is a general trend with sensitivity for both bacteria and pollutants, with higher sensitivity being seen at earlier life stages. In addition, the experiments carried out in the present study indicated that *V. tubiashii* is more virulent to mussel larvae than *V. alginolyticus*. It is also worth noting at this point that pathogenicity of bacteria may alter with the strain studied, hence it is important that no direct comparisons are made between studies unless the bacteria are from the same source. *V. alginolyticus* has been widely reported as pathogenic to bivalve larvae, however, a strain studied by Sugumar et al. (1998) did not produce any larval mortalities in *C. gigas* larvae.

Symptoms of vibriosis include: abnormal swimming behaviour, reduced feeding and general inactivity, due to proliferation of bacteria throughout the soft tissues (Gosling, 2003). Gross signs of vibriosis, preceding the high sudden mortalities, include initial settling of larvae in cultures and general decrease in larval motility, followed by decreased growth and velar abnormalities (Sindermann 1988). *Vibrio* bacteria proliferate throughout the larval tissues causing lysis and necrosis. After infection, death begins at 8 hours and complete mortality of the culture population may occur by 18 hours. The disease effects are enhanced by the secretion of water soluble toxins (Sindermann, 1988). Larvae probably die as a result of starvation (Ford and Tripp, 1996) as the velum is often the first site of infection. Nottage and Birkbeck (1987a) report the production of a proteinase that is toxic to bivalves by *V. alginolyticus* NCIMB 1339, the same strain used in this study. Nottage and Birkbeck (1987b) also noted that a mixture of *V. alginolyticus* collagenase and dispase is marketed by Boehringer, Mannheim Ltd for the disaggregation of tissues; the rapid damage to velar tissue associated with vibriosis is consistent with the action of a proteinase possibly secreted

by the bacteria. *Ostrea edulis* larvae that were 48 hours old were exposed to 10^6 *Vibrio alginolyticus* ml^{-1}, which resulted in the production of the proteinase (Nottage and Birkbeck 1987a). Subsequently, the protein was purified and 48 hour old larvae exposed to it with a resultant mortality of 42%. An antiserum was raised to the proteinase and was shown to reduce the toxicity of the purified proteinase but had no effect on the toxicity of culture supernatants to the larvae (Nottage and Birkbeck, 1987a). This indicates that the proteinase has a role to play in the toxicity of *V. alginolyticus* to bivalve larvae but other factors also contribute to its activity. Lee et al. (1996) also reported extracellular products (ECP) from *V. alginolyticus* that are lethal to both tiger prawns (*Peneaus monodon*) and kuruma prawns (*Peneaus japonicus*). These ECPs were shown to be proteolytic and haemolytic but again Lee et al. (1996) concluded that ECPs were only one factor involved in the toxicity of *V. alginolyticus* to the prawns.

Vibrio tubiashii has been reported to be highly pathogenic to bivalve larvae. Takahashi et al. (2000) found that it caused 100% mortality in cultures of D-shell *C. gigas* after experimental exposure for 24 hours at a concentration of 10^5 cfu (colony forming units) ml^{-1}. This supports the findings outlined here, namely that *V. tubiashii* is highly virulent, more so than *V. alginolyticus*. Takahashi et al. (2000) also noted that the *V. tubiashii* culture supernatant was pathogenic to larval oysters.

When considering the results of exposure of larvae to bacteria, empty D-shells are analogous to dead larvae and provide a reliable measure of mortality since they do not decay very rapidly. His et al. (2000) state that larvae which have been dead for more than 24 hours, are easily distinguished from moribund larvae, as bacterial degradation and feeding of protozoa on the dead tissue leave nothing but and empty transparent shell. In the present study it was found to take less than 24 hours for dead larvae to be consumed, degradation of moribund larvae may be accelerated due to the effects of vibriosis, which breaks the tissues down.

The results obtained, in the present study, when exposing D-shells to *V. tubiashii* show lower mortality in cultures exposed for 5 days than in cultures that were only exposed for 48 hours. The mortality in the larvae exposed to 10^6 *V. tubiashii* ml^{-1} for only 48 hours (253 ± 7.42 2SE) was more than four times that seen in larvae exposed to the

same level of *V. tubiashii* for 5 days (59, no SE as the results are from one vessel). Differences in mortalities in control cultures point to differences in the health of the larvae, the level of live animals in controls, i.e. not exposed to any stressors, is much higher in the 5 d experiment (210 out of 300 larvae) compared with the 48h experiment (188 out of 200 larvae). Unfortunately no statistical analysis can be carried out due to the fungal infection seen in the 5 days exposure vessels resulting in only one vessel being analysed. One possible explanation for the observation is that the adult mussels that spawned the D-shells for the 48 hour experiment were stressed. Bayne (1972), states that although gametogenesis may appear to be normal there is evidence that stress in the adult effects subsequent larval development. A second, perhaps more likely explanation, is that the differences in larval health may be due to the quality of eggs and sperm spawned. The 48h exposure was carried out with larvae spawned on the 28th June towards the end of spawning period for the population of mussels used. The gametes may therefore have been of lower quality. The 5 day experiment was carried out with larvae spawned on the 24th January, the start of the spawning season. Vitellogenesis takes place from November to January (Bayne et al., 1975), therefore oocytes spawned in January could contain more stored yolk than the later spawning oocytes. In the early stages of larval development, there is a complete dependence on the stored energy reserves acquired from the adults during vitellogenesis (Bayne et al., 1975).

The experiments have demonstrated that not only does mortality alter with the species of bacteria the larvae are exposed to, but the number of bacteria present also has a significant effect on virulence. Both bacteria species studied demonstrated dose-dependant larval responses in the D-shell exposures, with mortality levels being lower at lower bacterial concentrations. Exposure to *V. tubaishii* at the higher concentration of 1×10^6 *Vibrio* ml^{-1} resulted in a significant ($p<0.01$) increase in mortality levels compared with D-shells exposed to the lower concentration of 1×10^4 *Vibrio* ml^{-1}. Exposure of D-shell larvae to *V. alginolyticus* also showed a dose dependant response with 1×10^6 *Vibrio* ml^{-1} resulting in a significant ($p<0.05$) higher mortality levels compared with larvae exposed to the lower concentration of 1×10^4 *Vibrio* ml^{-1}. Sainz et al. (1998) noted a dose dependent response in the ability of *V. alginolyticus* to induce vibriosis in 6 day old larvae of the catarina scallop (*Argopecten ventricosus* = *circularis*). At concentrations below 0.5×10^5 CFU ml^{-1}, there was no effect seen but at

concentrations higher than 5.0 x 10^5 CFU ml^{-1} symptoms of vibriosis was seen leading to massive mortality. Elston and Leibovitz (1980) also reported dose dependant responses to bacterial infections. They found that mortality after experimental vibriosis of oyster larvae increases from 40% to 100% if the initial contamination rate increases from 10^3 *Vibrio* sp. ml^{-1} to 10^7 *Vibrio* sp. ml^{-1}. Perkins (1993) states that when larval culture conditions are sub-optimal, bacteria present in the system will multiply. He believed that if the total bacterial concentrations build to greater than 10^5, bivalve mortalities begin and generally progress to catastrophic levels. Sindermann (1988), however, reported that when *Vibrio* sp. concentrations in larval oyster cultures reach only 10^2, mortalities can be initiated. These differences in reported levels causing mortalities are probably simply down to the bacterial species studied, Perkins refers to "total bacterial concentrations", whereas Sindermann is referring to species of *Vibrio*. The bacteria levels used in the present experiments are achievable in the environment. Nogami and Maeda (1992) state that the stable maximum density of bacteria in the environment seems to be about 10^6 cells ml^{-1}, this suggests that the levels of bacteria used in these studies are environmentally realistic in terms of normal background levels. Concentrations of bacteria will exceed these levels during pollution incidents or planktonic blooms, however for the purpose of this study a concentration that is stable in the marine environment and may frequently be encountered by mussels was chosen as the maximum level for experimental exposure.

Clearly knowledge of the ontogeny of the bivalve immune system is important as mussel larvae appear to be susceptible to *Vibrio* infections. Adult mussels are more resistant to the infection which may be a result of increased immune development. Preliminary studies on immune ontogeny have revealed that not all immune capacities are present through all the life stages. Tirapé et al. (2007) studied the expression of immune-related genes during ontogenesis in the oyster *C. gigas*. Although RNA transcripts were detected in early stages (oocytes and 2-4 cell embryos) it was thought that they were not produced by the embryo itself but were of maternal origin. Expression levels varied depending on developmental stage and gene studied, RNA transcription levels increased from trochophore stage for some genes and D-shell stage for others. Tirapé et al. (2007) concluded that the maturation events leading to immunocompetence occur between the D-shell and veliger larval stages, with haemocyte generation/proliferation and induction of immune related genes being

concomitant. Mitta et al (2000d) studied the expression of antimicrobial proteins in early developmental stages of mussels. mRNA transcripts encoded by the mytilin B and MGD2 genes were undetectable from the eggs through most of the larval stages, with expression starting during, or shortly after, metamorphosis. Mytilin transcripts were the first antimicrobial peptides to be detected, at day 13 after fertilisation and reached a maximum at day 20 in the newly settled individuals. MDG2 transcripts were detected even later in the larval development, at day 25, during the period of metamorphosis of the newly settled larvae into juvenile mussels. These results clearly demonstrate that mussel larvae do not possess a mature fully developed immune system. Mitta et al. (2000b) showed that, after injection of adult *Mytilus galloprovincialis* with bacterial challenge (heat-killed *V. alginolyticus*), mytilin rich haemocytes migrated toward the injection site, the mytilins were thought to play a prominent role in killing the intracellular bacteria after phagocytosis. Furthermore, in the same study, it was shown that only haemocytes containing mytilin were capable of phagocytosing *Vibrio alginolyticus* that had been injected into the mussels. Examination of the haemocytes by electron microscopy showed that no bacteria were observed in mytilin-negative cells. Hence, the increased susceptibility of mussel larvae to *Vibrios* may be a result of an immature immune system, and, although they are thought to possess phagocytic cells, it may be that these cells cannot kill the bacteria in the same way as adult haemocytes.

Disaggregated cells from 7 day old larvae showed low levels of phagocytosis, when measured as the percentage of phagocytic cells, compared with adult haemocytes (Dyrynda, 1995). However, Dyrynda et al. (1995) point out that this may be due to fundamental differences in the assays being compared. The levels recorded in larval assays are a percentage of all larval cell types whilst levels of phagocytosis measured in adult assays are a percentage of haemocytic cells. Additionally, the disaggregation process itself may also affect the phagocytic ability of the cells (Dyrynda et al. 1995). Further weight may be lent to this observation being a dilution artefact by the work of Mitta et al., (2000b) who showed strong *in vitro* phagocytosis of bacteria by larval cells. The work by Dyrynda (1995) on the immune functions of 7 day old larvae showed that the enzymes phenoloxidase and arylsulphatase were present in disaggregated larval cells. The generation of superoxide anion was quantified in both trochophore and veliger larvae. Phagocytosis and the generation of superoxide anion were demonstrated in both whole larvae and disaggregated cells. The percentage of phagocytic cells

present showed very little difference between trochophore and veliger cells at approximately 4% (Dyrynda, 1995). This lack of correlation between a basic immune parameter, phagocytosis, and the different sensitivity of trochophores and veliger larvae to the bacteria studied implies that a range of factors, such as immunological development and larval morphology, combine to affect susceptibility. The ontogeny of immune function goes some way to explaining why larval mussels are more susceptible to bacterial challenge than adults, but clearly more work is needed before its development is fully understood.

Chapter 4. Susceptibility of postlarvae to bacteria.

4.1 Introduction

Postlarval mussel development

Once the larval pediveliger mussel comes into contact with a suitable substrate it will settle and metamorphose into the adult form. The first secretion of byssus threads and attachment to the substrate marks the termination of pelagic life, the process of metamorphosis then commences. Veliger stage larvae are positively phototrophic and are sensitive to pressure which serves to keep them in the surface waters. In contrast, pediveligers are negatively phototrophic, positively geotrophic and insensitive to pressure, which encourages them towards the sea bottom in preparation for metamorphosis (Bayne, 1976). In the absence of a desirable substrate the larva may postpone metamorphosis. *Mytilus edulis* has the ability to delay metamorphosis for several weeks (up to 40 days at 10°C), although during this time growth rates decline to zero (Bayne, 1965).

The act of "settlement" consists of the descent, by the pediveliger, from the plankton to the sea bottom, followed by a pattern of swimming and crawling behaviour ending with the secretion of byssus threads that attach the larva to the substrate and signals the beginning of benthic existence (Bayne, 1976). Lamellibranch larvae in general, have the ability to discriminate between different substrates prior to attachment by a series of crawling movements with the foot. If the stimuli are positive the crawling ceases and the pediveliger will secrete byssus threads and settle. If negative stimuli are encountered the foot is retracted and the larva will swim away from the substrate. The exact sequence of stimulus and response has not been described for mussel larvae, although some data on larval preferences for specific substrates are available (Bayne, 1976). Several studies report that mussel larvae settle most readily on filamentous substrates such as algae and hydroids (De Blok and Geelen, 1958; Bayne, 1964, 1965, 1976). Postlarval mussels that have successfully completed metamorphosis are referred to as plantigrades.

The secretion of the byssus threads signals the degeneration of the swimming and feeding organ, the velum, which begins within 48 hours of byssus secretion and marks the commencement of metamorphosis into the plantigrade mussel. The most dramatic of the morphological events that constitute metamorphosis in *Mytilus* are related to the transition into adult modes of feeding and locomotion. Not only does the velum degenerate the mouth and foot re-orientate, there is also growth of labial palps and gill filaments (Bayne, 1971). As the velum collapses and starts to disintegrate, it is invaded by phagocytes which absorb the velar cells (Kasyanov et al., 1998). During this period, the larval feeding mechanism is lost and the adult gill/palp feeding mechanism is developed; for these 1 to 3 days the plantigrade cannot feed and relies upon stored nutrients for metabolic energy (Bayne, 1976). With disintegration of the velum, the foot can grow in length and migrate forward in to the mantle cavity. This allows further changes within the mantle cavity, resulting in the formation of inhalant and exhalant siphons which allow the filtration of water by ctenidial filaments for the first time. The labial palps also develop rapidly so that particles that are now trapped by the ctenidia can be directed into the mouth. The movement of the foot also allows room for the growth of more gill filaments (Bayne, 1976). Changes in the structure of the shell represent the concluding stage of metamorphosis (Kasyanov et al., 1998). The microstructure, mineralogy and ornamentation of the shell drastically change. Layers of the new definitive shell, the dissoconch, are formed and a change in shape occurs, with the new shell assuming the adult form.

The settled larvae then undergo a period of growth, up to 2mm in shell length, before finally recruiting on to adult mussel beds (Gosling, 2003), this process is referred to as secondary settlement. Young postlarval mussels secrete monofilamental byssal threads that can reach up to 10cm in length (Lane et al., 1985), they drift on these byssus threads from the site of their primary settlement to new sites. Final settlement of the plantigrades tends to occur on mussel beds where the mussels will remain for the rest of their life. However, direct settlement has also been recorded in *Mytilus* (MacGrath et al., 1988; Cáceres-Martínez et al., 1993).

Postlarvae and bivalve culture

Mussels and oysters can be induced to settle on ropes or suspended cages hung from rafts. However, more commonly for mussels, the seed is gathered from natural settlements on coastal rocks and wound onto the ropes which are then hung from the rafts. In bivalve culture the supply of seed, also known as spat, is the critical element. (Gosling, 2003). Spat can be produced from broodstock maintained in the hatchery or, as previously mentioned (Chapter 1), from wild or cultured stocks in the field. Generally, mussels are cultured from collected spat whereas oysters are generally cultured from broodstock within the hatchery, this is due to financial considerations. Rearing larvae is costly and is often the most difficult part of cultivation. Owing to the lower price fetched by mussels compared with oysters (Mason, 1976) the cost of rearing mussels from larvae is not economically viable. As mussel aquaculture is dependant on a continued supply of healthy spat, it is therefore of interest to study the susceptibility of the spat to the same bacteria that have previously shown to be pathogenic to larvae (Chapter 3).

4.2 Exposure of postlarvae to bacteria: Development of exposure method.

As with the larval exposures, the bacteria used in the present study were obtained from NCIMB and had all been isolated from molluscs. In addition to the two bacterial species used in the larval studies (Chapter 3), the postlarvae were also exposed to *Alteromonas haloplanktis*. The details of *V. alginolyticus* and *V. tubiashii* are given in Chapter 3. *A. haloplanktis* is a Gram negative bacterium (Carrasquero-Verde, 1999), the bacterium obtained from NCIMB had been isolated from a mollusc and is reported to be pathogenic to oysters. However, antibacterial properties of this bacterium have also been reported by Riquelme et al. (1996) who found that it suppressed the growth of *V. alginolyticus* and *V. anguillarum*. This implies that under certain conditions *A. haloplanktis* may be beneficial to bivalve molluscs and has a potential role as a probiotic. The use of *A. haloplanktis* as a probiotic has been explored by Riquelme et al. (1996) and, although of interest, this application is outside the scope of the present study. The *A. haloplanktis* studied was NCIMB culture number 1545: isolated by

Colwell (1967) from *Crassostrea gigas*. NCIMB supply the bacteria in ampoules containing freeze-dried cultures, these were revived as detailed in Chapter 3.

The postlarvae (0.5-3mm) used in the present study were collected from Whitsand Bay, Cornwall. On transfer to the laboratory they were placed in tanks containing 3 litres of aerated seawater at 15°C and left overnight to acclimatise.

The present study aimed to assess the relative pathogenicity of three bacterial species to postlarval mussels. *V. alginolyticus* and *V. tubiashii* have previously been shown to be pathogenic to larval mussels (Chapter 3), the present study aimed to determine whether their pathogenicity alters with the mussel developmental stage studied.

4.2.1 Exposure vessel selection

Materials and Methods

Postlarvae were exposed to *V. tubiashii* at the same concentrations used in the larval exposure experiments (Chapter 3), that is 10^4 and 10^6 *Vibrio* ml^{-1}, with controls exposed to 5% Marine Broth alone. Two methods for exposure were investigated: (i) tissue culture vessels (Figs. 4.1 & 4.2) and (ii) stainless steel biopsy boxes (Fig. 4.3). The larvae in tissue culture vessels were exposed to *V. tubiashii* for 4 days, whilst the exposure time for the second experiment, with larvae in the stainless steel biopsy boxes, was increased to 7 days.

After 4 days it became difficult to see the postlarvae in the tissue culture vessels due to films of bacteria which had grown on the sides of the vessels, consequently the exposures were terminated at this point. No such problems were encountered with postlarvae exposed in stainless steel biopsy boxes, allowing the experiments to be run for the full 7 days.

(i) Tissue culture vessels (Figs 4.1 and 4.2): The postlarvae were placed in 50ml vented-cap tissue culture vessels (Fisher Scientific UK Ltd, Leicestershire, U.K), these were then filled with 40ml of 0.2µm filtered seawater containing 5% Marine Broth. The vessels were aerated via a 21G, 38mm needle. There were 3 replicates of each treatment (control, 10^4 *Vibrio* ml^{-1} and 10^6 *Vibrio* ml^{-1}) with each culture vessel containing 20 postlarvae. The culture medium was changed daily and the vessels were

checked under a dissection microscope for larval mortality. After 4 days, the postlarvae were fixed in EM fixative and removed from the culture vessels to assess the mortality levels, postlarvae that were gaping were considered dead. Fixation was completed in two steps; initially a drop of EM fixative was added to the culture vessels, the remainder of the fixative was then added. The addition of a drop of fixative causes all viable postlarvae to close their valves, thus when they are subsequently examined all gaping postlarvae can be considered dead. An intermediate stage, considered to be between healthy postlarvae and dead animals, was noted where animals had produced a large quantity of mucus that surrounded them.

(ii) Biopsy boxes (Figure 4.3): The postlarvae were put into stainless steel biopsy boxes, which were held inside plastic containers holding 300ml of 5% Marine Broth in 0.2μm filtered seawater. These plastic containers were aerated via a 21G, 38mm needle. Each plastic container held 4 biopsy boxes containing 25 postlarvae, again there were 3 replicates of treatment which were the same as for the tissue culture vessel exposures. The culture medium was changed daily, at which point the biopsy boxes removed from the plastic containers for assessment of larval mortality using a dissection microscope, exposure continued for 7 days. After 7 days the larvae were removed from the biopsy boxes and fixed in EM fixative.

High mortality levels were seen across all the treatments, including the control, in postlarvae exposed using the biopsy box exposures method (Fig 4.5). Close inspection of the postlarvae showed that they had damaged shells; this was thought to be due to mechanical damage during the daily examination of the larvae. Consequently, of the 2 methods of exposure for postlarvae investigated, the tissue culture vessel system was considered more satisfactory. Although the use of biopsy boxes allowed the postlarvae to be viewed with greater ease, it was found that individuals could be damaged when the lid of the boxes were being removed and replaced. The careful use of a cell scraper (Fisher Scientific UK Ltd, Leicestershire, U.K.) overcame the problem of a build up of bacteria on the walls of the tissue culture vessels. In subsequent experiments, the culture vessels were cleaned daily prior to the water changes allowing easy assessment of postlarval viability.

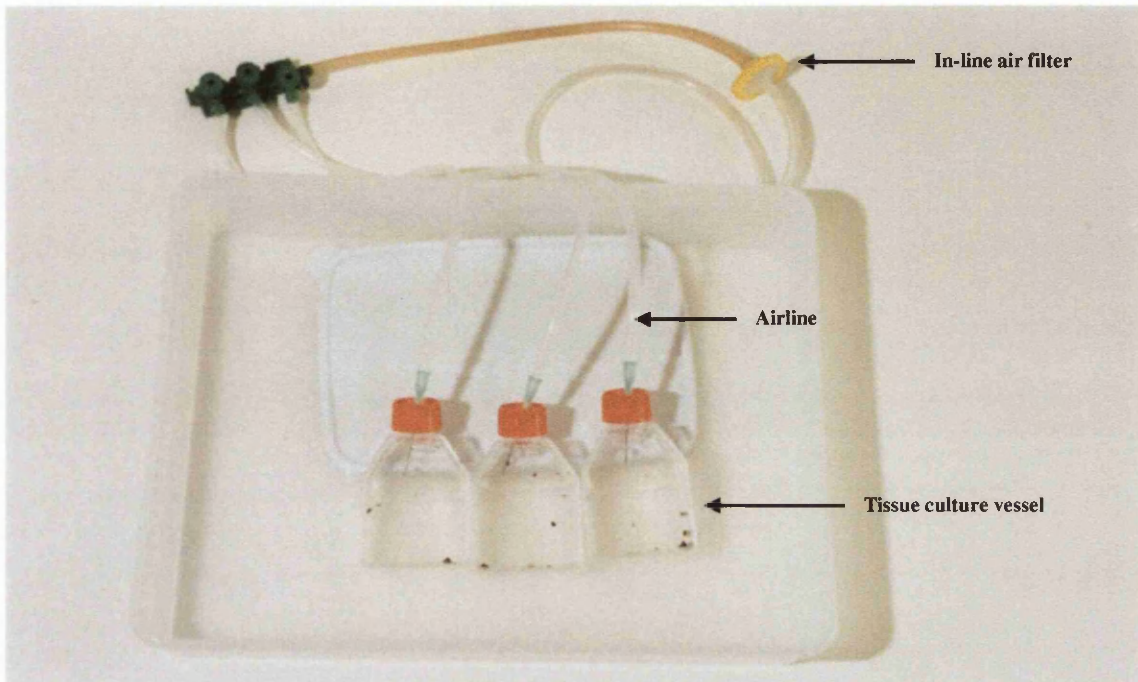


Figure 4.1 Tissue culture vessel postlarval exposure method, 3 vessels each containing 40ml seawater and 20 postlarvae.

Figure 4.2. Tissue culture vessel postlarval exposure method: detail showing hypodermic needles entering though the vessel lid to aerate the media.

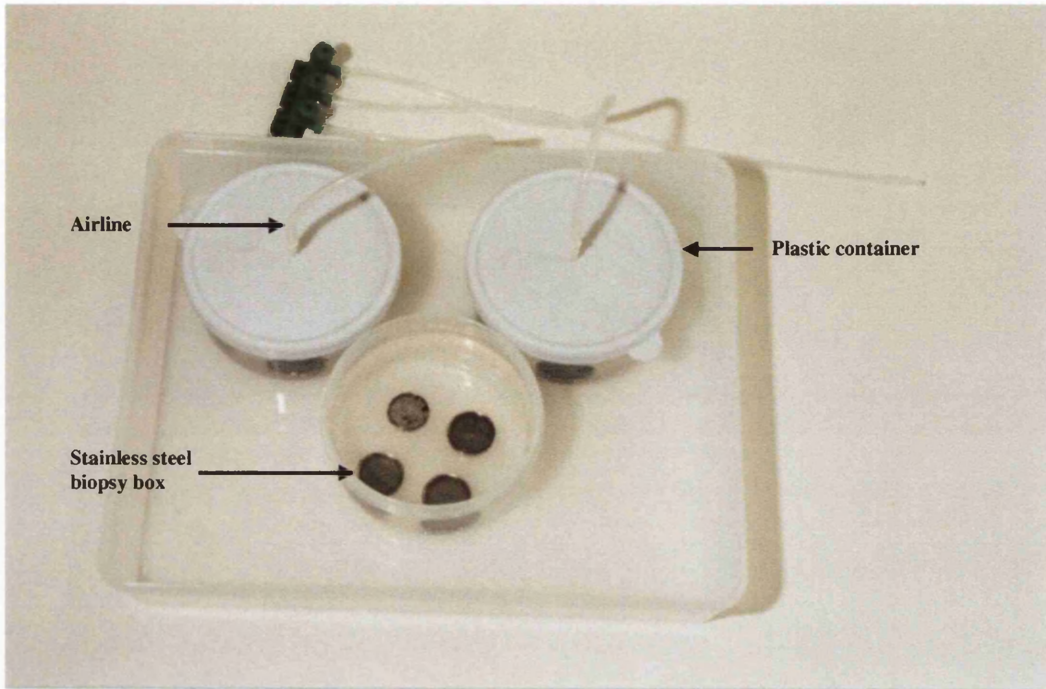


Figure. 4.3. Biopsy box postlarval exposure method showing airline entering the plastic container via a hypodermic needle and the biopsy boxes within one of the plastic containers (lid removed for photographic purposes).

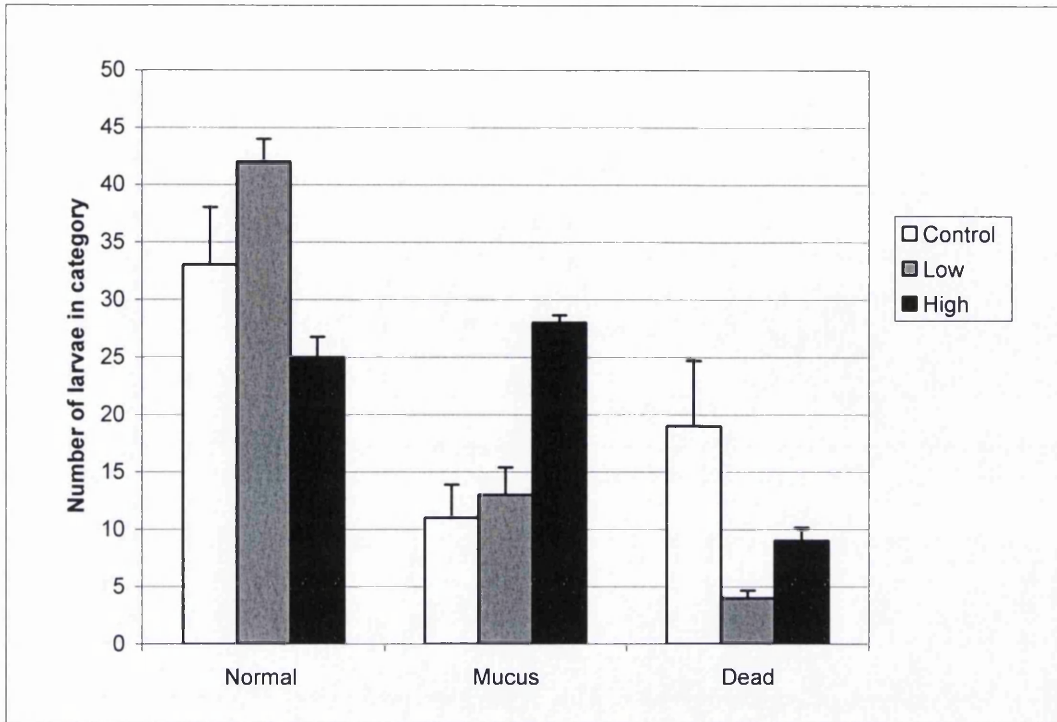


Figure 4.4. Postlarval viability after 4 day exposure (03/08/99), using tissue culture vessel method, to *Vibrio tubiashii*. (Control: No *Vibrio*. Low: 10^4 *Vibrio* ml^{-1}. High: 10^6 *Vibrio* ml^{-1}.). Postlarvae were determined to be either live, dead, or at an intermediate stage producing large quantities of mucus. Total counts are given, n=60 animals per treatment, 20 per vessel and 3 vessels per treatment.

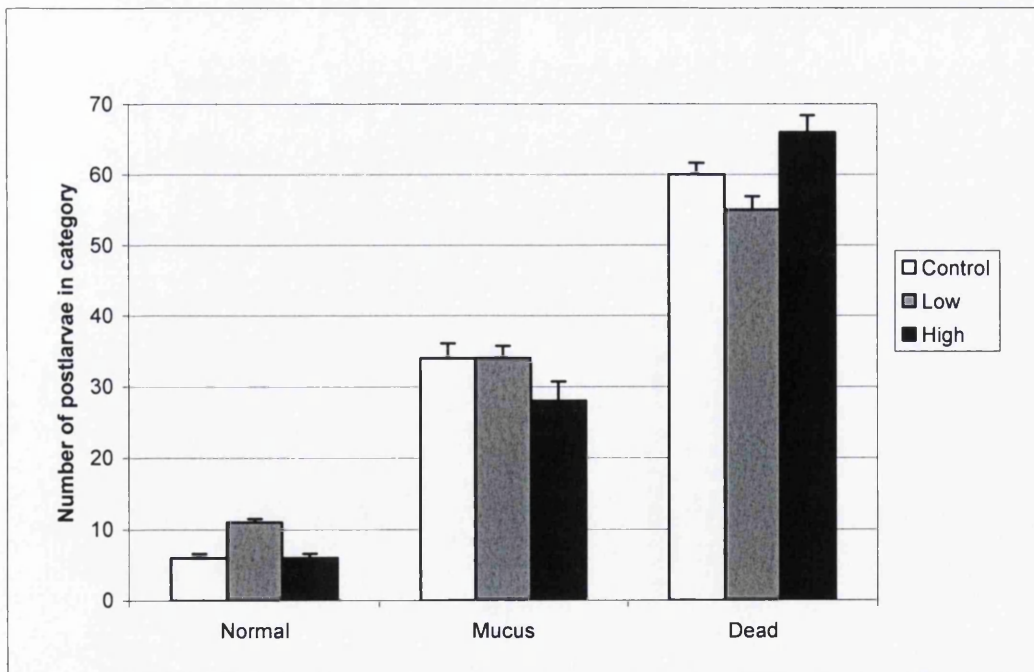


Figure 4.5. Postlarval viability after 7 day exposure (12/08/99), using biopsy box method, to *Vibrio tubiashii*. (Control: No *Vibrio*. Low: 10^4 *Vibrio* ml^{-1}. High: 10^6 *Vibrio* ml^{-1}.). Postlarvae were determined to be either live, dead, or at an intermediate stage producing large quantities of mucus. Total counts are given, n=10 animals per treatment, 25 per biopsy box and 4 boxes per treatment.

4.2.2 Indigenous bacterial contamination

The high level of mortality seen in the controls in experiments run while developing the exposure methodology (Figures 4.4 and 4.5) was of concern. This was thought to indicate that postlarvae collected from the environment have an indigenous bacterial flora. The introduction of the spat to the nutrient rich 5% Marine Broth media may then lead to a proliferation of these indigenous bacteria.

Consequently, studies were carried out to investigate whether the collected postlarvae harboured indigenous bacteria and, if this were found to be the case, to develop a method for eliminating these bacteria. Thiosulfite citrate bile sucrose (TCBS) agar was selected as a suitable medium for screening the postlarvae as it is used for the isolation and selective cultivation of *Vibrio cholerae* and other enteropathogenic *Vibrio* species including *V. alginolyticus* (Clesceri et al., 1998).

Postlarval screening

Fresh postlarvae were collected to analyse whether they harboured indigenous bacteria. On arrival at the laboratory, the postlarvae were washed in 0.2μm filtered seawater and placed in 3 litres of fresh filtered seawater. A subgroup of animals were then removed and placed, together with 20ml fresh filtered, UV treated seawater, onto TCBS agar plate which was incubated at 30°C overnight. The formation of yellow rings on the green TCBS agar around the postlarvae indicated that they contained indigenous *Vibrio* and may also contain other species of bacteria (Figure 4.6).

Antibiotic selection

In order to obtain results demonstrating the effect of bacterial exposure on postlarvae it was essential to eliminate the indigenous bacterial species. In light of the findings of Olafsen et al. (1993), showing that *Vibrio* species can survive UV depuration, it was considered necessary to treat the postlarvae with antibiotics. A number of antibiotic preparations were selected for testing. For each antibiotic, a solution of the strength recommended by the manufacturer was made up in 0.2μm filtered seawater, the postlarvae were immersed in this aerated solution and kept at 15°C. Samples of the media and the postlarvae were removed daily, when the solution was changed, and placed on TCBS agar plates overnight at 30°C.

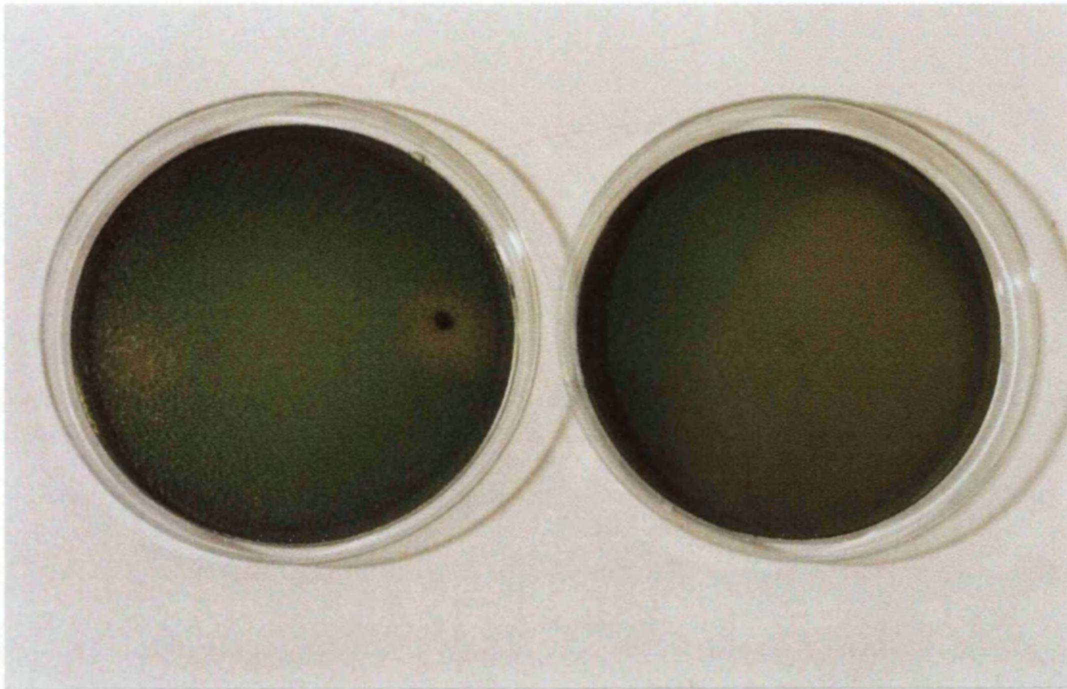


Figure 4.6. TCBS agar plates following exposure to postlarvae. The left hand plate contained , 2 postlarvae one has been removed leaving a yellow disc. The right hand plate was exposed to seawater the postlarvae were stored in on arrival at the laboratory.

Penicillin G, Streptomycin and Neomycin are all reported to be effective against *Vibrio* species (Jeffries, 1982) so a preparation containing all 3 was investigated (PSN Mixture 100X, GibcoBRL). A working solution of PSN containing 5mg Penicillin G, 5mg Streptomycin and 10mg Neomycin per litre was added to the tanks containing the postlarvae. After 7 days of treatment the TCBS agar tests showed that *Vibrio* species were still present.

Chloramphenicol, another antibiotic reported to be effective against *Vibrio* species (Matsubara et al., 2002; Robert et al., 1996), was then investigated. Provasoli's antibiotic concentrated solution (Sigma Aldrich Company Ltd.) contains a mix of antibiotics including Chloramphenicol. Provasoli's concentrate contains 12,000 units Penicillin G, 300 units Polymixin B, 50μg Chloramphenicol, 60μg Neomycin ml^{-1}. A working solution of 10ml Provasoli's concentrate per litre of filtered seawater was initially investigated. It was found that this eliminated the indigenous *Vibrio* species after 24 hours. In light of these findings, all postlarvae collected from the field were treated with the working solution of Provasoli's antibiotic mixture for 24 hours before use in exposure experiments.

A preliminary experiment was run using spat that had been treated with Provasoli's antibiotic concentrate on collection from the field. Postlarvae (0.5-3mm) were transferred to tanks containing 3 litres of Provasoli's antibiotic working solution (10ml concentrate L^{-1} seawater) in 0.2μm filtered, aerated seawater at 15°C and left overnight to acclimatise. Before use in the experiments, the postlarvae were removed from this solution and rinsed in fresh 0.2μm filtered seawater. Twenty postlarvae were then placed in each 50ml vented-cap tissue culture vessel containing 40ml of 5% Marine Broth in 0.2μm filtered seawater. The vessels were aerated via a 21G, 38mm needle. The treatments used were: control, 10^4 *V. tubiashii* ml^{-1} and 10^6 *V. tubiashii* ml^{-1}, with 2 replicates of each. The culture vessels were cleaned using a cell scraper and the media changed daily, at this point the vessels were checked under a dissection microscope for larval mortality. After 7 days, the postlarvae were fixed using EM fixative and removed from the vessel for assessment of mortality levels.

The results (Figure 4.7) show more normal postlarvae in the control vessels compared with the levels seen in the control vessels in the initial experiments involving postlarvae

with no antibiotic treatment (Figs. 4.4 and 4.5). The presence of *V. tubiashii* caused a decrease in the level of normal postlarvae, out of 40 postlarvae in each treatment 30 in the controls were normal at the end of the experiment compared with 12 and 16 respectively in the low and high dose exposures to *V. tubiashii*. However, there was no evidence of a dose dependant response in the numbers of dead postlarvae in the different treatments. It was thought that antibiotic residue was being transferred to the exposure vessels with the postlarvae reducing the number of bacteria present in the vessel; consequently a depuration period was introduced in all subsequent experiments.

4.3 Bacterial exposures

A depuration period of 4 days was introduced, during which time the postlarvae were kept in 0.2μm filtered UV sterilised seawater, which was changed daily. The use of filtered, UV-sterilised seawater ensured that there should be no re-colonisation of the postlarvae by bacteria before the exposure experiments.

The extensive methodological development, including antibiotic treatment and subsequent depuration, lead to the method described below which was used for all subsequent bacterial exposures studies.

4.3.1 Materials and Methods

Postlarvae (0.5-3mm) were collected and, on arrival at the laboratory, were transferred to tanks containing 3 litres of Provasoli's antibiotic working solution (10ml/L) in 0.2μm filtered, aerated seawater at 15°C. They were left in this solution for 24 hours to acclimatise and to eliminate indigenous bacteria. The postlarvae were then transferred to clean 0.2μm filtered UV sterilised seawater for a 4 day antibiotic depuration period, with the water being changed daily. After this depuration period, the postlarvae were fed daily with Liquifry Marine (Interpet Ltd, Dorking, England), a sterile commercial food for marine filter feeders, at the concentration recommended by the manufacturers (Stock solution = 1 drop in 10ml filtered seawater; add 220μl stock solution per 100ml), the culture medium was then changed an hour later.

The tissue culture vessels were filled with 60ml of 0.2μm filtered seawater containing 5% Marine Broth and aerated via a 21G, 38mm needle. There were 5 replicates of each treatment (control, 10^4 bacteria ml^{-1} and 10^6 bacteria ml^{-1}) with each culture vessel

containing 10 postlarvae, feeding commenced after the depuration period. At the end of the 7 day exposure period, the postlarvae were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 2 hours at 4°C, before being assessed using a dissection microscope. Postlarvae that were gaping were considered dead, an intermediate stage between healthy and dead animals was noted where larvae had produced a large quantity of mucus. The postlarvae were exposed to all 3 species of bacteria within one month (21/3/2000 to 18/04/2000) in order to minimise any possible seasonal effects.

Results were analysed using z-tests (Excel, Microsoft Office 2003), the data were log transformed before analysis. As the production of excess amounts of mucus was seen as a stress response and a precursor to mortality, levels of dead postlarvae and those in the mucus category were pooled for the purpose of statistical analysis

4.3.2 Results

The introduction of a depuration period removed the risk of antibiotic transfer to the exposure vessel. The levels of mortality in the postlarvae exposed to *V. tubiashii* were higher in the experiments run with depurated animals compared with those seen in the experiment run without a depuration period (Fig. 4.8 c.f. Fig. 4.7). This is summarised below in Table 4.1.

Treatment	No depuration step, % larval mortality	With depuration step, % larval mortality
Control	15	28
10^4 *V. tubiashii* ml^{-1}	15	28
10^6 *V. tubiashii* ml^{-1}	12.5	60

Table 4.1. Comparison of the levels of mortality (percentage) in experiments run for 7 days with or without a depuration period after antibiotic treatment.

The level of mortality in the control animals was increased with the introduction of a depuration period, this was thought to be due to food limitation as, initially, animals were not fed during the depuration period. Consequently, in all further studies the postlarvae were fed Liquifry Marine throughout the depuration period.

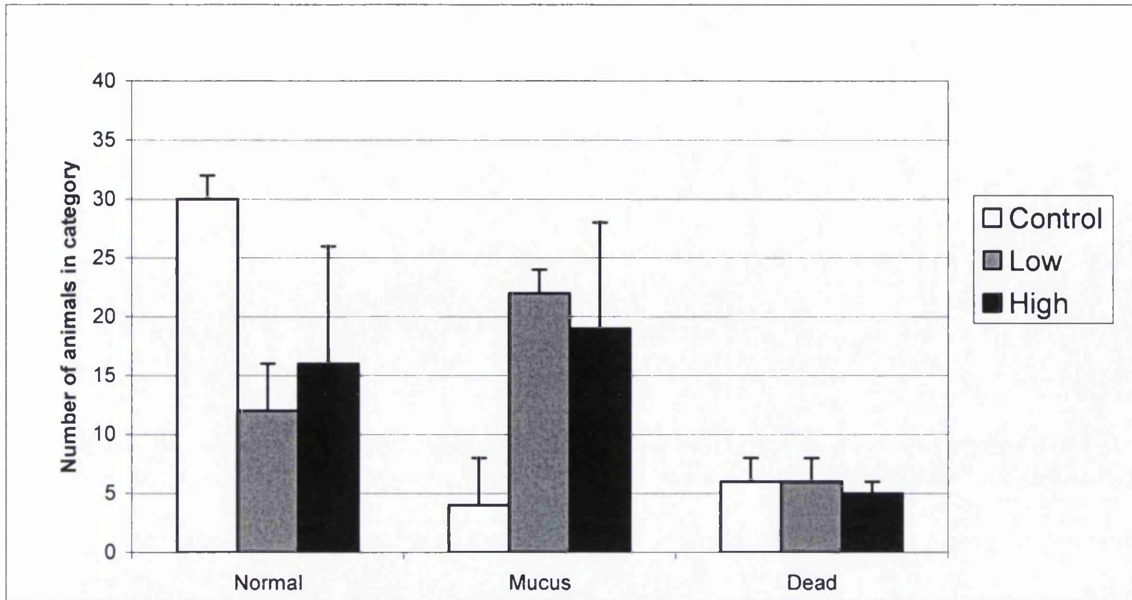


Figure 4.7. Antibiotic selection exposure experiment. Postlarval viability after 7 day exposure, with no depuration step, (01/11/99) to *Vibrio tubiashii*. (Control: No *Vibrio*. Low: 10^4 *Vibrio* ml^{-1}. High: 10^6 *Vibrio* ml^{-1}.). Postlarvae were determined to be either live, dead, or at an intermediate stage producing large quantities of mucus. Total counts are given, n=40 animals per treatment, 20 per vessel and 2 vessels per treatment.

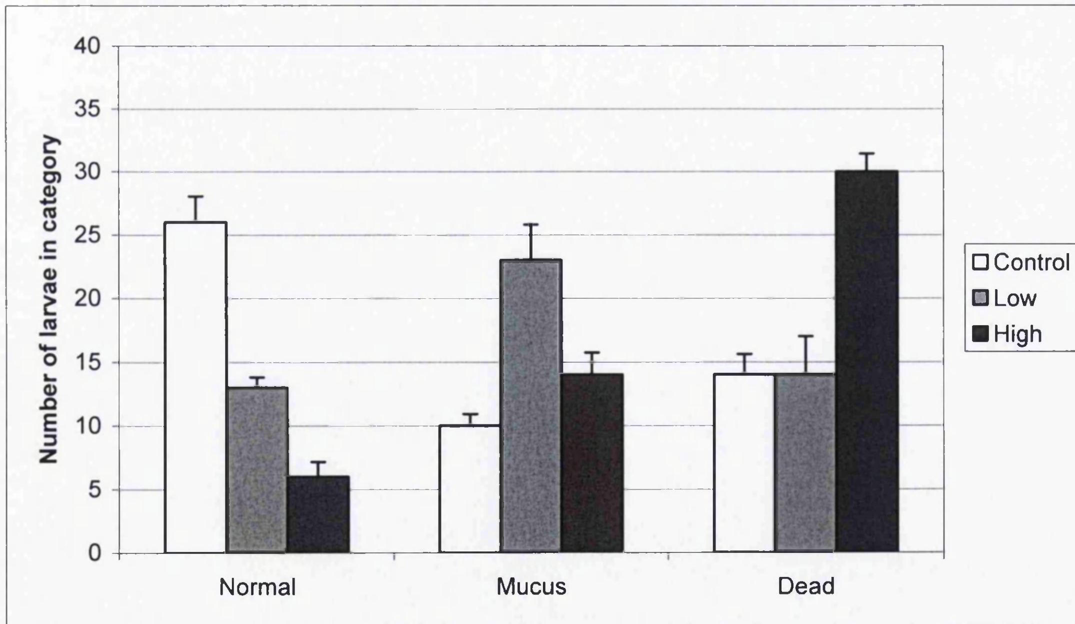


Figure 4.8. Postlarval viability after 7 day exposure (21/03/00) to *Vibrio tubiashii*. (Control: No *Vibrio*. Low: 10^4 *Vibrio* ml^{-1}. High: 10^6 *Vibrio* ml^{-1}.). Postlarvae were determined to be either live, dead, or at an intermediate stage producing large quantities of mucus. Total counts are given, n=50 animals per treatment, 10 per vessel and 5 vessels per treatment.

The postlarvae that were exposed to *V. alginolyticus* showed a high level of mortality in both the bacterial treatments (Fig. 4.9). However, there was also a very low level of normal postlarvae in the controls, 9 (±1.17, 2SE), and a high level of mucus production (29 ± 2.04, 2SE) by these animals. The decreased level of normal control spat was seen in all 5 replicates. This could be seen as an indicator of low quality seawater, however, this is unlikely as the seawater used throughout the experiments was routinely filter sterilised and UV purified. It is more likely that the postlarvae brought in from the field were in poor condition, possibly as a result of exposure to contaminants or food deprivation. Stressed individuals are often more susceptible to bacterial infection, which would lead to higher mortality in the exposed postlarvae, and higher mucus secretion in the control postlarvae. The nature of the stressor was not determined, however mussels from Whitsand Bay have been routinely analysed each month since 1995 and no unusual events, in the mussels annual cycle, were detected that would correlate with the periods larvae were collected for these experiments (Lowe, pers. comm.).

Exposure to 10^6 *V. tubiashii* ml^{-1} resulted in 30/50 dead postlarvae, twice the level (14/50) seen in those postlarvae exposed to 10^4 *V .tubiashii* ml^{-1} (Fig. 4.8). Those postlarvae exposed to the lower level *of V. tubiashii* produced the highest levels of mucus secretion.

Regarding *Alteromonas haloplanktis*, it appears that postlarvae used in the exposure experiment were stressed when brought in from the field. Those postlarvae exposed to 10^6 *A. haloplanktis* ml^{-1} developed a fungal infection, which could have been triggered by the additional stress of exposure to high levels of bacteria (Fig. 4.10). Those postlarvae exposed to both the lower dose of bacteria and the controls did not develop the infection.

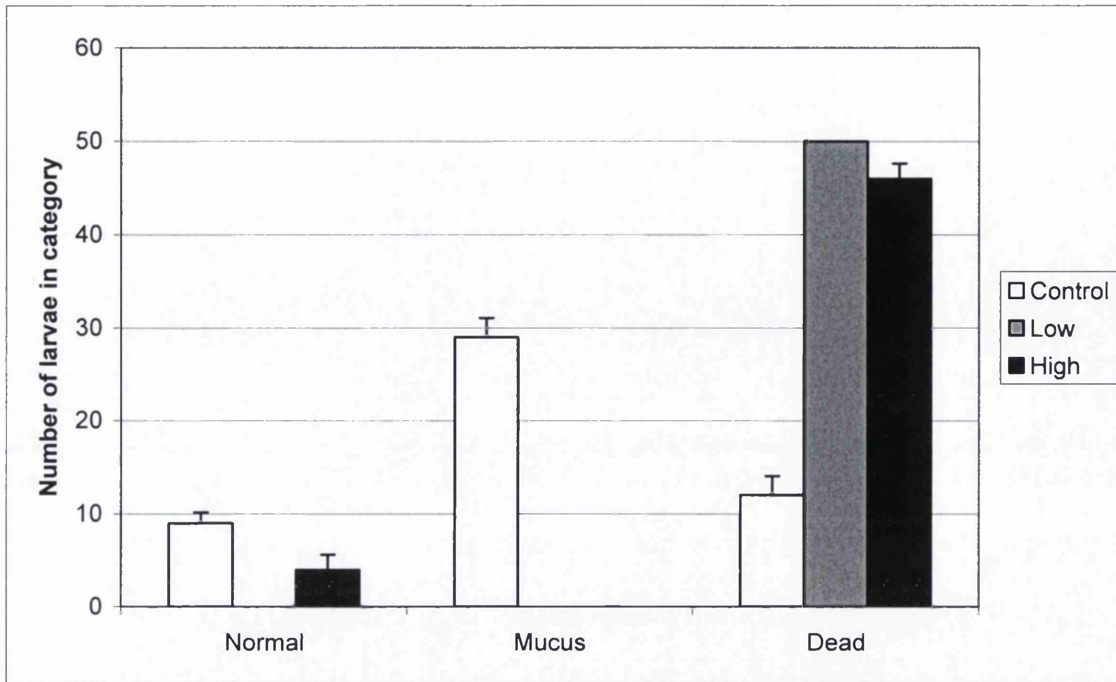


Figure 4.9. Postlarval viability after 7 day exposure (04/04/00) to *Vibrio alginolyticus*. (Control: No *Vibrio*. Low: 10^4 *Vibrio* ml^{-1}. High: 10^6 *Vibrio* ml^{-1}.). Postlarvae were determined to be either live, dead, or at an intermediate stage producing large quantities of mucus. Total counts are given, n=50 animals per treatment, 10 per vessel and 5 vessels per treatment.

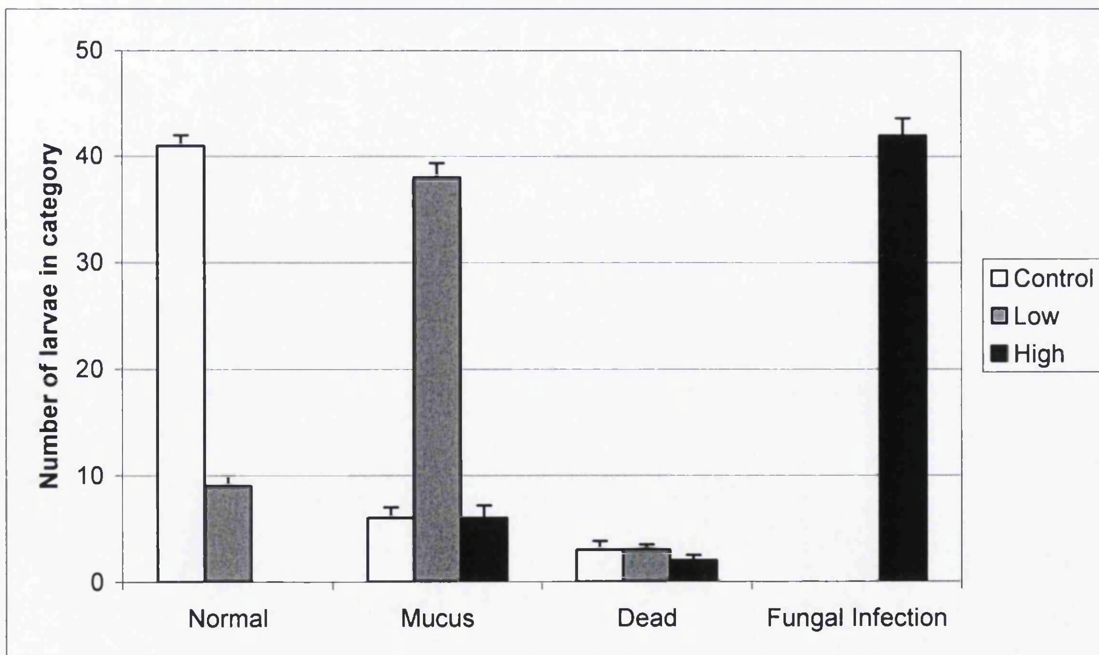


Figure 4.10. Postlarval viability after 7 day exposure (18/04/00) to *Alteromonas haloplanktis*. (Control: No *Alteromonas*. Low: 10^4 *Alteromonas* ml^{-1}. High: 10^6 *Alteromonas* ml^{-1}.). Postlarvae were determined to be either live, dead, or at an intermediate stage producing large quantities of mucus. Total counts are given, n=50 animals per treatment, 10 per vessel and 5 vessels per treatment.

Statistical analysis was not carried out on the results of the *A. haloplanktis* experiments due to the fungal infection. As previously mentioned, the levels of normal postlarvae in the control vessels of the *V. alginolyticus* experiments were also low (Fig. 4.10). An initial z-test was carried out to assess whether there was a significant difference between the control postlarvae in the *V. tubiashii* experiment compared with the *V. alginolyticus* experiment (Fig. 4.8 c.f. Fig. 4.9). There were significantly more normal postlarvae in the *V. tubiashii* control vessels (p=0.000) compared with those in the *V. alginolyticus* vessels at the end of the experiments. As a result of their being significant differences in the level of mortality in the control vessels of the two experiments it is impossible to carry out detailed statistical analysis to compare the results of these two exposures and assess the respective virulence of each species. However, the literature does contain a number of studies on *Vibrio* infections in bivalves and these studies have been considered in the discussion and related to the findings of this study wherever possible. As the levels of normal postlarvae in both the *Vibrio* exposure experiments was low z-tests were not run to assess whether the addition of *Vibrio* bacteria had a significant effect on the survival of postlarvae. However, z-tests were run to assess whether there were any significant differences resulting from exposure to high bacterial concentration versus low bacterial concentration. Exposure to 1 x 10^6 *V. tubashii* ml^{-1} did not significantly ($p>0.05$) increase postlarval mortality compared with exposure to 1 x 10^4 *V. tubashii* ml^{-1}. This was also the case with *V. alginolyticus* exposure, a z-test showed that there was no significant ($p>0.05$) difference resulting from exposure to 1 x 10^6 *V. alginolyticus* ml^{-1} compared with exposure to1 x 10^4 *V. alginolyticus* ml^{-1}.

4.4 Discussion.

In the present study postlarvae collected from the environment were shown have an indigenous bacterial flora. The existence of indigenous bacteria in wild bivalves has been widely reported, these bacteria are often present at higher concentrations in individual bivalves compared with the surrounding environment (Kueh & Chan, 1985). Colwell and Liston (1962) were the first to study extensively the natural bacterial flora of marine invertebrates, finding that Gram-negative bacteria predominated. The composition of indigenous bacteria in bivalves varies with geographical location, species and season. In the case of *Crassostrea gigas,* Kueh and Chan (1985) found that *Pseudomonas* dominated the bacterial flora, while Olafsen et al. (1993) reported that

Pseudomonas, Alteromonas, Vibrio and *Aeromonas* organisms were equally dominant. Olafsen et al. (1993) demonstrated the *Vibrio* species were present in the haemolymph and tissues of Pacific oysters and horse mussels. Ripabelli et al. (1999) studied the bacterial flora of *Mytilus galloprovincialis* harvested from approved shellfish waters in the Adriatic Sea, as well as samples purchased from markets. Although no *Campylobacter, E. coli* or *Salmonella* bacteria were isolated many *Vibrio* species were, with the most frequently isolated being *V. alginolyticus*. Multiple *Vibrio* species were reported to coexist in some individuals (Canesi et al., 2001). Bacteria have different sensitivities to the depuration process that shellfish are exposed to before being sold; some *Vibrio* species have been reported to be resistant to the process and are able to persist and multiply within shellfish tissues (Jones et al., 1991; Canesi et al., 2001). Olafsen et al. (1993) reported that *Vibrios* persist in shellfish haemolymph and soft tissues after depuration in UV treated seawater. Some of these *Vibrio* species are opportunist pathogens that proliferate, invade tissues and cause disease when the animals are exposed to stressors such as increased water temperature (Grischkowski & Liston, 1974). Although *Crassostrea gigas* possess lectins that agglutinate *Vibrio cholerae*, they were not found to agglutinate any of 79 other strains of bacteria tested, including *Vibrio alginolyticus* (Tamplin & Fisher, 1989). This lack of agglutination may be symptomatic of a lack of immune response to the presence of *Vibrio* species and account for their proliferation in shellfish tissues. The indigenous bacteria present in the postlarvae used in this study were eliminated using an antibiotic mix before exposure experiments were carried out.

Although the postlarvae used in the present study were found to contain associated *Vibri*o species it is unclear whether these species would go on to cause mortalities. Perkins (1993), however, reported that population explosions of opportunistic bacteria such as *Vibrio* are especially apparent in confined habitats, for instance in hatcheries, where they can cause mortalities. These explosions are thought to be related to nutrient enrichment and the introduction of postlarvae to the Marine Broth media also provides nutrient enrichment leading to the proliferation of indigenous bacteria. Perkins (1993) went on to consider whether eutrophication of estuaries may lead to an elevation of numbers of *Vibrio* species, which could then cause mortalities of bivalve larvae in the estuary. However, given the lower density of postlarvae in the field, compared to hatcheries, he concluded that the concentrations of *Vibrio* species needed to induce

disease in the field are probably much higher than those which cause disease in a hatchery.

Postlarvae exposed to bacteria were shown to produce large quantities of mucus. The use of mucus to isolate an animal from its environment is common in marine molluscs (Davies & Hawkins, 1998). Any water-borne element entering a bivalve first encounters the copious mucus covering all external surfaces of the soft tissues (Fisher, 2004). Mucus forms a line of defence in many marine invertebrates (Astley & Ratcliffe, 1989) often containing antibacterial factors (McDade & Tripp, 1967). In *M. edulis*, mucus is involved in normal feeding as well as the production of pseudofaeces, that is the rejection of excessive or unsuitable particles. Mucus binds particles on the gill filaments and enables their transfer in mucus strings that eventually enter the oesophagus and stomach (Jørgensen, 1981). However, excessive secretion of mucus can be considered a stress response and is often associated with exposure to metals (Fisher, 2004) and hydrocarbons (Davies & Hawkins, 1998). Increased mucus secretion by mussels as a response to copper exposure has also been noted by several authors (Scott & Major, 1972; Hvilsom 1983; Sze & Lee 1995), who concluded it was a means of detoxification.

Elston et al. (1982) conducted the first study investigating reports of bacterial disease in juvenile shellfish in large scale shellfish culture. Losses of juvenile stage animals is considered a far more insidious problem than the losses of larval stage animals because of difficulties in monitoring and enumerating chronic losses once individuals are transferred to the growing beds. Elston et al. (1982) studied juvenile (from 0.8 to 3.0mm in shell height) *Crassostrea virginica, Ostrea edulis* and *Mercenaria mercenaria*, collecting samples of the juvenile bivalves, cultured algae and water to determine the source of bacterial contamination. It was thought that juveniles contract bacterial infection upon settlement, as most bacteria found in the shellfisheries studied had strong surface affinity. The bacteria found in the hatcheries were identified to the genus level and were found to be Vibrios (Elston et al. 1982) The juvenile bivalves seem more resistant to infection of soft tissues than their larval counterparts, however, opportunistic infections still occur. Previous studies had demonstrated vibriosis to be an important problem in large scale commercial culture of larval bivalves (Pallaird et al., 2004), but the study by Elston et al. (1982) was the first to indicate postlarvae were also

susceptible to the disease. The present study also indicated that postlarval mussels are susceptible to *Vibrio* infections although it wasn't possible to determine whether one species of bacteria was more virulent than another.

The emergence of *Vibrio* species as etiological agents of disease in cultured bivalves will be undoubtedly one of the major problems in the future years (Pallaird et al., 2004). Although many virulence factors of human and fish pathogenic *Vibrio* species have been identified, little knowledge exists regarding the *Vibrio* spp. pathogenic to bivalves. *Vibrio vulnificus* is an important human pathogen causing vomiting and in immunocompromised persons, it can infect the bloodstream, causing a life-threatening septicaemia, but it has not been reported to affect shellfish (Sokolova et al, 2005). Bacterial diseases affect bivalves differently according to their life stages and in general, adult bivalves do not suffer high mortality when experimentally challenged with bacteria that are pathogenic to larvae. *Vibrio vulnificus* concentrations measured on a per gram basis were found to significantly decrease with increasing tissue weight of adult *Crassostrea virginica* (Sokolova et al, 2005). Tubiash (1974) found that *V. anguillarum* is highly pathogenic to experimentally infected week old *Crassostrea virginica* larvae causing 90% mortalities after 48 hours. However, no significant mortality was seen in adult oysters injected with 8 x 10^7 *V. anguillarum*. Tubiash (1974) sacrificed the adult oysters 5 months later, finding that, aside from a blister at the site of injection, the animals were in good condition. Jeffries (1982) found that exposure to three unidentified *Vibrio* strains produced shell anomalies and damages shell ligaments in juvenile *Crassostrea gigas*. The juvenile *C. gigas* ceased feeding and abnormal sloughing of absorptive cells of the digestive tract occurred. The exposure resulted in mortality of 20-70% of juvenile populations (Jeffries 1982). This is considerably lower than the levels of mortality seen in larval cultures of *C. gigas* exposed to *Vibrio*. Prieur et al. (1990) reported that in adult molluscs *Vibrio* species are considered as bacteria of secondary infection rather than strict primary pathogens. Even under ideal conditions for vibriosis, ie. high temperature, poor physical condition of the mollusc and large level of bacterial contamination, mortality of adult molluscs is relatively low, at around 30%.

It is clear that the response of bivalves to *Vibrio* is species specific; in the present study two species of *Vibrio* were examined, *V. alginolyticus* and *V. tubiashii*. *V. alginolyticus*

has been reported in *M. galloprovincialis* in Italy (Ripabelli et al. 2003), it is part of the normal marine flora which, during warm periods, can reach concentrations in the shellfish sufficient to cause disease in humans. This species is the most common *Vibrio* isolated from mussels and seawater (Hervio-Heath et al. 2002). *V. alginolyticus* has been associated with both wound infections and gastrointestinal infections in humans, however, its exact role as an enteric pathogen is unclear. Ripabelli et al. (2003) report that the strain of *V. alginolyticus* they isolated from mussels was resistant to both ampicillin and streptomycin. Gómez-León et al. (2005) isolated a strain of *V. alginolyticus* (TA15) from moribund carpet shell clam larvae (*Ruditapes decussatus*). They then went on to experimentally induce mortalities in 6mm spat using this isolated bacterium at 10^6 colony forming units (CFU) ml^{-1}, mortalities reached 50% after 30 days. Extracellular products (ECPs) from *Vibrio alginolyticus* TA15 were extracted and shown to have cytotoxic activity that significantly diminished adult clam haemocyte survival after 24 hours. There have been many reports regarding the production of ECPs by *Vibrio*, as mentioned in Chapter 3.

Although *Vibrio alginolyticus* is a commonly occurring, if not ubiquitous, marine bacterium, some strains are more pathogenic to shellfish than others (Elston and Lockwood, 1983). Elston and Lockwood (1983) isolated a *V. alginolyticus* strain from a number of moribund juvenile abalone subsequent histological observations confirmed that a bacterial infection was associated with the morbidity seen. *V. alginolyticus* has also been reported to be pathogenic to white shrimp (*Litopenaeus vannamei*) causing anorexia, poor growth and necrotic musculature (Cheng et al., 2005). The same bacterium has been shown to be lethal to the small abalone (*Haliotis diversicolor supertexta*) as were its extracellular products (Lee et al., 2001). The veliger larvae of red abalone (*Haliotis rufescens*) were found to be more susceptible to this pathogen than the postlarvae, massive mortality occurred within 24 hours at concentrations above 10^5 cells ml^{-1} but a concentration of 10^6 cells ml^{-1} was required to produce the same effect in postlarvae (Anguiano-Beltrán et al., 1998). High levels of mortality were seen in mussel postlarvae exposed in the present study to both 10^4 and 10^6 *V. alginolyticus* ml^{-1}, however, as mentioned earlier (section 4.3.2), these results are inconclusive as no statistical analysis can be carried out due to the low levels of normal postlarvae in the controls.

Regarding the bacteria examined in the present study, Nottage and Birkbeck (1986) studied the effects of 12 *Vibrio* strains on *Crassostrea gigas* and *Ostrea edulis* spat up to 7mm shell size. Two of the strains studied by Nottage and Birkbeck (1986) were the same as those used in the present study, NCIMB 1336 *V. tubiashii* and NCIMB 1339 *V. alginolyticus*. Nottage and Birkbeck (1986) exposed the spat in 25 well tissue culture Petri plates for maximum of 120h, examining them at 24 hour intervals. All 12 Vibrio strains examined were toxic but to varying degrees, Nottage and Birkbeck (1986) suggested that this was due to different strains producing variable amounts of toxin, the maximum toxicity of strains 1336 and 1339 was 93%. They reported that *Vibrio* infections typically produce larval mortalities in excess of 90% within 24 hours. The results obtained by Nottage and Birkbeck (1986) support the contention that oysters become more resistant to *Vibrio* infections as they grow larger. The 7mm *C. gigas* spat were 6 times less sensitive to the *Vibrio* toxin than 2-3mm spat. No significant inter-species differences in sensitivity to the toxin were reported between *O. edulis* and *C. gigas* spat of equivalent size, thus it may be the case that the results obtained by Nottage and Birkbeck (1986) could be extrapolated to *M. edulis* spat of the same size. No information was given regarding whether or not there were significant differences in toxicity between the strains 1336 and 1339.

In addition to studies on pathogenicity of *Vibrio* species to larval and postlarval bivalves the effects of exposure to *Alteromonas* species has also been studied by a number of researchers. Garland et al. (1983) found that two strains of *Alteromonas* species caused mortalities in *C. gigas* larvae at $1 \times 10^7 - 5 \times 10^7$ bacteria after 7 days of exposure, at the lower dose of $1 \times 10^5 - 5 \times 10^5$ bacteria the disease took 2-3 days longer to develop. The bacteria were isolated from diseased larvae in a hatchery and then identified to genus level but no further. Riquelme et al. (1996), however report that *Alteromonas haloplanktis* has probiotic properties. *Argopecten purpuratus* larvae were pre-incubated with a stationary phase inoculum of 5×10^6 cells ml^{-1} *Alteromonas haloplanktis* for 1 hour before being exposed to *Vibrio anguillarum*. The results indicated that *A. haloplanktis* was able to protect larvae from the infection with *Vibrio anguillarum* at a concentration of 10^3 cells ml^{-1} but at a higher concentration of 10^6 cells ml^{-1} the level of protection was reduced. Screening revealed that *A. haloplanktis* has a broad inhibitory spectrum, inhibiting Gram-positive as well as Gram-negative bacteria. The inhibitory compound appeared to be proteinaceous in nature and is produced or excreted by living

cells and was contained intracellularly. It is possible that the differences in the findings reported by Garland et al. (1983) and Riquelme et al. (1996) are due to the different workers using different species of *Alteromonas*. Alternatively the differences in virulence seen between the studies of Garland and Riquelme may relate to the growth phase of the bacterial culture used in the experiments, as the bactericidal components produced in the studies undertaken by Riquelme et al. (1996) are secondary metabolites and are only excreted in the stationary phase.

The results obtained in the present study indicate that there are potentially differences in virulence to postlarval mussels between bacterial species. Additionally there appear to be differences in bacterial virulence depending on the developmental stage studied. Unlike the larvae, *V. alginolyticus* appears to be more virulent to postlarvae than *V. tubiashii,* however this is not a definitive observation as the results were not statistically analysed and are inconclusive due to the low survival of control spat in the *V. alginolyticus* experiment. The fungal infection seen in postlarvae exposed to *A. haloplanktis* only occurs in those individuals exposed to the high (10^6 bacteria ml^{-1}) bacterial treatment. It may be that the bacteria are not usually pathogenic but they may suppress immune function sufficiently to allow opportunistic fungal infection.

Chapter 5. Effects of food availability on postlarval disease susceptibility.

5.1 Introduction

The amount of food available to filter feeding bivalves is partly determined by the efficiency with which the particles are retained. Mussels, *Mytilus edulis*, are capable of removing particles down to 2-3 µm with 80-100% efficiency (Møhlenberg & Riisgård, 1977). Mussels can also regulate their diet by altering both filtration rate and selective retention of particles. In experiments with pure algal cultures, several bivalve species have been observed to adjust their clearance rate in response to food concentrations (Prins et al., 1991). Both the inhalant and exhalent siphons of mussels possess a velum which can regulate the current flows (Bayne et al., 1976). Food particles that enter the mussel are driven by cilia and bound into mucus strings on the gill lamellae where they are carried along the ciliated grooves to the labial palps. The labial palps regulate the amount of food which enters the mouth and transfer surplus or inedible particles on to the rejection tracts of the mantle surface, from where it is expelled via the exhalent siphon as pseudofaeces. There is both spatial and seasonal variation in the quantity and quality of suspended particles (seston) in coastal waters (Gosling, 2003). The precise nutritional contribution that each component of the seston makes to the diet varies seasonally. Mussels ingest a wide range of seston including phyto- and zoo-plankton, bacteria and detritus and have been shown to alter their pumping rate in response to particle concentration.

At very low particle concentrations, *Mytilus* do not feed and in order for filtration to start the concentrations must exceed a critical threshold level. This critical threshold level varies with the type of seston the mussels are exposed to. Filtration rate also decreases at high particle concentrations; this is thought to be related to gut passage time and satiation of the digestive system (Riisgård, 1991). In nature, mussels are exposed to low concentrations of phytoplankton and are dependent on the supply of algae transported with water currents. Only the phytoplankton in the water up to 0.5m above the mussel beds are available to the animals. There is evidence that the growth of

benthic suspension feeding bivalves may be food-limited because the phytoplankton concentrations above dense populations may be depleted (Riisgård, 1991). The food requirements of bivalve spat are reported to be relatively low in comparison with those of adult animals. Laing (1995) found that *C. gigas* spatfall was maximal at 0.6μg food larva^{-1}, which is equivalent to a feeding rate of about 1250 *Isochrysis* cells hour^{-1}. Adult mussels and oysters have typical feeding rate of around 1000 times higher than the spat with adult *M. edulis* clearing approximately 20 x 10^6 *Rhodomonas* cells hour^{-1}(Riisgård, 1991). When feeding is withheld (over a period of up to 10 days), *C. gigas*, *C. virginica* and *Ostrea edulis* spat are able to utilise lipid reserves (Holland & Spencer, 1973; Laing, 1987,1995).

There is little information regarding the relative retention efficiency of particles by larval and juvenile bivalves (Wilson, 1980). It is thought that an upper limit of 10μm is imposed by the size of the larval oesophagus in lamellibranchs (Thorson, 1950). Walne (1965) demonstrated that cells of 3 to 10μm were efficiently retained by *Ostrea edulis* larvae (200-300μm in shell length). However, Baldwin and Newell (1991) reported that late umbo-stage *Crassostrea gigas* larvae ingested particles ranging from 0.2 right up to 30μm. Size retention of food particles by bivalve veligers is partly related to their mouth and oesophagus diameters, which get larger as they grow (Raby et al., 1997), the larvae studied by Baldwin and Newell were late umbo-stage and ready to metamorphose. Wilson (1980) noted that selectivity of particles increased with *Ostrea edulis* spat size, observing young juveniles at 1.3mm and older spat at 10mm, however, particle size preference remained constant. Wilson (1980) also compared the feeding behaviour of spat and larval *Ostrea,* noting that there was a difference in the optimal particle concentrations for larvae and spat, which he attributed to differences in the methods of filtration by the two stages. Juvenile *M. edulis* (1-4mm shell length) have been shown by Riisgård et al. (1980) to have 100% retention efficiency for particles down to 4μm but this decreases to 20% for 1μm particles.

It is generally assumed that mussels rely on phytoplankton as their main energy source but new evidence from field and laboratory studies shows that *M. edulis* can capture and ingest mesozooplankton such as crustacean and bivalve larvae from 10-1000μm (Davenport et al., 2000). Given that mussels are capable of sorting and rejecting particles in the form of pseudofaeces, the ingestion and discovery by Davenport et al.

(2000) of *Artemia* and larval barnacles in the stomach of mussels implies that they are extracting energy from the mesozooplankton. In addition to phyto- and mesozoo-plankton, dissolved amino acids have been shown experimentally to provide supplementary sources of energy for both larval and adult mussels (Manahan & Crisp, 1982; Seed & Suchanek, 1992).

Bacteria were originally reported to be cleared from suspension by mussels (Zobell & Feltham, 1938). They found that *Mytilus californianus* reduced the numbers of *Rhodococcus agilis* and *Bacillus marinus* from 500 - 5000 million bacteria ml^{-1} to only a few thousand ml^{-1} within 6 hours (Zobell & Feltham, 1938). Some of the bacteria were rejected in the pseudofaeces but many were ingested. The mussels were fed exclusively on these bacteria for up to 9 months and their weight monitored over this period; mussels fed on *R. agilis* gained 12.4% weight, *B. marinus* gained 9.7% and the fasting controls lost 16.3%, demonstrating that the bacteria were of nutritional value to the mussels (Zobell & Feltham, 1938). The oyster *Tiostrea chilensis* has also been reported to digest bacteria; they have been shown to digest *Vibrio parahaemolyticus*, a bacterium that causes gastroenteritis in humans (Cabello et al., 2005). Bacterial contributions to bivalve nutrition are thought to be of increasing importance, particularly at times of low phytoplankton abundance (Langdon & Newell, 1990). Bacterial concentrations can be high (up to 4×10^6 bacteria ml^{-1}) and can reach an average of 20% of the planktonic primary production, but since bacteria are very small the retention efficiency of the mussel may not be very high. Prieur et al. (1990) calculated that given a 20% retention efficiency by bivalves and a heterotrophic bacterial production of around 20% of the phytoplankton production, free-living bacteria may meet only 4% of an adult bivalve's needs. McHenery and Birbeck (1985) found that 5 (*Arctica islandica, Cerastoderma edule, Mya arenaria, Mytilus edulis* and *Ostrea edulis*) out of the 6 bivalves they studied were able to remove bacteria from an axenic suspension, only *Chlamys opercularis* was unable to do so, although it could when the bacteria were in a mixed suspension with the alga, *Tetraselmis suecica*. The bacteria were not attached to the *Tetraselmis*, it was thought that the increased clearance of bacteria in the presence of the alga was due to the triggering of a feeding response by the alga.

As mentioned in previous chapters, bivalves harbour an exceptionally rich microflora in which *Vibrio* species predominate. Healthy bivalves may have bacteria in their soft tissues and haemolymph and can act as specific carriers of some vibrios, such as *Vibrio parahaemolyticus* and other human pathogens (Zampini et al., 2003). Adult bivalves are normally populated by opportunistic pathogens without contracting disease. Opportunistic vibrios appear to be prevalent on the external surfaces and in the digestive tract of healthy oysters (Pujalte et al., 1999), and the number of vibrios increase in compromised hosts. The aim of this chapter is to assess whether the addition of a stressor, food deprivation, affects the virulence of bacteria to postlarval mussels. The bacteria used in the present study have been shown to be pathogenic to larval mussels (Chapter 3) but exhibit less of an effect on postlarval mussels (Chapter 4). It is of further interest to determine whether these bacteria can be utilised by postlarval *M. edulis* as potential food sources in the absence of phytoplankton.

5.2 Materials and methods

Postlarvae (0.5-3mm) were collected from Whitsand Bay, Cornwall and, exposed to bacteria as detailed in Chapter 4 (section 4.3.1). However a number of alterations were made, the postlarvae were fed throughout the depuration period but were not fed during the 7 day exposure to bacteria.

There were 2 replicates of each treatment (control, 10^4 bacteria ml^{-1} and 10^6 bacteria ml^{-1}) with each culture vessel containing 20 postlarvae, with the exception of the *V. tubiashii* exposure experiment commencing on the 07/03/2000; which had 5 replicates of each treatment with 10 postlarvae in each vessel. At the end of the 7 day exposure period, the postlarvae were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 2 hours at 4°C, before being assessed using a dissection microscope. Postlarvae that were gaping were considered dead, an intermediate stage between healthy and dead animals was noted where larvae had produced a large quantity of mucus. The postlarvae were exposed to three species of bacteria, *Vibrio tubiashii, Vibrio alginolyticus* and *Alteromonas haloplanktis* supplied by NCIMB, again as detailed in Chapter 4. Experiments exposing postlarvae to *Vibrio tubiashii* were started on the 20/12/1999 and 07/03/2000. In addition, experiments exposing postlarvae to all three species of bacteria concurrently were carried out on the 21/02/2000, with the

postlarvae for all three experiments being collected from the field at the same time. It was hoped this would minimise any possible environmental effects and allow direct comparison of the pathogenicity of the different bacteria.

Results were analysed using z-tests (Excel, Microsoft Office 2003), the data were log transformed before analysis. As the production of excess amounts of mucus was seen as a stress response and a precursor to mortality (see Chapter 4), levels of dead postlarvae and those in the mucus category were pooled for the purpose of statistical analysis.

5.3 Results

A z-test was run to assess the effects of food availability on postlarval survival, the results from the experiments run in the present chapter were compared with the results from experiments run in Chapter 4, which exposed postlarvae to the same bacterial species, however, they were fed Liquifry Marine throughout the exposures. Exposure of postlarvae to bacteria in the absence of food (this Chapter) significantly decreased ($p=0.0001$) the number of normal individuals seen at the end of the exposure period compared with those exposed to bacteria in the presence of food (in Chapter 4).

Figures 5.1 – 5.3 show the results of exposure to *Vibrio tubiashii* in the absence of feeding. This experiment was run three times and produced differing results each time it was run; this is thought to be a result of seasonal effects. Those postlarvae exposed to *V. tubiashii* in the winter (20/12/1999) show a clear dose dependant response, with the number of normal animals declining and the number of dead animals increasing with increasing bacterial concentration.

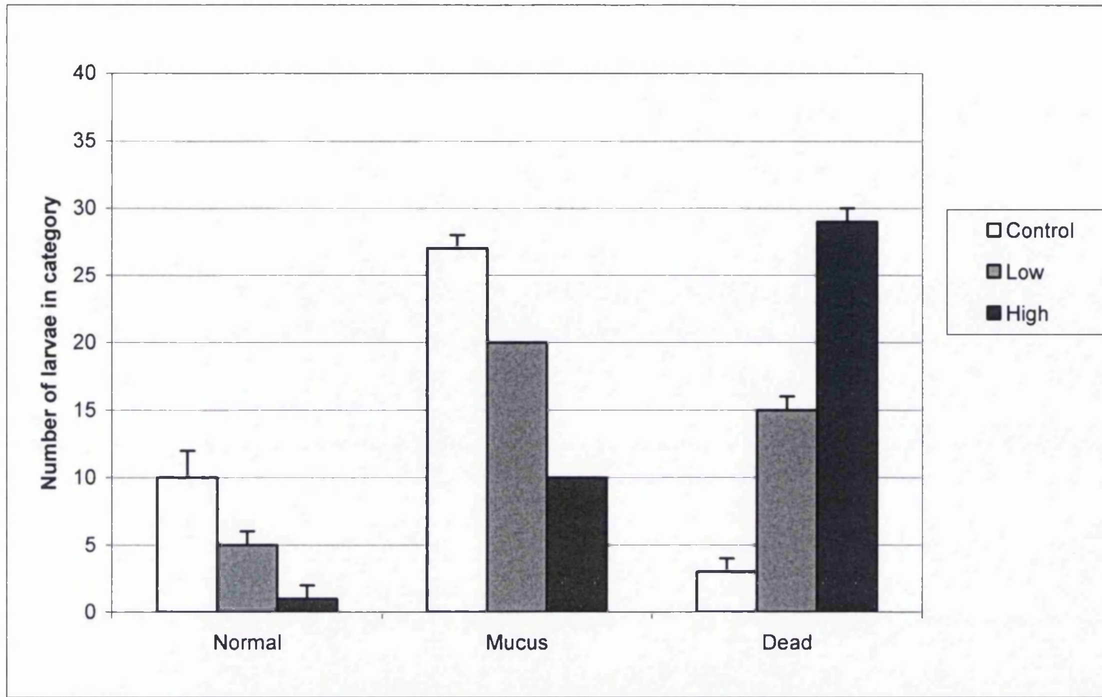


Figure 5.1. Postlarval viability after 7 day exposure to *Vibrio tubiashii* in the winter (20/12/99). (Control: No *Vibrio*. Low: 10^4 *Vibrio* ml^{-1}. High: 10^6 *Vibrio* ml^{-1}). Postlarvae were determined to be either live, dead, or at an intermediate stage producing large quantities of mucus. Total counts are given ±2 standard errors, n=40 animals per treatment, 20 per vessel and 2 vessels per treatment.

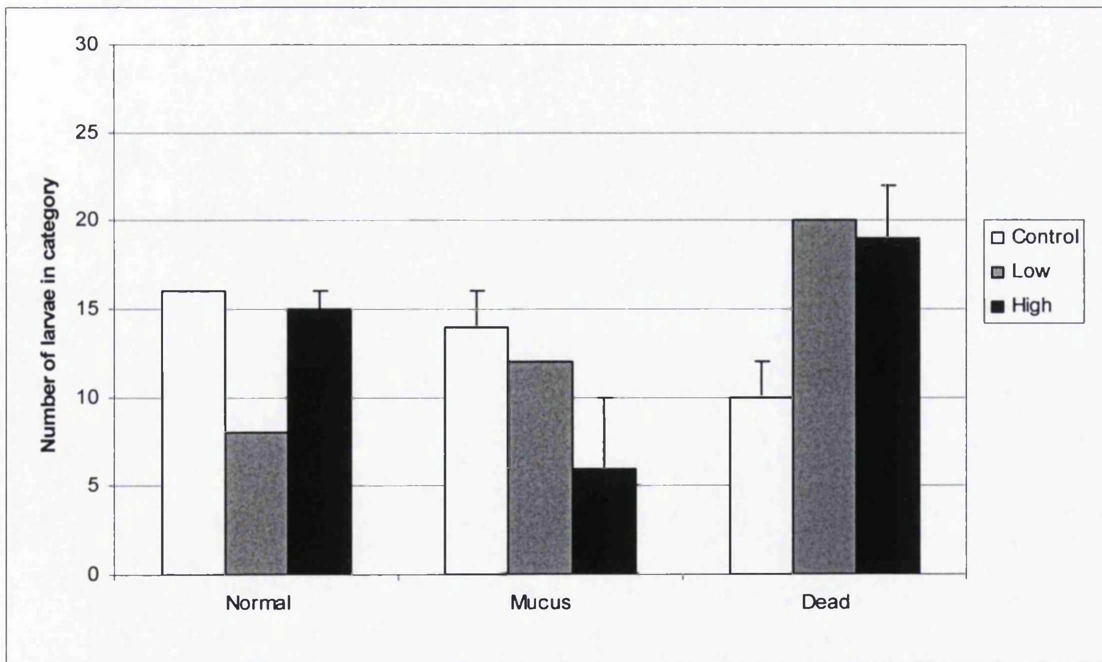


Figure 5.2. Postlarval viability after 7 day exposure to *Vibrio tubiashii*, early spring exposure (21/02/00) run concurrently with *V. alginolyticus* and *A. haloplanktis* exposures. (Control: No *Vibrio*. Low: 10^4 *Vibrio* ml^{-1}. High: 10^6 *Vibrio* ml^{-1}.). Postlarvae were determined to be either live, dead, or at an intermediate stage producing large quantities of mucus. Total counts are given ±2 standard errors, n=40 animals per treatment, 20 per vessel and 2 vessels per treatment.

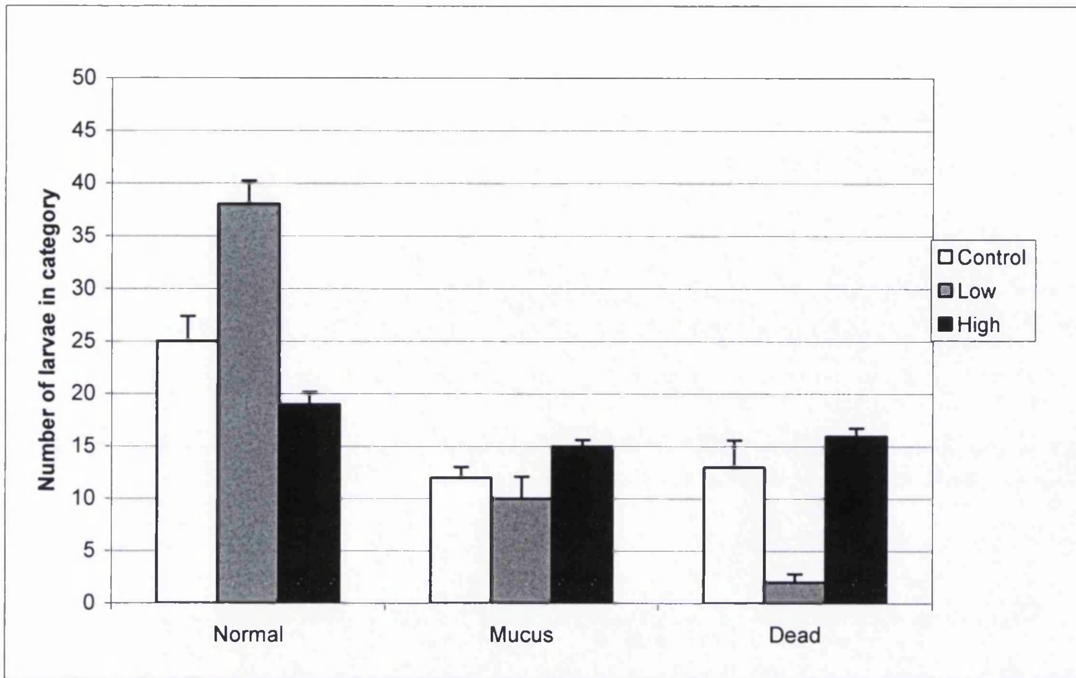


Figure 5.3. Postlarval viability after 7 day exposure to *Vibrio tubiashii*, late spring exposure (07/03/00). (Control: No *Vibrio*. Low: 10^4 *Vibrio* ml^{-1}. High: 10^6 *Vibrio* ml^{-1}.). Postlarvae were determined to be either live, dead, or at an intermediate stage producing large quantities of mucus. Total counts are given ±2 standard errors, n=50 animals per treatment, 10 per vessel and 5 vessels per treatment.

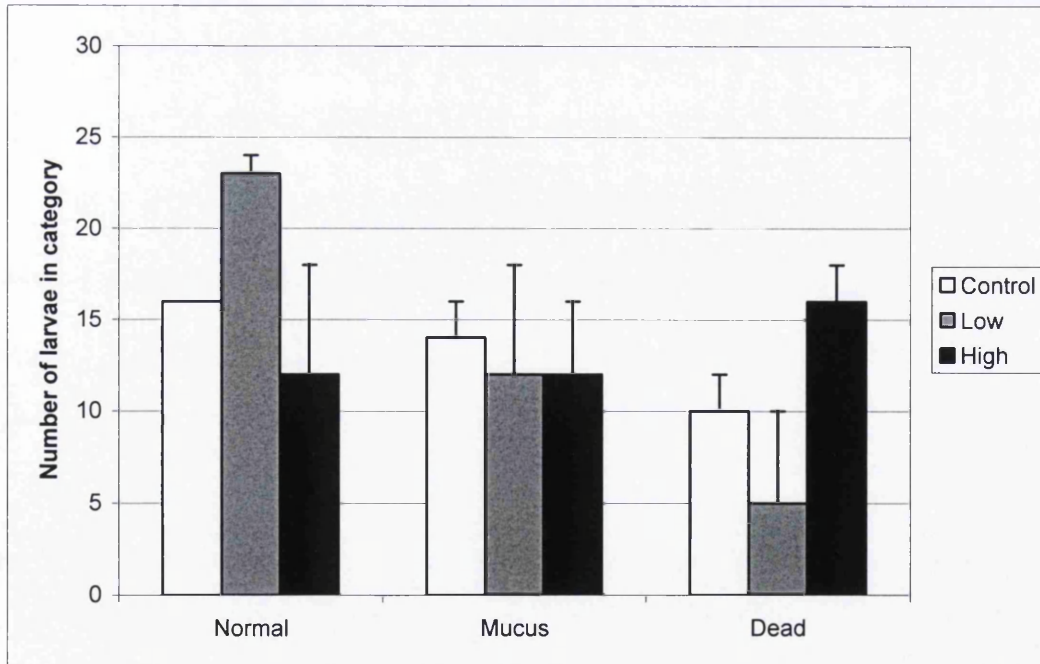


Figure 5.4. Postlarval viability after 7 day exposure to *Vibrio alginolyticus*, early spring exposure (21/02/00) run concurrently with *V. tubiashii* and *A. haloplanktis* exposures. (Control: No *Vibrio*. Low: 10^4 *Vibrio* ml^{-1}. High: 10^6 *Vibrio* ml^{-1}.). Postlarvae were determined to be either live, dead, or at an intermediate stage producing large quantities of mucus. Total counts are given ±2 standard errors, n=40 animals per treatment, 20 per vessel and 2 vessels per treatment.

However the number of normal individuals at the end of the exposure experiment in the control vessels is very low (10 ± 2, 2SE). Those postlarvae exposed to *V. tubiashii* in the spring months (21/02/2000 and 07/03/2000) showed a much higher level of normal individuals in the control vessels at the end of the exposure period, however there was no longer any clear dose dependant effect. A z-test was run to assess the effects of the presence of *V. tubiashii* in the absence of food, the low and high bacterial doses, from all three experiments, were combined and contrasted with the control. The presence of bacteria did not significantly ($p=0.0944$) affect the number of normal postlarvae seen at the end of the experiment compared with those in the control. A further z-test was then run to assess whether the level of bacterial exposure was of importance, $p=0.0571$ showing that there was no significant difference between those postlarvae exposed to 10^4 *V. tubiashii* ml^{-1} and those exposed to 10^6 *V. tubiashii* ml^{-1}.

Figure 5.4 shows the results of the exposure of postlarvae to *Vibrio alginolyticus* in the absence of food. No dose dependant response can been seen resulting from exposure to *V. alginolyticus*, although exposure to 10^6 *V. alginolyticus* ml^{-1} increases the percentage of dead postlarvae seen at the end of the experiment. Two z-tests were also run on the results of this exposure, neither were significant. The z-test which was run to assess the effects of the presence of *V. alginolyticus* in the absence of food generated a p value of 0.698, showing that the presence of bacteria did not affect the number of normal postlarvae seen at the end of the experiment compared with those in the control. There was no significant effect of concentration of bacterial exposure, $p = 0.1394$.

Figure 5.5 shows the results of exposure of postlarvae to *Alteromonas haloplanktis* in the absence of food. The addition of high bacterial dose (10^6 *A. haloplanktis* ml^{-1}) appears to increase the numbers of normal larvae at the end of the experiment compared with both the control and the low (10^4 *A. haloplanktis* ml^{-1}) bacterial dose. In order to see if this was a statistically significant result z-tests were run. The first z-test assessed the effects of the presence of *A. haloplanktis*, regardless of the concentration, in the absence of food, consequently the low and high bacterial doses were combined and contrasted with the control. The presence of bacteria alone did not significantly ($p=0.1272$) affect the number of normal postlarvae seen at the end of the experiment compared with those in the control.

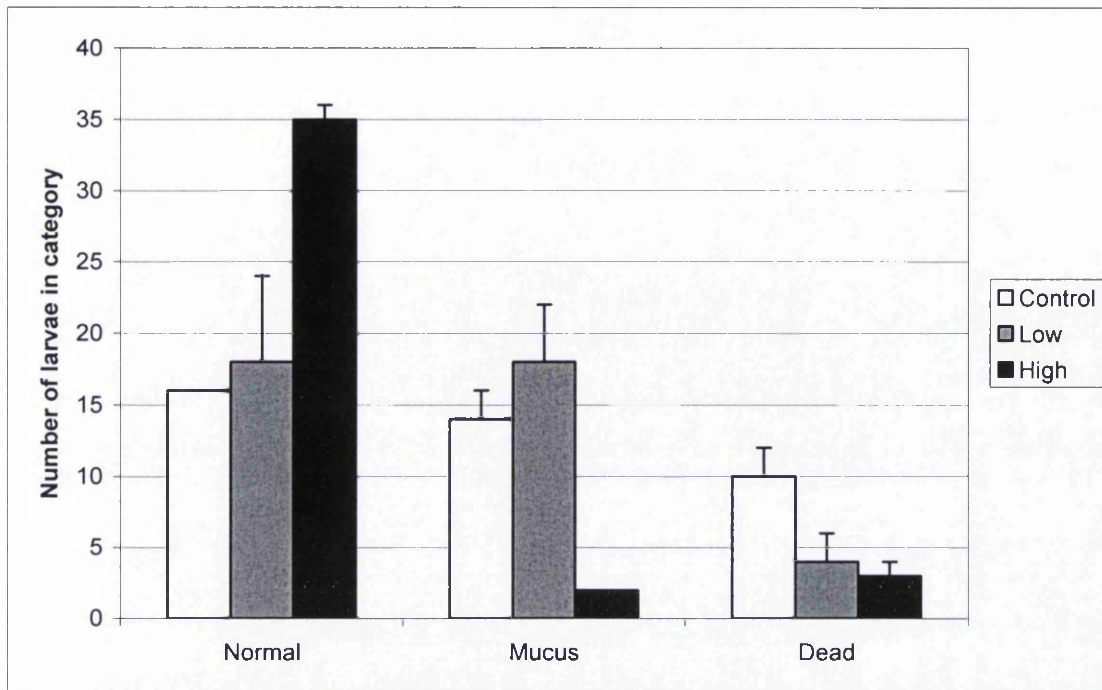


Figure 5.5. Postlarval viability after 7 day exposure to *Alteromonas haloplanktis*, early spring exposure (21/02/00) run concurrently with *V. tubiashii* and *V. alginolyticus* exposures. (Control: No *Alteromonas*. Low: 10^4 *Alteromonas* ml^{-1}. High: 10^6 *Alteromonas* ml^{-1}.). Postlarvae were determined to be either live, dead, or at an intermediate stage producing large quantities of mucus. Total counts are given ±2 standard errors, n=40 animals per treatment, 20 per vessel and 2 vessels per treatment.

A further z-test was then run to assess whether the level of bacterial exposure was of importance, contrasting the low bacterial dose with the high bacterial dose. At the end of the exposure period, when the level of normal postlarvae were compared with dead and mucus producing postlarvae, there was found to be a significant difference ($p=0.0109$) between those postlarvae exposed to 10^4 *A. haloplanktis* ml^{-1} and those exposed to 10^6 *A. haloplanktis* ml^{-1}.

5.4 Discussion.

The aim of these experiments was to see whether the absence of food acted as a stressor increasing the susceptibility of postlarvae to bacterial infection. Stress has been shown to affect disease susceptibility and is thought to be one of the main causes of summer mortality in oysters (Dégremont et al., 2007). The precise stressor thought to cause summer mortality is still unclear, but it is currently thought that high temperatures or anoxia increase the susceptibility of oysters to pathogenic bacteria. Temperature has been linked to outbreaks of vibriosis in the red abalone, *Haliotis rufescens* (Lee et al., 2001) with the causative agent being *Vibrio alginolyticus*. Lee et al. (2001) reported that the mortality of the small abalone, *Haliotis diversicolor supertexta*, is increased at raised temperatures, the abalone become much more sensitive to the bacterium and a much lower dosage will kill animals at higher temperatures compared with those held at lower temperatures. Temperature and levels of food availability have been shown by Hawkins et al., (2005) to alter the metabolism of the scallop, *Pecten maximus*. Alterations in the metabolism were shown to lead to accumulation of some metabolites, such as lactate and L-alanine, which were in turn shown to inhibit phagocytic activity (Hauton et al., 2001). The presence of elevated levels of ammonia has been linked with decreased phagocytic and clearance efficiency of *V. alginolyticus* in the white shrimp, *Litopenaeus vannamei* (Tseng et al., 2004). Under ordinary conditions, the immune responses of *L. vannamei* prevent *V. alginolyticus* infection taking hold and the resultant mortality (Tseng et al., 2004).

In the present study, postlarvae were exposed to potentially pathogenic bacteria in the absence of food to determine whether food deprivation acted as a stressor altering the susceptibility of postlarvae to these bacteria. However, it is also possible that, in the absence of other food sources, the bacteria may act as a carbon and nitrogen sources, as

demonstrated by Douillet (1993) and Lane and Birkbeck, (2000). The presence *V. tubiashii* or *V. alginolyticus* did not increase the survival of postlarval *M. edulis* exposed to these bacteria in the absence of phytoplankton, in fact exposure to these species were generally deleterious, as seen in previous chapters (Chapters 3 & 4). However, exposure of postlarvae to *A. haloplanktis* at high levels (10^6 bacteria ml^{-1}) significantly increased (p=0.0109) the number of normal postlarvae at the end of the 7 day period.

Three separate experiments were run using *Vibrio tubiashii* on the following dates 20/12/99 (Fig 5.1), 21/02/00 (Fig. 5.2) and 07/03/00 (Fig 5.3), producing differing results possibly as a result of seasonal effects. These results imply that postlarvae are more susceptible to pathogens at different times of the year. There appears to be a much lower percentage of normal animals in the control vessels in the winter exposure (Fig. 5.1) compared with the spring experiments (Fig. 5.2 and Fig. 5.3). It is possible that the differences in susceptibility of the postlarvae to the pathogen are due to the nutritional status of the postlarvae, the spat used in the winter experiments may already be food deprived before being brought into the laboratory and starved. L4 is a sampling station located 10 nautical miles southwest of Plymouth; weekly samples have been collected from this site since 1989. From L4 dataset, (http://www.pml.ac.uk/L4/), phytoplankton carbon levels are shown to be low, below 20mg C m^{-3}, from October 1999 until March 2000 when they start to rise again, doubling to 40 mg C m^{-3} by April. This seasonal pattern of variation in phytoplankton levels is seen annually.

Not only does there appear to be a much higher mortality of spat exposed to the high *V. tubiashii* levels in the winter compared with the spring, but within the two experiments run in spring there were also differing results. Higher mortality was seen in the earlier experiment, again lending support to the theory that as the spring progressed there was more food available in the environment so the animals that were brought in had more reserves and were therefore better able to cope with food deprivation. The differences in survival are not a result of the reproductive cycle as the postlarvae used in this experiment were unlikely to be sexually mature, the earliest gonad development and gamete storage in *M. edulis* occurs at 12-15mm shell length (Toro et al., 2002).

The diet of larvae can have an appreciable effect on the vitality of juveniles (Walne, 1970). It is obviously very important to consider the nutritional status of the postlarvae

brought in from the field as it clearly influences the responses seen, especially when the animals are being put under such severe stress by being starved at the same time as being exposed to potential pathogens. It is of most use to compare the results of the *V. tubiashii* experiment run in early spring with those exposing postlarvae to the two other bacterial species as these experiments were run concurrently, postlarvae for all three experiments being collected from the field at the same time. All of the species of bacteria can be seen to have different effects on the level of normal postlarvae at the end of the exposure period. As shown in the results, only high doses (10^6 bacteria ml^{-1}) of *Alteromonas haloplanktis* significantly ($p=0.0109$) increase the number of normal postlarvae at the end of the experiment.

Lane and Birkbeck, (2000) found that bacteria can be degraded by *M. edulis* at rates of up to 10^9 cells h^{-1}, and the bacterial components may provide a substantial part of the carbon and nitrogen requirement of some bivalves, especially in the absence of other food sources. It is therefore possible that high levels of *A. haloplanktis* are of nutritional benefit to postlarvae in the absence of phytoplankton. Douillet (1993) demonstrated that D-shell *C. gigas* larvae can ingest bacteria and assimilate bacterial particulate organic carbon. Of 21 strains of bacteria tested by Douillet and Langdon (1993) most were found to be detrimental to larval growth and survival, however, one (CA2) consistently enhanced larval survival and growth. The bacteria were added to axenic *Isochrysis galbana*. The bacterial strains tested did not provide all the nutritional requirements for the larvae but appeared to partially satisfy larval metabolic requirements. Larvae in cultures inoculated with single bacterial strains or mixtures of naturally occurring marine bacteria had higher survival rates than starved larvae, but lower growth rates than those fed on algal diets. Further studies by Douillet and Langdon (1994) demonstrated that the addition of CA2 at 10^5 cells ml^{-1} to cultures of algal-fed larvae increased larval growth, the proportion of larvae that set to produce spat and the subsequent size of larvae. It was thought that CA2 enhanced larval cultures by either providing essential nutrients not present in the algal diet or by improving their digestion by supplying digestive enzymes to the larvae. Tomaru et al. (2000) have shown that *Escherichia coli* and *Synechococcus* sp. act as food sources for the larvae of the Japanese pearl oyster (*Pinctada fucata martensii*). Hidu and Tubiash (1963) have also reported that larvae from clams (*Mercenaria mercenaria*) and oysters (*Crassostrea viginica*) are able to utilise bacterial flora as a food source.

Short term periods of starvation alone do not appear to cause negative effects in postlarvae, in fact Strömgren and Cary (1984) reported that postlarval *M. edulis* (shell length 12-22mm) starved for 5-8 days continued to grow slightly, with average shell growth rates of approximately 0.4μm h^{-1}. This implies that the absence of food alone for the period of exposure used in these studies should not have deleterious effects on the postlarvae. Fidalgo et al (1994) also demonstrated that spat can survive periods of starvation, reporting no mortality amongst *Mytilus galloprovincialis* spat (6-13mm in length) that were starved for 20 days, however, they lost 67% of initial dry flesh weight. However, in both these reported studies the effect of this stress on resistance to infection was not considered, but may be a significant factor in disease susceptibility and increase the susceptibility to opportunistic pathogens.

Previous nutritional history has been shown to have an effect on subsequent exposure to stressors. Beaumont and Toro (1996) demonstrated that postlarval *M. edulis* that were fed, survived significantly longer when exposed to copper than mussels that were starved and exposed to copper. Sixty three percent mortality was seen in the starved spat exposed to 100ppb copper in just 7 days, those spat that were fed reached 68% mortality after 30 days, animals that were starved, but not subjected to copper stress, reached 72% mortality after 52 days, animals fed and not subjected to copper stress had a mortality of <2% after 52 days. Without food, mussels must draw directly upon their food reserves for the metabolic energy required to detoxify copper and the availability of microalgae as food enabled mussels in the fed group to replenish energy lost in the process of detoxification. Immune system functions are thought to be influenced by the nutritional status of animals, including humans (de Pablo et al., 2000). The starvation of juvenile shrimps, *Litopenaeus vannamei*, for as little as 7 days results in a reduction of physiological and immunological indicators (Pascual et al., 2006). In experiments involving pre-conditioning with high protein diets, shrimp that were fed low protein diets showed the quickest reduction in immune responses such as respiratory burst and phenoloxidase activity. Thus, tolerance to food deprivation is closely related to previous nutritional condition. Compromised nutritional status may be correlated with the onset of or with increased susceptibility to diseases (Elston 1984).

The effects of any previous food deprivation may be enhanced by subsequent exposure to bacteria. McHenery and Birkbeck (1986) found that exposure to high concentrations

of marine *Vibrio* species in seawater (5 x 10^6 cells ml^{-1}) rapidly inhibited filtration by *Mytilus*. However, this inhibitory effect on filtration, and hence feeding, is specific to the species of bacteria the mussels are exposed to. When *Mytilus edulis* were fed *Pseudomonas* 1-1-1 the bacterium was rapidly cleared from suspension, clearing 90% of the initial concentration of bacteria (5 x 10^6 cells ml^{-1}) in 1.6h ±0.2h. In contrast, when the mussels were exposed to 4 strains of *Vibrio anguillarum* (isolated from moribund oyster larvae) after 10 to 20 minutes of normal pumping activity the mussels partially closed their shells. The bacteria were still cleared from suspension, although less than 80% were cleared over a period of 6 hours. The effect was also mediated by washed bacteria cells, implying the involvement of bacterial surface components. It is possible, therefore, that exposure to the *Vibrio* species used in the present study may reduce the mussel filtration rates and therefore minimise any possible nutritional benefits conferred by their ingestion.

As a result of bacterial presence causing alterations in filtration rate, the spat exposed to potentially pathogenic bacteria in the presence of food (Chapter 4) may be exposed to a higher concentration of bacteria than the spat exposed in the present study. McHenery and Birkbeck (1985) have shown that bivalves preferentially select algae in the presence of bacteria, indeed algal presence enhances the rate of bacterial uptake. The presence of *Tetraselmis suecica* increased the clearance coefficients of *Escherichia coli* from suspension although the bacteria were not attached to the algal cells. It was thought that the increased clearance of bacteria in the presence of algae could be due to triggering of feeding response by the algae.

The species of bacteria to which *Mytilus* are exposed is clearly of importance, with both *Vibrio* species studied here having a negative effect on postlarval survival whilst *A. haloplanktis* was beneficial at high doses. Additionally different strains of the same species of bacteria can vary in their pathogenicity. Nottage and Birkbeck (1990), compared two strains of *V. alginolyticus*, NCIMB 1339 (as used in this study) which is pathogenic to juvenile bivalves and an environmental isolate, obtained from the bacterial flora present in the tanks of a commercial aquaculture company. The environmental isolate exhibited >99% similarity to *V. alginolyticus* ("Bactid" scheme of identification, Jilly, 1988), however, the bivalve pathogenic strain (NCIMB 1339) was 2.5 times more lethal to haemocytes extracted from adult *Mytilus* than the

environmental isolate. *Vibrio alginolyticus* was shown to induce the loss of pseudopodia and cause cell rounding of *M. edulis* haemocytes. At lower numbers the *Vibrio* are agglutinated and killed when incubated with haemolymph fractions, with haemocytes appearing to be responsible for most of this antimicrobial activity, producing toxic oxygen intermediates (as measured by the reduction of NBT) when exposed to *Vibrio* cells. In addition they are able to degrade ingested *vibrio*, albeit more slowly than other marine bacteria. The effectiveness and rate of development of the internal defence system of juvenile bivalves has been little studied. Consequently it is possible that young bivalves are susceptible to V*ibrio* infections only while their internal defence systems are maturing.

In conclusion, there were significantly ($p=0.0001$) fewer normal postlarvae at the end of these experiments compared with those described in Chapter 4 when the postlarvae were fed. The addition of a high dose of *Alteromonas haloplanktis* (10^6 cells ml^{-1}) significantly increased the level of normal postlarvae present after the exposure period. The lower level of *A. haloplanktis* (10^4 cells ml^{-1}) did not significantly increase the level of normal postlarvae: it is possible that the lower level of dosing was not nutritionally rich enough to make a difference. This demonstrates that potentially pathogenic bacteria may be effectively used as a food source in the absence of other food but this is dependant on the virulence of the bacterial strain. It should be noted however that no tests were carried out to assess whether postlarvae harboured potentially pathogenic agents such as fungi or viruses in addition to the indigenous bacteria. Neither fungi nor viruses would be eliminated by the use of Provasoli's antibiotic concentrate. Consequently a caveat should be added stating it is possible the beneficial effect of *A. haloplanktis* noted here may be a result of the suppression of unknown pathogenic fungi or viruses present in the postlarvae collected from the field.

Chapter 6. Effects of copper on postlarval disease susceptibility.

6.1 Introduction

Metals are natural substances that become pollutants as a result of human activity. Unlike most other sources of anthropogenic pollution, such as organic pesticides, heavy metals are non-biodegradable. Copper is essential for the normal function of several enzymes such as superoxide dismutase, cytochrome oxidase and lysine oxidase (Nies, 1999). It is a component of the oxygen-binding haemolymph pigment, haemocyanin, present in some marine molluscs. The marine mussel, *Mytilus edulis*, does not possess haemocyanin, however, copper is still an essential constituent of some *Mytilus* haemolymph proteins (Davenport & Redpath, 1984). Although essential for normal metabolism in all marine organisms, at elevated levels copper is one of the most toxic heavy metals (Davenport & Redpath, 1984).

Copper enters the marine environment from many sources that can broadly be classified as runoff, aeolian input, and geothermal sources (Miranda & Rojas, 2006). Runoff is probably the most important of these, in terms of gross tonnage input, providing localized input into coastal waters which are major centres of population and sources of seafood (Lewis & Cave, 1982). Copper may be added to the sea as a result of mining operations, by power stations, desalination plants and as a by-product of industrial processes, including oil refining; dredging operations may also release copper from metalliferous sediments (Davenport & Redpath, 1984). Copper may also leach directly into the marine environment from Cu/Ni alloys used in marine structures; this alloy is widely used as not only is it resistant to seawater corrosion but, in addition, the copper also infers a high resistance to biofouling (Warnken et al., 2004).

Until recently, there was a tendency, in the United Kingdom at least, for copper pollution to be thought of as a problem of the past associated with the declining mining and smelting industries (Davenport & Redpath, 1984). However, since the discovery that tributyl tin (TBT) causes imposex (the development of false male organs in females) in gastropod molluscs and shell deformation in oysters, the use of copper-

based antifouling paints has increased and consequently the awareness of copper as a pollutant has risen. Copper has been used to control biofouling since the early 18th century when copper sheathing was applied to the hulls of boats (Valkirs et al., 2003). TBT-based antifouling paints were banned for use on crafts under 25m in length in 1987 (Walker et al., 1996) forcing a return to the traditional paints with copper oxide as their active component. The copper-containing compound acts as a biocide, preventing the settlement of fouling organisms or killing them after they have settled (Warnken et al., 2004). The antifouling paints are designed to provide long-term leaching of copper, producing a toxic environment on or next to the painted surface (Valkirs et al., 2003).

A historical study by Claisse and Alzieu (1993) on seawater copper concentrations from 1979 to 1991 found that there had been a perceptible increase that may have been attributable to the re-introduction of copper-based antifouling paints in 1982. The International Maritime Organization (IMO) banned the application of TBT, on any craft, on or after January 1st 2003. Furthermore, the IMO have agreed to the removal or over-coating of TBT antifouling systems on all vessels by 2008, so there is likely to be another increase in the use of copper as an antifouling agent in the near future (Valkirs et al., 2003). To assess the relative input from copper-based paints, Warnken et al. (2004) studied waters around popular anchorages in unspoilt areas of Australia, with the aim of separating inputs from boating sources and inputs from urban runoff into marinas. The results obtained showed that in open water areas, at sites used by 10 to 30 vessels, copper release from conventional tin-free antifouling paints were sufficient to significantly raise copper concentrations above normal background levels. A study by Matthiessen et al. (1999) also concluded that the single largest source of copper into estuarine waters of Essex and Suffolk was boating traffic employing copper-based antifouling paints. Valkirs et al. (2003) estimated that approximately 70% of the total annual copper loading in San Diego Bay originates either from leaching process or from the mechanical cleaning of antifouling coatings. Both Valkirs et al. (2003) and Warnken (2004) highlighted that although total levels of copper were increasing, due to the use of these paints, the environmental significance of copper loading does not solely depend on total mass input to the harbour but also on the speciation and complexation of copper. Thus further studies are necessary to fully investigate the impact of copper-based paints.

Copper levels in nearshore waters, are generally higher than those of the open oceans due to the proximity of copper sources (Lewis & Cave, 1982). The levels of copper vary with season due to runoff fluctuations (being higher in the winter), and levels also fluctuate with tidal cycle and depth. Reports of copper levels in UK coastal waters show wide variation from as low as 1.4ppb up to 8 ppb (Davenport & Redpath, 1984). Levels of 230-250 ppb copper have been recorded in waste discharged by the Los Angeles outfall systems (Martin et al., 1975) but values exceeding 30 ppb are unusual and very localised. Sedimentary levels of copper can be much higher. Geffard et al. (2002) reported sediment levels of copper in Arcachon Bay, France, of 33 $\mu g\ g^{-1}$ dry weight (±3), however, the associated water levels were only 0.4ppb. The EC Dangerous Substances Directive led to the designation of Environmental Quality Standards (EQSs) with the EQS for copper in seawater now set at $5\mu g\ L^{-1}$, equivalent of 5ppb, as an annual average (DEFRA, 2005). In 1995, no sites in the UK, as surveyed by the Environment Agency, exceeded this limit, and copper concentrations in 2001 were at similar levels, reflecting a lack of reduction in inputs from rivers, industrial, recreational, and sewage effluents to estuaries (DEFRA, 2005). DEFRA (2005) found that concentrations of copper are highest in industrial estuaries and coastal sediments around England and Wales, with the highest sediment copper concentration recorded in the Tamar (western English Channel) at 203 $mg\ kg^{-1}$, due to local mineralization and industrial activity.

The feeding habit of *M. edulis,* filtering large quantities of water, and their nearshore habitat (occurring to a maximum depth of 10 metres) exposes this species to many pollutants. They are long-lived and sedentary in nature which serves to increase their exposure to pollutants and consequently they often accumulate a wide range of toxicants. Molluscs, particularly *M. edulis,* have been used as indicator organisms to monitor pollution in the marine environment, most notably as part of the "Mussel Watch" programme. The "Mussel Watch Project" analyses chemical and biological contaminant trends in sediment and bivalve tissues collected in the USA and has been running from 1986 to present (National Centers for Coastal and Ocean Science NCCOS, 2007). Mussels show many different behavioural, physiological and metabolic effects in response to copper pollution. Elevated levels can cause respiratory and cardiovascular depression, inhibit ciliary activity and decrease the number of byssal threads produced (Scott & Major, 1972; Davenport & Redpath, 1984). Elevated copper

levels have also been shown to affect protein metabolism in *M. galloprovincialis*, reducing amino acid uptake by gills, mantle and digestive gland by 90-95 % in 7 days (Davenport & Redpath, 1984). *M. edulis* show "testing" behaviour in response to increased copper levels in the environment, this behaviour involves a series of alternate adductions and parting of the shell valves allowing sampling of the external medium by the mussel. The threshold for detection by mussels is about 0.02ppm total copper, little more than one order of magnitude higher than the background concentration in the Menai Straights, North Wales (Davenport & Manley, 1978). At copper concentrations of 0.2ppm or more, mussels show complete valve closure, thus isolating themselves from the environment (Davenport & Manley, 1978). Valve closure in *Mytilus* can lead to a decrease in oxygen tension in the mantle cavity and disruption in cardiac activity (Curtis et al., 2000). This valve closure is thought to allow a relatively sedentary bivalve such as *Mytilus* to temporarily avoid contamination and withstand episodic environmental threats. Since growth results from a range of behavioural, physiological and biochemical functions, it is not surprising that copper pollution also reduces growth rate. Sustained concentrations of 200ppb copper (administered for 1 month) or more kill *Mytilus*, while 100ppb is sufficient to alter behaviour, affect physiological processes and inhibit growth (Davenport & Redpath, 1984). Concentrations as low as 2 to 5 ppb produce measurable growth inhibition (Davenport & Redpath, 1984), indicating that background environmental levels are likely to affect mussels.

Bivalve molluscs concentrate metals by several orders of magnitude, relative to concentrations in the surrounding water, and tissue burdens often reflect the degree of contamination in the environment (Serafim et al., 2002). Boyden (1977) found that there was a size effect on copper concentration in *Mytilus*, with smaller mussels having higher concentrations than larger animals. The deleterious effects of elevated copper levels on marine organisms may be controlled by physiological and biochemical factors, some of which vary from species to species (Lewis & Cave, 1982). *M. edulis* appears to be able to excrete copper, and it has been hypothesized that there might be specific copper transporting sites (possibly on the gill) that have evolved because of the biological importance of copper to *Mytilus* (Phillips, 1976). However, it has also been shown that mussels taken from areas with different copper concentrations contain different levels of copper. The digestive gland of mussels living in an enclosed harbour

contained 127 mg Cu kg^{-1} dry tissue compared with 16 mg Cu kg^{-1} in coastal animals (Davenport & Redpath, 1984).

Mussels appear to distribute copper generally throughout the body. This is in contrast to other metals which are concentrated in particular tissues, with cadmium, for example, stored in metal containing granules in the kidney (George & Pirie, 1980). In invertebrates, two major mechanisms of detoxification involving intracellular metal ligands have been well documented. First, by metal-binding to cytosolic compounds including metallothioneins and, second, by biomineralisation, the incorporation of metals into membrane bound vesicles similar in appearance to lysosomes (Harrison et al., 1983). Depending on the species, the relative importance of these two detoxification mechanisms varies considerably. Extreme examples have been observed for particular metals in particular species, such as the storage of insoluble silver in *C. gigas* exposed in the laboratory or the worm, *Hediste diversicolor,* which when exposed to high levels of copper in the field stores 90% of its copper body burden in membrane bound vesicles (Amiard et al., 2006). In these two cases, only a fraction of cytosolic metal is bound to metallothioneins. Metallothioneins (MT) appear to play a role in the routine handling of essential metals like copper and zinc, especially during periods of normal growth and development (Ringwood & Brouwer, 1995). Additionally, they are involved in the detoxification of excess intracellular amounts of these essential metals and also of non-essential metals such as cadmium, silver and mercury (Amiard et al., 2006). Metallothionein is a cytosolic protein of low molecular weight that has been shown, both in the laboratory and *in situ*, to be induced by metal contamination in many taxa, including mammals, fish and marine invertebrates (Geffard et al., 2002). It has been demonstrated that in mussels, the induction of metallothionein synthesis plays an important role in metal detoxification (Viarengo et al., 1987). Limited or negligible involvement of metallothioneins in metal binding has been reported in other bivalve molluscs such as *Scrobicularia plana* (Langston et al., 1998). Oysters (*Crassostrea gigas* and *Ostrea edulis*) that were exposed to high concentrations of copper accumulated this metal in particular haemolymph cells, the granular amoebocytes, which were able to store it by a compartmentation process within membrane bound vesicles (George et al., 1978).

It is also well known that different stages of the life history of organisms exhibit different sensitivities to pollutants, and it has often been suggested that larval stages are less tolerant than adults (Coglianese & Martin, 1981; Davenport & Redpath, 1984; Roesijadi et al., 1997). Bioassays have shown that different ontogenetic stages in marine species may differ in their sensitivity to pollutants (Coglianese & Martin, 1981). As a general trend, a higher sensitivity to toxicants is found at earlier life stages and in bivalves the relative sensitivities are embryo>veliger>pediveliger>juvenile>adult (Ringwood, 1993; His et al., 2000). Davenport and Redpath (1984) reported that exposure to 200ppb (0.2ppm) copper for 2 weeks failed to cause significant mortality in veliger and pediveliger stages of *Mytilus edulis*. However, Davenport and Manley (1978) showed that a lower concentration (15ppb copper) resulted in a 50% mortality of adult *M. edulis* in a flow through system after 1 month. More recently, Hoare and Davenport (1994) also found that veliger mussels are relatively insensitive compared with the adults. Beaumont et al. (1987) postulated that this difference in sensitivity may be as a result of larval metabolism, which is based on lipid rather than glycogen storage, being more readily able to produce copper-binding metallothionein.

Physiological stress caused by the presence of a toxicant has been shown to reduce the resistance of the organism to bacterial challenge, thus decreasing the response thresholds as reviewed by His et al. (2000). In the present study, we examined the effects of copper exposure on the disease susceptibility of postlarval mussels. For the purpose of this investigation, a copper concentration of 0.02ppm was used as an environmentally relevant concentration representing acute exposure, as experienced in the Fall estuary in Cornwall following the Wheal Jane tin mine closure (Langston, pers. comm.). The postlarvae were co-exposed to *Vibrio tubiashii*, which is potentially pathogenic to postlarval mussels.

6.2 Materials and methods

Postlarval mussels (0.5-3mm) were collected from Whitsand Bay, Cornwall and, exposures were carried out as detailed in Chapter 4 (Section 4.3.1), with postlarvae being fed throughout the depuration and exposure periods. The postlarvae were only exposed to one bacterial species, *Vibrio tubiashii*, due to time constraints.

For this study, preliminary experiments were carried out in order to establish whether or not combining bacterial and copper exposure would lead to synergistic effects. These preliminary experiments were conducted to ensure that the combination of 0.02ppm copper with bacteria at levels used previously (Chapters 4 and 5) resulted in significant effects without inducing mass mortality. In addition to the *Vibrio tubiashii,* the postlarvae were also exposed to copper sulphate solution (Sigma-Aldrich C2284) at 0.02ppm. For the preliminary experiments there were 5 replicates of each treatment (control, 10^4 bacteria ml^{-1} and 10^6 bacteria ml^{-1}) with each culture vessel containing 10 postlarvae and 0.02ppm copper. At the end of the 7 day exposure period, the postlarvae were processed and assessed as detailed in Chapter 4. The results of these preliminary experiments are shown in the results section (Section 6.3).

The preliminary experiments showed that, despite co-exposure to copper and *V. tubiashii*, mortalities remained very low. Therefore the exposure experiments, together with full controls, were run with the following conditions:

- Control = 5% Marine Broth in 0.2μm filtered UV sterilised seawater, no copper and no bacteria.
- Copper control = as control, however also containing 0.02ppm copper but no bacteria.
- Low bacterial dose = containing 0.02ppm copper and 10^4 *V. tubiashii* per ml.
- High bacterial dose= containing 0.02ppm copper and 10^6 *V. tubiashii* per ml.

Postlarvae were exposed in tissue culture vessels, with 10 individuals per vessel and 5 replicates for each treatment. The experiments were run for 7 days and larval survival was assessed as detailed above.

Results were analysed using z-tests (Excel, Microsoft Office 2003), the data were log transformed before analysis. As the production of excess amounts of mucus was seen as a stress response and a precursor to mortality, levels of dead postlarvae and those in the mucus category were pooled for the purpose of statistical analysis.

6.3 Results

The results from the preliminary experiments are shown in Figures 6.1 and 6.2. The relatively low level of mortality in all the treatments indicated that the combination of copper and *V. tubiashii* was at sufficient levels not to cause immediate mass mortality. The preliminary experiments were run twice (27/09/2000 and 10/10/2000) due to the low level of normal postlarvae in the copper control from the experiment started on 27/09/2000. The results imply that the addition of bacteria increases survival of postlarvae, the levels of normal individuals at the end of exposure period appears to be higher in the low and high bacterial treatments compared with the copper controls. A z-test was run to assess the presence of bacteria in addition to copper in these preliminary experiments, the low and high bacterial doses were combined and contrasted with the copper control. The presence of bacteria significantly ($p<0.0000$) increased the number of normal postlarvae seen at the end of the experiment compared with those exposed to copper alone. A further z-test was then run to assess whether the level of bacterial exposure was of importance, $p=0.079$ showing that there was no significant difference between those postlarvae exposed to 10^4 *V. tubiashii* ml^{-1} and those exposed to 10^6 *V. tubiashii* ml^{-1}.

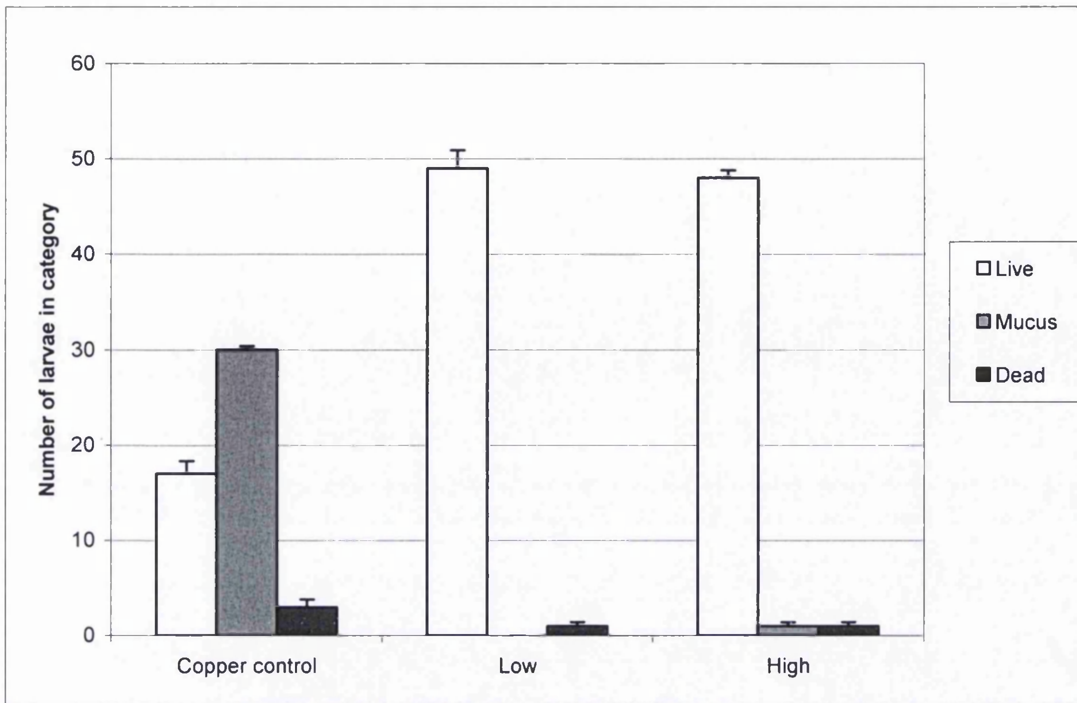


Figure 6.1. Postlarval viability after 7 day exposure (27/9/00) to *Vibrio tubiashii* and 0.02ppm copper. (Copper control: 0.02ppm copper, No *Vibrio*. Low: 0.02ppm copper and 10^4 *Vibrio* ml^{-1}. High: 0.02ppm copper and 10^6 *Vibrio* ml^{-1}). Postlarvae were determined to be either live, dead, or at an intermediate stage producing large quantities of mucus. Total counts are given ±2 standard errors, n=50 animals per treatment, 10 per vessel and 5 vessels per treatment.

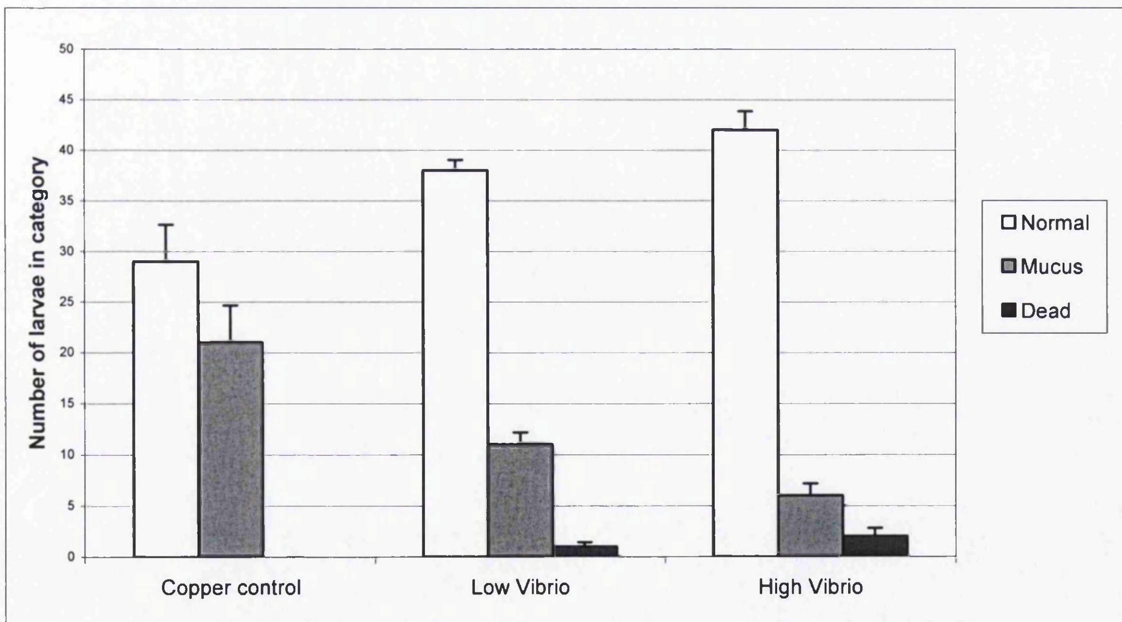


Figure 6.2. Postlarval viability after 7 day exposure (10/10/00) to *Vibrio tubiashii* and 0.02ppm copper. (Copper control: 0.02ppm copper, No *Vibrio*. Low: 0.02ppm copper and 10^4 *Vibrio* ml^{-1}. High: 0.02ppm copper and 10^6 *Vibrio* ml^{-1}.). Postlarvae were determined to be either live, dead, or at an intermediate stage producing large quantities of mucus. Total counts are given ±2 standard errors, n=50 animals per treatment, 10 per vessel and 5 vessels per treatment.

The exposure experiments were run with these copper and bacterial exposure levels again for a period of 7 days, but this time including a control, as well as the copper control, to ensure that any effects of copper alone could be monitored. The experiments were again run twice (18/05/2001 and 07/06/2001) to ensure consistency, results of the exposure experiments are shown in Figures 6.3 and 6.4. respectively. As with the preliminary experiments, a z-test was run to assess the presence of bacteria in addition to copper; the low and high bacterial doses were combined and contrasted with the copper control. The presence of bacteria did not significantly ($p=0.336$) affect postlarval health. To assess whether, when the two bacterial doses were considered individually, the level of *V. tubiashii* the postlarvae were exposed to was significant a second z-test was run. This test considered postlarvae exposed to 10^4 *V. tubiashii* ml^{-1} and 10^6 *V. tubiashii* ml^{-1} separately, but again the result was not significant ($p=0.739$).

The results from the preliminary experiments appeared to differ from those obtained in the exposure experiments so a z-test was run to see whether these differences were statistically significant. The z-test looked purely for overall differences in postlarval health on exposure to copper; the copper control, low and high dose bacterial exposures were pooled. There is a significant difference in postlarval health between the preliminary experiments and the exposure experiments $p<0.0000$.

A final z-test was run to examine whether there was any significant effect of exposure to copper on postlarval health. The results for the preliminary and exposure experiments were combined and contrasted against the results obtained when exposing postlarvae to *V. tubiashii* alone (Chapter 4); again the copper control, low and high bacterial doses were combined. There was a significant difference ($p<0.0000$) between spat exposed to *V. tubiashii* alone and those exposed to *V. tubiashii* in combination with copper, co-exposure to copper increases the survival of the animals.

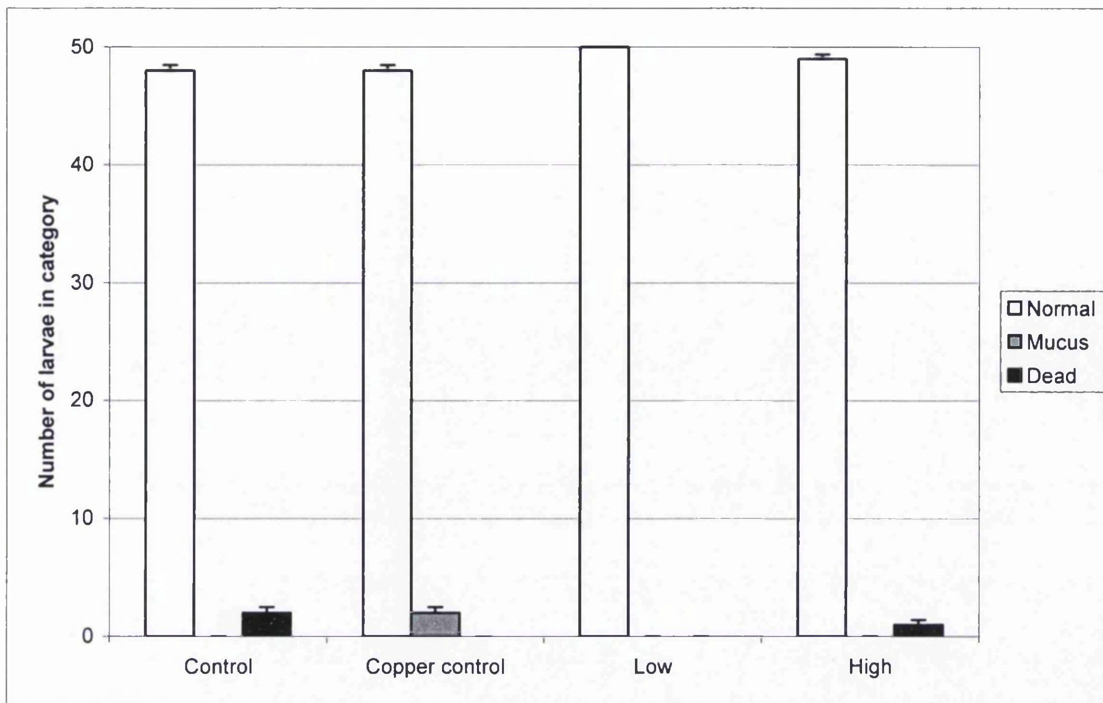


Figure 6.3. Postlarval viability after 7 day exposure (18/05/01) to *Vibrio tubiashii* and 0.02ppm copper. (Control: No copper, no *Vibrio*. Copper control: 0.02ppm copper, no *Vibrio*. Low: 0.02ppm copper and 10^4 *Vibrio* ml^{-1}. High: 0.02ppm copper and 10^6 *Vibrio* ml^{-1}) Postlarvae were determined to be either live, dead, or at an intermediate stage producing large quantities of mucus. Total counts are given ±2 standard errors, n=50 animals per treatment, 10 per vessel and 5 vessels per treatment.

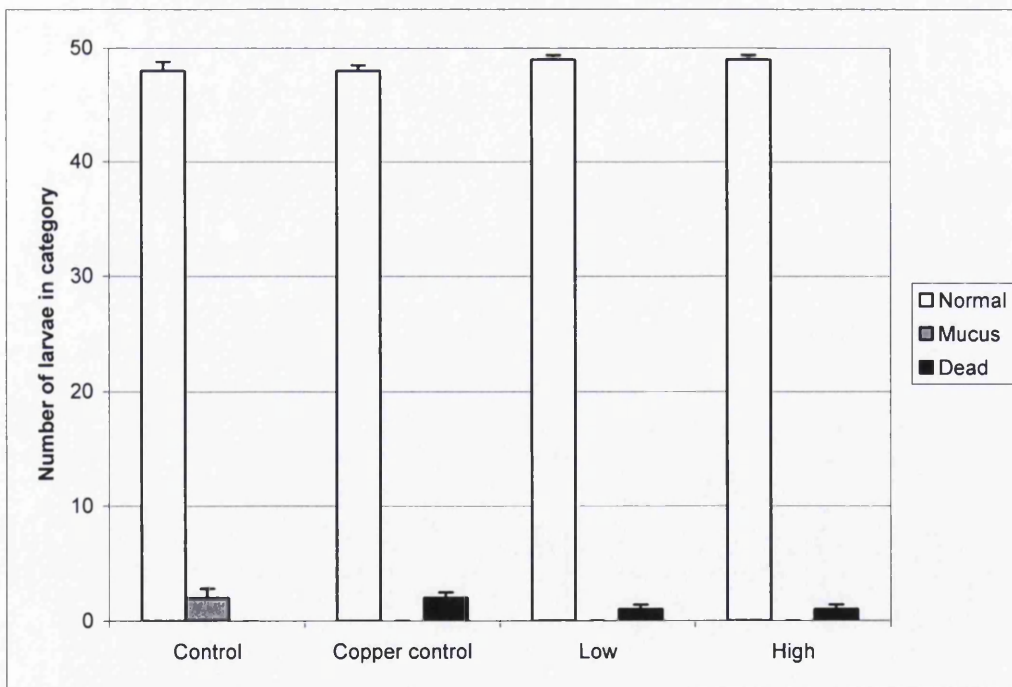


Figure 6.4. Postlarval viability after 7 day exposure (07/06/01) to *Vibrio tubiashii* and 0.02ppm copper. (Control: No copper, no *Vibrio*. Copper control: 0.02ppm copper, no *Vibrio*. Low: 0.02ppm copper and 10^4 *Vibrio* ml^{-1}. High: 0.02ppm copper and 10^6 *Vibrio* ml^{-1}). Postlarvae were determined to be either live, dead, or at an intermediate stage producing large quantities of mucus. Total counts are given ±2 standard errors, n=50 animals per treatment, 10 per vessel and 5 vessels per treatment.

6.4 Discussion

The initial hypothesis was that the mussel postlarvae would be deleteriously affected by exposure to copper and that this would have an impact on their susceptibility to *Vibrio tubiashii*, an opportunistic pathogen of marine bivalves. Heavy metals have been shown by various authors to affect developmental stages of marine bivalves. Ringwood (1993) found that there were age-specific differences in the sensitivity to cadmium in a range of life-history stages of *Isognomon californicum*, a Hawaiian bivalve. There was an inverse relationship between age and sensitivity, so the scale of relative sensitivity was embryos>veligers>pediveligers>postlarvae>adults. Davenport and Redpath (1984) report that exposure to 200ppb (0.2ppm) copper for 2 weeks fails to cause significant mortality in veliger and pediveliger stages of *Mytilus edulis*. Prytherch (1934) examined the effects of copper on *Ostrea edulis* veligers, particularly looking at its effects on settling and metamorphosis. Exposure to copper concentrations ranging from 3-20ppm for periods of 3 to 5 minutes were always fatal to the larvae, resulting in gradual disintegration of the tissues through the cytolytic action of the copper. Solutions containing 0.5ppm or less of copper were non-toxic to the *Ostrea edulis* veligers even when they were exposed continuously. As 0.2ppm copper failed to cause significant mortality in *M. edulis* veligers mortalities in the postlarvae as a result of exposure to copper alone were not anticipated. The interactive effects between the copper and *V. tubiashii* and whether the postlarvae were affected sub-lethally leaving them more vulnerable to opportunistic bacterial infection were the focus of this study.

In contrast to the age-specific findings of Ringwood (1993), Strømgren & Nielsen, (1991) found that although the growth rate in juvenile and larval mussels (*M. edulis*) was reduced by exposure to copper (5-6ppb Cu for 10 days), the larval stage was no more sensitive to copper than the juvenile. In addition Beaumont et al. (1987), Hoare and Davenport (1994) and Hoare et al. (1995), found that, contrary to the inverse relationship between age and sensitivity (reported by Ringwood, 1993 and His et al., 2000), *M. edulis* veliger larvae showed the highest resistance to copper of any stage in their life cycle. Hoare et al. (1995) found that veliger larvae had a 15 day LC_{50} of 500 ppb ("50% Lethal Concentration", the concentration which causes 50% mortality within the specified period). Beaumont, et al. (1987), found that veliger larvae of *M. edulis* were from 7 to 10 times more tolerant of copper than juveniles or adults, which was "an

unexpected finding in relation to the recent literature on copper toxicity and accumulation in mussels". The *M. edulis* veligers also appeared to be less sensitive to copper than veligers of other species of bivalves such as *Pecten maximus, Mercenaria mercenaria and Crassostrea virginica.* Hoare and Davenport (1994) investigated the timing of change from the relative insensitivity of veliger mussels to copper to the greater sensitivity seen in adults. The increased sensitivity may be anticipated to appear around metamorphosis and settlement, when metabolic and physiological changes occur in the mussel. However, it appears that there is no distinct cut off point relating to developmental stage, with veliger level sensitivity being seen before settlement and adult sensitivity seen directly after. Hoare and Davenport (1994) found that resistance to copper toxicity begins to decline after metamorphosis from the high levels shown by veligers. The decline continues, although more slowly after a shell length of 3mm is reached, until the adult level of resistance is attained at 5mm. The postlarvae used in the present study were 0.5 to 3mm so a level of resistance intermediate between the adult and veliger levels may be expected. Hoare and Davenport (1994) found an LT_{50} of between 12.5 days for 1mm *M. edulis* and 6.8 days for 3mm *M. edulis* when exposed to 400ppb added copper. The exposure level in the present study was more than order of magnitude lower than that studied by Hoare and Davenport (1994), and mortalities, as a result of exposure to copper alone, were not seen.

Although exposure to copper may not cause mortalities in postlarval and adult *M. edulis* it may cause alterations in their immune capabilities. Brown et al., (2004) found that even though adult *M. edulis* appeared to be tolerant to copper exposure, there were effects at the cellular level at 68.1ppb Cu, however no effects were seen using neurotoxic and physiological biomarkers. In contrast, common limpets (*Patella vulgata*) showed effects at all levels of biological organisation at just 6.1ppb Cu, in the shore crab (*Carcinus maenas*) cellular and neurotoxic endpoints were apparent at 68.1ppb Cu. Harrison et al. (1983) also found that exposure to sublethal concentrations of copper produced cytotoxic responses that induce stress symptoms in mussels. Bivalve molluscs have been shown to produce metallothioneins as a result of exposure to metals including copper, Table 6.1 gives details of experiments exposing different developmental stages of mussels and oysters to copper and cadmium resulting in metallothionein production. The presence of relatively high constitutive levels of metallothioneins and similar proteins has been documented in eggs and larvae of

Bivalve species	Developmental stage	Exposure concentration	Reference
Crassostrea virginica	Trochophore larvae	100ppb Cu	Ringwood & Brouwer, 1993.
	Trochophore larvae	3.8ppm Cu	Calabrese, 1973.
	D-shell larvae	37ppb Cd	Roesijadi et al., 1995.
Mytilus edulis	Adult	0.025ppm Cu	Harrison et al., 1983.
Mytilus galloprovincialis	D-shell larvae	100ppb Cu	Pavičič, 1985.
	Adult	40ppb Cu	Viarengo et al., 1981.

Table 6.1. Metallothionein expression in metal exposed bivalves.

bivalve molluscs (Roesijadi et al., 1982; Pavičič et al., 1985). Metallothioneins (MT) in *C. virginica* become inducible between 8 and 12 hours after fertilization (Roesijadi et al., 1997). Negative relationships between tissue weight and MT concentrations have been observed previously in adult *Crassostrea gigas* (Mouneyrac et al. 1998). However, Serafin et al. (2002) found that there was a twofold higher increase in MT induction in large mussels (5.2±0.7cm), when exposed to cadmium, compared with small mussels (3.5±0.5cm). It is clear that both larvae and postlarval mussels possess metallothioneins and are therefore capable of detoxifying the copper in the exposure experiments undertaken in this study.

Davenport and Redpath (1984), report that, for adult mussels, continuous exposure to 200ppb added copper or more can produce 100% mortality within 1 month; while 100ppb added copper can cause significant changes in behaviour and growth. Pipe and Coles (1995) exposed adult *M. edulis* to 0.2ppm copper for 7 weeks and found them to be more resistant than the mussels Davenport and Redpath (1984) studied, with only 22% mortality after 7 weeks. There are clearly differences in heavy metal responses, not only between life-stage studied and species, but also between research groups looking at the same species and life stages. These differences may well be due to the experimental set up of the laboratories. In addition seasonal effects, and differences in the populations studied, may have some influence on the apparent sensitivity of bivalve adults and larvae to copper. A clear seasonal effect can be seen in the present study, individuals used in the exposure experiments were shown to have a significantly higher survival rate than those in the range finding experiments. The preliminary experiments were run in early autumn (27/9/00, Fig. 6.1, and 10/10/00, Fig. 6.2) whereas the exposure experiments were run in the early summer (04/05/01, Fig. 6.3, and 07/06/01, Fig. 6.4). Seasonal effects have also been seen in previous experiments carried out as part of the present study (Chapter 5). Damiens et al. (2006), demonstrated a strong seasonal effect of metallothionein induction by both copper and cadmium. In early May exposure to these metals does not cause an induce MT. However, by the end of May, exposure of *Crassostrea gigas* larvae, of the same age, to the same levels of copper resulted in induction of MT. There have been many reports of temporal changes in MT levels in *Mytilus*, MT concentrations tend to peak in mid summer, May to July, although it is unclear whether this is linked to temperature effects or reproductive cycle (Viarengo et al., 1987; Serra et al., 1999; Geffard et al., 2005). The level of

metallothionein induction may be one of the reasons a reduced level of survival was seen in the controls of the preliminary experiments compared with the exposure experiments, remembering that the animals in the controls of the preliminary experiment were exposed to copper but not bacteria. The postlarvae studied in these experiments were not reproductively mature (see Chapter 5), so reproductive cycle unlikely to be a factor in the seasonal effects seen. In the exposure experiments the postlarval survival levels in the control vessels was nearly 100% at the end of the experiment. There was no longer a statistically significant increase in survival with the addition of bacteria, a result seen in the preliminary experiments, as there was little room for improvement. However, there was no deleterious effect as a result of co-exposure to copper and *V. tubiashii*; this contrasts with exposure to *V. tubiashii* alone, which reduced postlarval survival (Chapter 4).

White shrimp (*Litopenaeus vannamei*) juveniles have been shown to be more susceptible (96h LC50) to *V. alginolyticus* when reared in water containing Cu^{2+}, although the copper level they were exposed to was extremely high, at 5ppm (Yeh et al., 2004). However, the study also documented that a concentration of Cu^{2+} as low as 1ppm increased the susceptibility of *L. vannamei* to *V. alginolyticus* infection by a reduction in immune response parameters such as total haemocyte count, phenoloxidase activity, phagocytic activity and clearance efficiency. Pipe et al. (1999) reported that exposure to copper alters assays of immune function in adult *M. edulis* at more environmentally realistic levels of 0.02ppm and 0.05ppm. The present investigation aimed to study, indirectly, whether 0.02ppm copper altered immune function by assessing susceptibility of postlarvae to a facultatively pathogenic bacterium. Preliminary experiments were carried out to determine any synergistic effects of co-exposure to copper and *V. tubiashii.* The results of both the preliminary and exposure experiments showed that the addition of copper appeared to increase larval survival. There were synergistic effects of co-exposure, however, rather than copper increasing the susceptibility of postlarvae to *V. tubiashii* there was a significant benefit conferred by co-exposure. It appears that there is some interaction between the copper, *V. tubiashii* and the postlarvae that reduces the pathogenicity of this bacterium to the postlarvae.

It has been known for some time that microorganisms are capable of releasing organic metabolites into the environment which are able to complex metals. This complexation is of importance as, not only can it, enhance the bioavailability of essential metals it can also reduce the ionic concentration of deleterious metals such as copper (Jardim & Pearson, 1984). Elevated copper concentrations induced the natural estuarine microbial communities to produce copper-complexing ligands which dramatically reduced the free Cu^{2+} ion concentrations in the water column (Dryrden et al., 2004). The copper concentrations investigated by these authors did not exceed those seen in the environment (100 to 200nmol L^{-1}). The authors believe that this interactive response to copper stress represents a feedback system through which microbial communities can potentially buffer dissolved Cu^{2+} ion concentrations, thereby regulating copper bioavailability and toxicity.

More specifically, Gordon et al. (2000) have demonstrated that marine bacteria of the genus *Vibrio* produce copper-binding ligands, the concentration which increased as the copper concentration was increased. Gordon et al. (1993) describe a response of marine *Vibrios* to copper stress which involves excretion of an extra-cellular copper-complexing molecule which is an essential component of the physiological mechanisms mediating recovery from copper toxicity. Miranda and Rojas (2006) reported that cellular accumulation is one of the principal copper resistance mechanisms for a *Vibrio* species.

The marine bacterium *Vibrio alginolyticus* ATCC51160 has been extensively studied and shown to respond to copper stress by producing extracellular copper binding proteins (CuBP) that complex and detoxify copper in the growth medium (Harwood-Sears & Gordon, 1990; Schrieber et al., 1990; Gordon et al., 1993). Gordon et al. (1994) examined the production of CuBP by a range of bacterial strains. They found that CuBP-like proteins were present in copper-stressed *Vibrio* cultures but similar extracellular copper complexing proteins were absent in the control cultures or *E. coli* and *Bacillus* cultures, showing that CuBP-like proteins are not produced by all bacteria in response to copper stress. A protein with a molecular mass similar to those observed in the *Vibrio* cultures was detected in copper-stressed, recovered cultures of *Pseudomonas*. Harwood-Sears and Gordon (1990) demonstrated that the addition of copper to exponentially growing bacterial cultures resulted in a temporary inhibition of

growth followed by a recovery into exponential growth. The growth rate in the second, recovery, exponential phase was generally retarded showing copper inhibition was not entirely overcome. The lag in growth (temporary inhibition of growth followed by recovery) was proportional to the amount of copper added. The copper concentrations that allowed recovery ranged from 4 to 24ppm (Harwood-Sears & Gordon, 1990; Gordon et al., 1993). In copper challenged cultures, each of the *Vibrio* species and strains examined demonstrated an extracellular protein of the same electrophoretic mobility as CuBP from *V. alginolyticus* ATCC51160, this protein was absent in control cultures (Harwood-Sears & Gordon, 1990). This observation suggests that CuBP-like proteins are commonly produced by *Vibrio* species, and it is likely that the species used in the present study, *V. tubiashii*, is also a producer. The expression of CuBP was induced by as little as 0.16ppm $CuSO_4$ in wild type *V. alginolyticus*, and concentrations higher than 0.4ppm inhibited growth in broth cultures (Harwood & Gordon 1994). However, the copper resistant mutant studied by Harwood and Gordon (1994) was a constitutive producer of CuBP, the concentrations of CuBP varied between vessels but the lowest concentration was comparable to CuBP concentrations in 8ppm copper-stressed wild-type cultures. It may be that the strain of *Vibrio tubiashii* used in the present study was a constitutive producer of CuBP which binds the free copper in the exposure vessels.

Analysis of incorporation of labelled ^{14}C glucose into the extracellular copper-binding compound indicated that it was produced actively by the cells during recovery from copper toxicity, and was not present as a result of non-specific release by lysed bacterial cells (Schreiber et al., 1990). CuBP can be detected as soon as 2 hours after the addition of copper, at 8ppm, to cultures of *V. alginolyticus* (Harwood-Sears & Gordon 1990). These proteins are not detected in supernatants from copper-challenged, chloramphenicol-treated cultures, supporting the contention that they are the product of *de novo* synthesis (Harwood-Sears & Gordon 1990). The toxicity of *Vibrio* bacteria is, at least in part, mediated by production of extracellular proteinases (Nottage & Birkbeck, 1987b; Lee et al., 1996; Gómez-León et al., 2005), as discussed in Chapter 3.

The reduction in pathogenicity, seen when postlarvae are co-exposed to copper and *Vibrio tubiashii,* may result from the synthesis of CuBP by the bacteria in preference to

the extracellular proteinases that induce vibriosis. In conclusion, exposure to copper increases the survival of postlarvae when they are co-exposed with *V. tubiashii*.

Transition metals are widely reported to have antibacterial, antifungal and anticancer activities (Chohan et al., 2006). Copper has been shown to have strong antibacterial activities against both Gram-negative and Gram-positive bacteria (Sharma et al., 2006). It has been woven into fabrics for use in healthcare textiles and wound care products (Cupron, 2007) and is reportedly effective in the reduction of MRSA survival in the healthcare environment (Noyce et al., 2006). The direct effect of copper on *Vibrio tubiashii* in the absence of postlarvae was not investigated in this study, consequently a caveat must be added to state that the increased survival of postlarvae when co-exposed to copper and *V. tubiashii* may be due to the antibacterial properties of copper.

Chapter 7. Effects of phenanthrene on postlarval disease susceptibility.

7.1 Introduction

Hydrocarbons, compounds composed of the elements carbon and hydrogen, are the main constituent of petrochemicals, with other components such as sulphur, oxygen and nitrogen present in varying quantities (Neff & Anderson, 1981). Anthropogenic pollutants can be ranked in order of their relative toxicity to organisms, in the marine environment, His et al. (2000) gave the following list of chemicals ranked by decreasing toxicity: TBT, heavy metals, chlorine, organics and pesticides, detergents, petroleum products, urban effluents and sediments. Hydrocarbons are divisible into two classes: i) the alkanes, alkenes and alkynes and ii) the aromatic hydrocarbons containing at least one benzene ring (Walker et al., 1996). Benzene rings contain six carbon and hydrogen atoms, with the carbon atoms having alternating double bonds. Molecules containing two or more benzene rings linked directly together are known as polycyclic aromatic hydrocarbons (PAHs) (McElroy et el., 1989). Like metals, petrochemical compounds, such as polycyclic aromatic hydrocarbons, are naturally occurring substances. Petrochemicals became pollutants as a result of the activities of man leading to levels in the environment becoming elevated. PAHs occur in crude oils, they are also formed by incomplete combustion of petrochemicals, tobacco or tar and some of them are known or suspected carcinogens, with their toxicity being structure dependant. PAHs are amongst the most water-soluble of the hydrocarbons, thus allowing them to be accumulated to high concentrations in the tissues of many marine organisms (Wootton et al., 2003b).

Although metals and PAHs are naturally derived, elevated levels in urban and industrial discharges resulting from anthropogenic activities lead to environmental pollution (Fisher, 2004). Oil and oil based contaminants reach the marine environment from a variety of sources, the most important of which are; rivers and run off from land, atmospheric fallout, the offshore oil and gas industry and accidental spills (DEFRA, 2005). The greatest single input of oil-based contaminants originates on land and is carried to sea by rivers, run off and the atmosphere with contamination of sediments

greatest in nearshore and estuarine environments. Polycyclic aromatic hydrocarbons, as a fraction of oil-based contaminants, are of particular concern due to their persistence, tendency to bio-accumulate, toxicity and mutagenic potential (DEFRA, 2005).

Levels of PAHs and other hydrocarbons in the marine environment have also been elevated by oil spills from tankers which lead to temporally and spatially discreet peaks of pollution. These pollution events are generally well studied and the effects on native wildlife well documented. Not only have effects on marine birds and mammals been studied but research has also been undertaken on marine invertebrates and the long term effects of oil spills. Two large scale shipping spillages have occurred in British waters in the recent years. The "Braer" grounded off the southern tip of Shetland in January 1993 and the "Sea Empress" grounded in the entrance to Milford Haven, West Wales, in February 1996. The "Braer" released around 85,000 tonnes of North Sea crude oil, but, as a consequence of the light nature of this oil and the turbulent conditions at the time, a conventional slick did not form and almost no oil was stranded on the shoreline. The "Sea Empress" released around 72,000 tonnes of Forties crude oil and 480 tonnes of heavy fuel oil. Despite a rapid response at sea, employing oil dispersants, oil came ashore along a 200km stretch of coastline and bans were imposed on commercial and recreational fishing in the region. The fishing bans were removed within 4 months of the spill and within 18 months all restrictions on shellfisheries were also removed.

PAHs are of concern, from a human health perspective, in shellfish that are sold commercially because some can be biotransformed to carcinogenic metabolites which are then bioaccumulated within the shellfish (Moore et al., 1989; DEFRA, 2005). Although a large body of literature exists on the toxicity to humans and carcinogenicity of PAHs, primarily benzo[a]pyrene, toxicity data for phenanthrene is very limited. Phenanthrene has been identified in seafood collected from contaminated waters and in smoked and charcoal-grilled food (IARC, 1983), although human exposure occurs primarily through smoking of tobacco or inhalation of polluted air. The UK NMMP (National Marine Monitoring Programme) routinely analyses sediments from dedicated sites for their PAH burdens, and the PAH burdens in biota are also monitored by the Food Standards Agency. In 2000, intakes for both adults and children were two to five times lower than they were in 1979 for two (Benzo[a]pyrene and benz[a]anthracene) of the three most harmful PAHs. The other most harmful PAH (dibenz[ah]anthracene) was

at such a low level that it could not be detected in 2000 (Food Standards Agency, 2002). In the UK, for the 8 PAHs routinely screened, mussel tissue concentrations were well below the provisional EAC (ecotoxicological assessment criteria), in spite of PAH concentrations exceeding the upper EACs in the environment at several of the sites monitored (DEFRA, 2005).

Mussels filter large volumes of water while feeding and can bioconcentrate lipophilic chemicals, such as PAHs, from water by 2-5 orders of magnitude (Neff, 2002). In order for bioaccumulation of PAHs to occur they must be bioavailable i.e. they must be present in the water column or particulate organic and inorganic matter. During pollution events filter feeding organisms are exposed to both PAHs in the water and in suspended particulate matter, although the relative importance of each source for PAH accumulation in mussels is uncertain (Webster et al., 1997). Mussels, unlike many other invertebrates, and all vertebrates, have a limited ability to metabolise PAHs and passively release accumulated hydrocarbons into the surrounding water when ambient concentrations decrease (DiSalvo et al., 1975; Page et al., 2005). Phenanthrene is a tricyclic PAH, composed of three benzene rings, and is a known carcinogen. Research has shown phenanthrene to bioaccumulate in bivalves (Law et al., 1999; Auffret et al., 2004).

The enzymatic metabolism of most lipophilic xenobiotics occurs in two phases, finally resulting in the generation of a more water soluble product that can then be excreted. The Phase I biotransformation involves oxidation, hydrolysis, hydration or reduction producing a metabolite containing a hydroxyl group (Walker et al., 1996). Phase II of detoxification involves a range of enzyme activities on this hydroxyl group that conjugate the metabolite to endogenous substrates (Sheehan & Power, 1999). The cytochrome P450 enzymes are responsible for the Phase I metabolism of a variety of lipophilic xenobiotics e.g. drugs, pesticides, PAHs and plant allochemicals (Rewitz et al., 2006). Functions of P450s in the metabolism of endogenous compounds (e.g. steroids and fatty acids) and xenobiotics have been extensively studied in the last 30 years (Gonzalez, 1989). Some products of P450 metabolism are, however, more reactive, and thus more cytotoxic, leading to cellular damage and in certain cases to the initiation of carcinogenesis (Hong & Yang, 1997). Typically, total P450 protein and associated enzymatic activities are found to be ten-fold lower in invertebrates than

mammals (Livingstone, 1991). The biochemical evidence of relatively low activity for cytochrome P-450 monooxygenase in molluscan digestive-gland cells, when considered together with the ability of these cells to accumulate and retain very high concentrations of PAH, indicates that their loss by metabolic transformation is limited (Livingstone, 1985).

DiSalvo et al. (1975) consider the nature of the hydrocarbon exposure to be important in determining the length of time taken for depuration. Mussels exposed to a short-term hydrocarbon insult are able to readily discharge their body burden over a few weeks, however, those exposed longer-term to hydrocarbons do not rid themselves of the burden as rapidly. Long term monitoring of mussel populations has revealed a cyclical pattern of hydrocarbon concentration (Jacob et al., 1997; Webster et al., 1997; Dyrynda et al., 2000). Tissue levels of combustion-derived PAHs increase during autumn/winter reflecting higher inputs from land run-off due to increased rainfall and increased demand for power generation during the winter months. This elevated PAH input coincides with the period when mussels store lipids in preparation for spring spawning and this increase in lipid content in turn permits the retention of higher amounts of lipophilic compounds such as PAHs (Webster et al., 1997). The tissue levels of PAHs decrease during early spring and summer, coinciding with the mussel spawning period. Spawning is thought to serve as a depuration process for molluscs due to their minimal ability for metabolism of hydrocarbons (Lee et al., 1972; DiSalvo et al., 1975; Page et al., 2005). Oysters may lose up to 50% of their body burden of certain PAHs in a single spawn (Ellis et al., 1993). As a result of spawning acting as a depuration mechanism PAH pollution may impair the reproductive success of the adults. The PAH load may decrease the quality and/or quantity of the gametes, which in turn may affect the fertilization success, embryo development, and larval viability (Jeong & Cho, 2005a).

Crude petroleum is a complex mixture of thousands of different hydrocarbons and is generally less toxic to marine organisms than its refined products, although crude oils from different sources vary enormously in their toxicity. The differing toxicity is due to the variability in crude oil composition from different sources which in turn affects dispersal, solubility and persistence in the marine environment. Solubility is inversely related to molecular weight, (Neff & Anderson, 1981) although it is not a strictly linear relationship and is also affected by molar volume. Low molecular mass PAHs are

among the most acutely toxic components of crude oil, while some of the high molecular mass PAHs are potent carcinogens (Dyrynda et al., 2000). The low molecular weight of phenanthrene produces a highly water-soluble PAH which is readily bioavailable and thus highly toxic to marine animals (Neff & Anderson, 1981). Few investigations have been conducted on the effects of PAH contaminants on immune defences in natural marine invertebrate populations (Dyrynda et al., 2000), although several laboratory based studies have been carried out (Sami et al., 1992; Viarengo et al., 1992; Coles et al., 1994; Krishnakumar et al., 1997; Wootton et al., 2003b). Contaminant PAHs seldom occur in environmental isolation (Oros & Ross, 2005) and this therefore poses the question of interactive biological effects resulting from multiple xenobiotic challenge (Moore et al., 1987).

Marine organisms vary in their sensitivity to oil pollutants (Neff & Anderson, 1981) with larval and juvenile stages being more sensitive than adults (Pelletier et al., 1997). Many adverse effects of PAHs have been recorded including biochemical, cellular and physiological effects which are ultimately reflected in the growth, reproduction and survival of individuals (Moore et al., 1987). PAHs are known to be immunotoxic (Dyrynda et al., 1997), exerting adverse effects on immunity in invertebrates which can in turn affect disease resistance. In the wake of the "Sea Empress" oil spill no mussel mortalities were recorded, however, significant changes in immune function were detected. The alterations included decreased phagocytosis and superoxide generation (an internal killing mechanism of haemocytes) which could compromise the resistance of mussels to pathogenic bacteria and parasites (Dyrynda et al., 2000).

In the present study the effects of phenanthrene exposure on the disease susceptibility of postlarval mussels were examined. For the purpose of this investigation a phenanthrene concentration of 0.2ppm was used as a concentration that has previously been shown to cause alterations in immune parameters measured in adult *M. edulis* but not to cause mortalities (Wootton et al., 2003b). The concentration of phenanthrene used in the present study was also lower than the water concentrations of PAHs found in a chronically polluted marine environment (0.5ppm) (Madany et al., 1994). The postlarvae were co-exposed to *Vibrio tubiashii*, which is potentially pathogenic to mussels.

7.2 Materials and methods

Postlarval mussels (0.5-3mm) were collected from Whitsand Bay, Cornwall and, exposures were carried out as detailed in Chapter 4 (Section 4.3.1), with postlarvae being fed throughout the depuration and exposure periods. The postlarvae were only exposed to one bacterial species, *Vibrio tubiashii*, due to time constraints.

Previous experiments (Chapters 4 and 5) have shown that *Vibrio tubiashii* affects postlarvae at the levels selected for the present study. As for the copper exposures in Chapter 6, preliminary experiments were carried out to assess the effects of combining this bacterium with phenanthrene exposure. The preliminary experiments were conducted to assess synergistic effects and ensure that the combination of 0.2ppm phenanthrene with bacterial levels used previously (Chapters 4 and 5) resulted in measurable effects without inducing mass mortality.

In addition to the *Vibrio tubiashii*, the postlarvae were also exposed to phenanthrene (Sigma P2528), which was dissolved in acetone to give a stock solution of 20mg ml^{-1}. This stock solution was added to the 5% Marine Broth to give a final concentration of 0.2ppm. For the preliminary experiment, started on the 26/10/2000, there were 5 replicates of each treatment (phenanthrene control, 10^4 bacteria ml^{-1} and 10^6 bacteria ml^{-1}) with each culture vessel containing 10 postlarvae and 0.2ppm phenanthrene. At the end of the 7 day exposure period, the postlarvae were processed and assessed as detailed in Chapter 4. The result of the initial range finding experiment is shown in the results section (Section 7.4).

The preliminary experiments showed that no mass mortalities occurred as a result of co-exposure to phenanthrene and *V. tubiashii* and effects of exposure could be seen. Therefore the exposure experiments, together with full controls, were run. Vehicle controls (control plus acetone) were used for the exposure experiments to ensure there were no effects caused by the acetone carrier, the following conditions were used for the exposure experiments:

- Control = 5% Marine Broth in 0.2μm filtered UV sterilised seawater, no phenanthrene or bacteria.

- Acetone Control = as Control but also containing acetone, in equal volume to the phenanthrene/acetone solution (10μl L^{-1} in 5% Marine Broth), no bacteria.
- Phenanthrene Control = as Control but also containing 0.2ppm phenanthrene solution, no bacteria.
- Low = 5% Marine Broth in 0.2μm filtered UV sterilised seawater, 0.2ppm phenanthrene solution and 10^4 *V. tubiashii* ml^{-1}.
- High = 5% Marine Broth in 0.2μm filtered UV sterilised seawater, 0.2ppm phenanthrene solution and 10^6 *V. tubiashii* ml^{-1}.

Postlarvae were exposed in tissue culture vessels, with 10 individuals per vessel and 5 replicates of each treatment. The experiments were run for 7 days and larval survival was assessed as detailed above. The exposure experiments were run twice, to ensure consistency, beginning on the 4/05/2001 and 19/06/2001.

Results were analysed using z-tests (Excel, Microsoft Office 2003), the data were log transformed before analysis. As the production of excess amounts of mucus was seen as a stress response and a precursor to mortality, levels of dead postlarvae and those in the mucus category were pooled for the purpose of statistical analysis.

7.3 Results

The result of the preliminary experiment is shown in Figure 7.1. The results show that, highest mortalities were less than 65% (32 postlarvae from a total of 50 exposed, ± 1.62, 2SE) and that these occurred with the low concentration of *V. tubiashii* (10^4 bacteria ml^{-1}) following 7 days exposure to the pollutant and pathogen. Exposure to bacteria in the presence of phenanthrene reduced the number of normal individuals seen at the end of the exposure period. The effects do not appear to be dose-dependant with exposure to 10^4 *V. tubiashii* ml^{-1} resulting in a more mortalities and a lower level of normal individuals compared with postlarvae exposed to 10^6 *V. tubiashii* ml^{-1}.

A z-test was run to assess the presence of bacteria in addition to phenanthrene in the preliminary experiment; the low and high bacterial doses were combined and contrasted with the phenanthrene control. The presence of bacteria significantly ($p<0.0000$) decreased the number of normal postlarvae seen at the end of the experiment compared

with those exposed to phenanthrene alone. A further z-test was then run to assess whether the level of bacterial exposure was of importance, p=0.083 showing that there was no significant difference between those postlarvae exposed to 10^4 *V. tubiashii* ml^{-1} and those exposed to 10^6 *V. tubiashii* ml^{-1}.

The exposure experiments were run with these phenanthrene and bacterial exposure levels again for a period of 7 days, but this time including full controls to ensure that any effects of the acetone vehicle carrier alone and the acetone/phenanthrene solution could be monitored. The experiments were run twice to ensure consistency, the results of the exposure experiments are shown in Figures 7.2 (04/05/2001) and 7.3 (19/06/2001). The results show that there is little difference between the Control and the Acetone Control. This suggests that the addition of acetone alone does not affect postlarvae allowing any changes in mortality seen when exposing postlarvae to phenanthrene/acetone solution to be attributed to phenanthrene. The addition of phenanthrene alone (Phenanthrene Control exposure) appears to result in increased mucus production, whilst co-exposure to phenanthrene and *V. tubiashii* leads to increased mortality. The increased mortality with co-exposure to the pollutant and bacteria exhibits a dose-dependent response; higher mortality being seen when the postlarvae are exposed to the higher *V. tubiashii* concentration (10^6 bacteria ml^{-1}).

The first step in analysing the results of the exposure experiments was to run z-tests to assess whether there were any statistical differences between the controls. A z-test was run to analyse any differences between the Control and the Acetone Control to determine if the addition of acetone altered the survival of postlarvae, there was no significant difference between these two treatments $p = 0.2904$. A z-test was also run to assess whether the presence of phenanthrene, in addition to the acetone, altered the survival of postlarvae, with the Acetone Control results being contrasted with the Phenanthrene Controls. Again the results were not significant, $p = 0.1021$. The analyses of these controls allows any further postlarval effects seen to be attributed to the co-exposure of the postlarvae to *V. tubiashii* and phenanthrene and not a result of exposure to the acetone carrier.

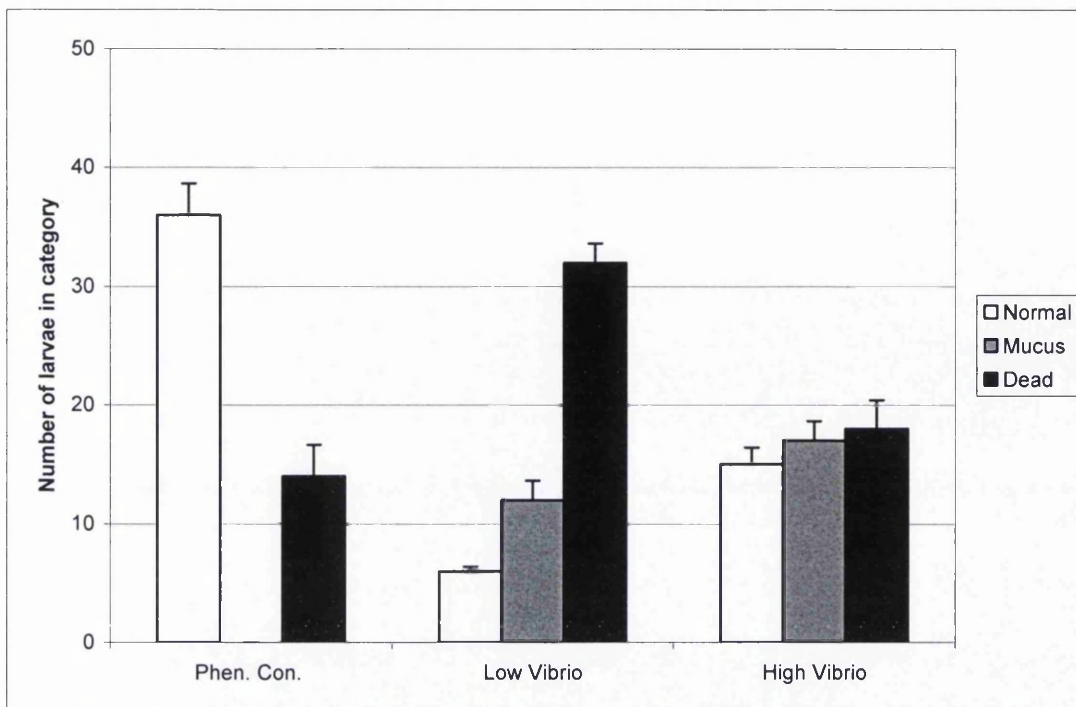


Figure 7.1. Postlarval viability after 7 day exposure (26/10/2000) to *Vibrio tubiashii* and 0.2ppm phenanthrene. (Phen Con: 0.02ppm phenanthrene, No *Vibrio*. Low: 0.2ppm phenanthrene and 10^4 *Vibrio* ml^{-1}. High: 0.2ppm phenanthrene and 10^6 *Vibrio* ml^{-1}). Postlarvae were determined to be either live, dead, or at an intermediate stage producing large quantities of mucus. Total counts are given ±2 standard errors, n=50 animals per treatment, 10 per vessel and 5 vessels per treatment.

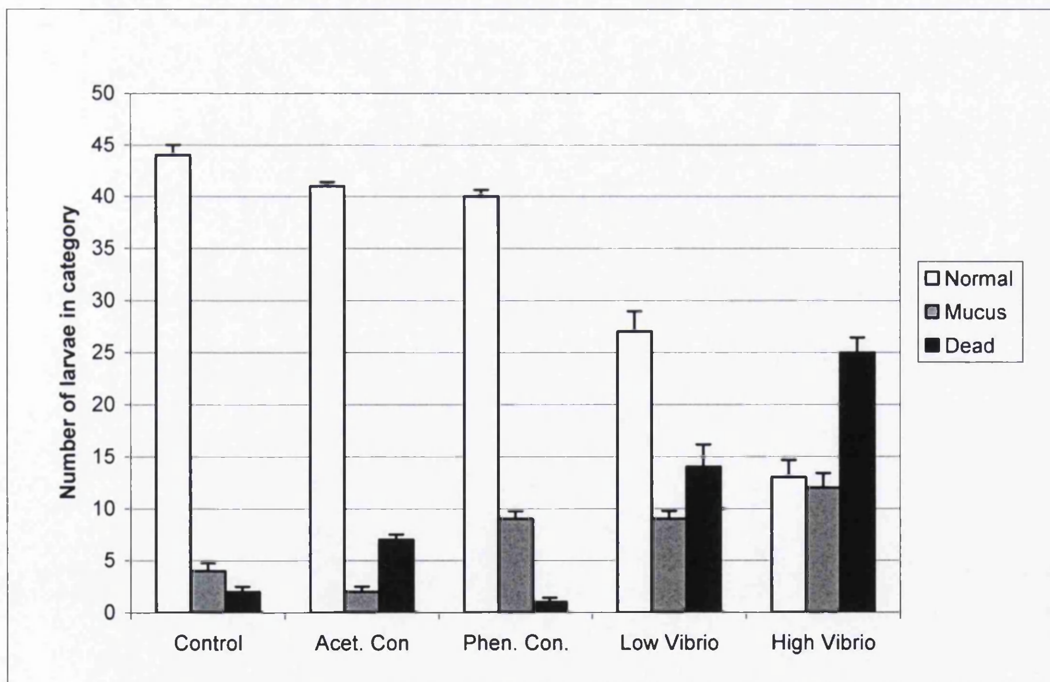


Figure 7.2. Postlarval viability after 7 day exposure (04/05/01) to *Vibrio tubiashii* and 0.2ppm phenanthrene. (Control: No phenanthrene, no *Vibrio*. Acet Con: No phenanthrene, no *Vibrio*, with acetone. Phen Con: 0.2ppm phenanthrene, no *Vibrio*. Low: 0.2ppm phenanthrene and 10^4 *Vibrio* ml^{-1}. High: 0.2ppm phenanthrene and 10^6 *Vibrio* ml^{-1}.). Postlarvae were determined to be either live, dead, or at an intermediate stage producing large quantities of mucus. Total counts are given ±2 standard errors, n=50 animals per treatment, 10 per vessel and 5 vessels per treatment.

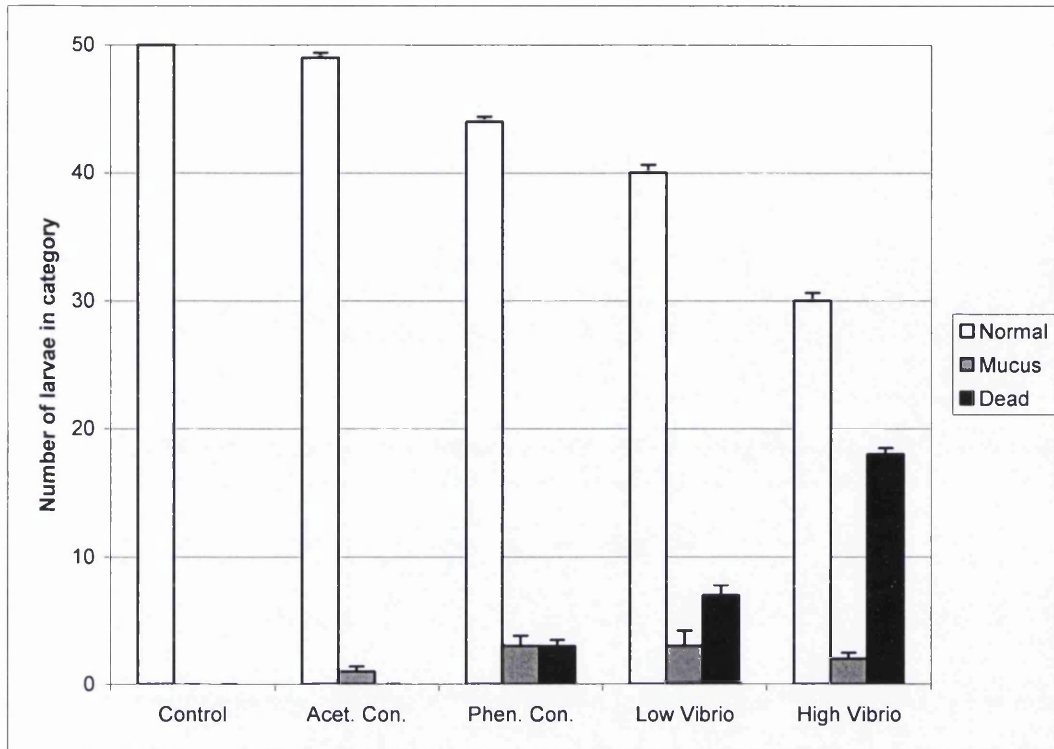


Figure 7.3. Postlarval viability after 7 day exposure (19/06/01) to *Vibrio tubiashii* and 0.2ppm phenanthrene. (Control: No phenanthrene, no *Vibrio*. Acet Con: No phenanthrene, no *Vibrio*, with acetone. Phen Con: 0.2ppm phenanthrene, no *Vibrio*. Low: 0.2ppm phenanthrene and 10^4 *Vibrio* ml^{-1}. High: 0.2ppm phenanthrene and 10^6 *Vibrio* ml^{-1}). Postlarvae were determined to be either live, dead, or at an intermediate stage producing large quantities of mucus. Total counts are given ±2 standard errors, n=50 animals per treatment, 10 per vessel and 5 vessels per treatment.

As with the preliminary experiment, a z-test was run to assess the presence of bacteria in addition to phenanthrene; the low and high bacterial doses were combined and contrasted with Phenanthrene Controls (containing acetone/phenanthrene at 0.2ppm). The presence of bacteria significantly ($p<0.0000$) affected postlarval health, causing a reduction in the percentage of normal postlarvae seen at the end of the exposure period. To assess whether the level of *V. tubiashii* the postlarvae were exposed to significantly affected survival a second z-test was run. This test considered postlarvae exposed to 10^4 *V. tubiashii* ml^{-1} and 10^6 *V. tubiashii* ml^{-1} separately; the result was significant ($p=0.0037$) demonstrating that there was a dose-dependant response, with postlarval mortality increasing with increasing bacterial concentration.

7.4 Discussion.

The results obtained in the present study imply that the presence of phenanthrene increases the susceptibility of postlarval mussels to *Vibrio tubiashii*. Exposure to phenanthrene alone, at 0.2ppm for 7 days, did not lead to increased postlarval mortality; however, co-exposure to the potentially pathogenic bacterium *Vibrio tubiashii* led to a significant ($p<0.0000$) decrease in the number of normal postlarvae at the end of the experiment.

Excess mucus production by postlarvae was seen in the experiments, as it has been in previous experiments (Chapters 4, 5 and 6) when postlarvae are co-exposed to potentially pathogenic bacteria and pollutants, as well as bacteria alone. Excessive mucus production by invertebrates as a result of exposure to hydrocarbons has been seen by other investigators (Neff & Anderson, 1981; Axiak & George, 1987). Axiak and George, (1987) reported that clams (*Venus verrucosa*) exposed to water-accommodated fractions of oil, at concentrations of 0.18 and 0.36ppm, showed a conspicuous increase in mucus production on the gills. When mucus production was enhanced many oil droplets were found to be rapidly incorporated within strands of mucus, these large mucus masses were brought to the edge of the gill and rejected. Mucus production by *Montastrea annularis* (reef coral) was also stimulated by exposure to the water-soluble fractions of south Louisiana crude oil (Neff & Anderson, 1981). Naphthalenes were detected in the mucus and it was suggested that the mucus served to bind or absorb aromatic hydrocarbons. Surface mucus may protect the underlying coral

tissues from aqueous hydrocarbons and the secretion of mucus may be an avenue of hydrocarbon release from contaminated corals (Neff & Anderson, 1981). It may also serve a similar function in the clams studied by Axiak and George (1987), with the mucus binding and removing oil droplets to prevent ingestion.

Although mechanisms exist to prevent the ingestion of pollutants, bivalves have been shown to accumulate PAH. This accumulation can occur both directly, by the uptake of water-solubilised PAH (generally 2-3 ring PAHs) through their gills, and indirectly, by ingestion and assimilation through the digestive tract (generally 4-6 ring PAHs) (Oros & Ross, 2005). The uptake routes reflect the water chemistry of the PAHs, the lower molecular weight PAHs are more readily water soluble. Exposure of *C. gigas* embryos and larvae to PAH polluted sediments (Geffard et al., 2003) led to bioaccumulation of low molecular weight PAHs, only the soluble fractions of PAHs appeared to be accumulated by the embryos despite the direct contact between the embryos and the sediment. Geffard et al. (2003) found that there was a maximum body burden of PAHs, of 0.3μg g^{-1}, above which larval abnormalities were observed. These larval body burdens were achieved with exposure to whole sediment at more than 1.25 g PAH L^{-1} and sediment elutriate at more than 163 g PAH L^{-1}. Larval and juvenile stages of marine invertebrates are more sensitive to PAHs and petroleum than adults (Pelletier et al., 1997). Strømgren & Nielsen, (1991) found that diesel oil is at least one order of magnitude more toxic for larvae than for juveniles. Strømgren (1986) showed that exposure to hydrocarbon fractions also resulted in sublethal effects in juvenile *M. edulis* (11-16mm shell length), causing a strong inhibitory effect on growth. The postlarval mussels used in the present study were 0.5-3mm in shell length and it was therefore expected that exposure to 0.2ppm phenanthrene would cause sublethal effects that may result in altered susceptibility to a potential bacterial pathogen.

In addition to life stage differences in response to pollutants there are also between species differences, meaning that it is not possible to extrapolate results obtained when exposing one species to potential effects in another species. *M. edulis* populations were able to survive extensive and heavy oiling in the wake of the "Sea Empress" oil spill, in contrast to other bivalves, such as *Ensis ensis*, *Patella* spp., *Cerastoderma edule* and *Littorina* spp., which all suffered mortalities (Dyrynda et al., 2000). Wootton et al. (2003b) studied the response of three bivalve species to phenanthrene exposure in the

laboratory and found that immunomodulation in *M. edulis* was not comparable to that observed in the cockle, *Cerastoderma edule* and razor shell *Ensis siliqua.* The resilient nature of *M. edulis*, to environmental stress, was illustrated by its survival of 0.4ppm phenanthrene, a concentration that resulted in 100% mortality in *C. edule* and *E. siliqua.*

Immunomodulation associated with hydrocarbon exposure has been demonstrated in bivalve molluscs by several researchers (Coles et al., 1994), in both controlled laboratory investigations and field studies. In field studies the effects of the stressor can be difficult to distinguish from those of other physical influences, such as fluctuations in environmental temperature, physiological stressors, such as oxygen or salinity perturbations and seasonal influences, such as spawning. Demonstration of the link between haemocyte parameters and immune activities requires experiments involving exposure to pollutants and pathogens (Gagnaire et al., 2006). The decreased survival rates of postlarvae co-exposed to phenanthrene and *Vibrio tubiashii* in this study imply that phenanthrene affects some aspects of juvenile mussel immune function. Several authors have previously demonstrated that the alteration of haemocyte parameters in bivalves exposed to pollutants could be associated with an increase in disease susceptibility (Anderson et al. 1981; Gagnaire et al., 2006).

Initial results obtained 2 months after the "Sea Empress" oil spill showed impaired immune activity in mussel haemocytes, which coincided with the extremely high hydrocarbon levels in the tissues (Dyrynda et al., 1997). As the hydrocarbon levels declined, immune capability in oiled mussels increased to levels similar to those mussels from the reference site (Dyrynda et al., 1997) demonstrating that immunosuppression following pollution incidents in the field may be severe, but not necessarily permanent. Results obtained in the present study suggest that the immunosuppression may lead to increased mussel mortalities if the exposure to PAHs occurs at the same time as exposure to elevated levels of pathogenic bacteria. Anderson et al. (1981) found that the exposure of the clam *Mercenaria mercenaria* to PAH and organochlorides as single contaminants impaired the ability of haemocytes to clear bacteria. Exposure to benzo[a]pyrene significantly reduced the bacterial clearance by haemocytes, as measured by the number of viable bacteria in the circulating haemolymph.

Studies on the exact nature of the immunomodulation seen in bivalves on exposure to PAHs have also been undertaken by several authors. Coles et al., (1994) found that exposure to fluoranthene at 0.2 and 0.4ppm resulted in significant stimulation in the release of superoxide radicals in *M. edulis* haemolymph and circulating blood cell numbers, indicating an overall stimulation of the immune capability. However, it was concluded that the energetic costs to the individual are likely to be high, implying that the results of long-term, chronic exposure could well be detrimental. Krishnakumar et al., (1997) found that 6 and 30 day exposure of *M. edulis* to a mixture of PAHs (including phenanthrene) at a concentration of 0.15ppm resulted in significant bioaccumulation of these chemical contaminants, alterations in lysosomal membrane function and induction of some detoxifying enzymes in the digestive tissue. Sequestration and accumulation of aromatic hydrocarbons is a well-established property of cellular lysosomes (Moore, 1979). Mussels exposed to 0.2ppm phenanthrene for 22 hours showed alterations to the lysosomal membranes of digestive cells at EM level, these changes were not seen in mussels exposed to 0.2ppm of the isomeric hydrocarbon anthracene (Nott et al., 1985). Lysosomes are considered to play an important role in invertebrate defence reactions involving bacterial destruction; their functional impairment may affect host resistance to pathogen infection (Gagnaire et al., 2006). In accordance with previous literature reports Gagnaire et al. (2006) found that *in vitro* exposure of *C. gigas* haemocytes to PAHs, including phenanthrene, significantly decreased the number of lysosomes seen and esterase activity.

Phagocytosis by haemocytes has been widely shown to be affected by PAH exposure. It was shown to be exceptionally low in the immediate wake of the "Sea Empress" oil spill, and has generally been found to decrease with increasing exposure to PAH or water/oil mixes (Dyrynda et al., 2000). In December 1999 the oil tanker "Erika" broke in two off the coast of the Atlantic coast of Brittany spilling 15,000 tonnes of crude oil. A subsequent study by Auffret et al. (2004), conducted 2 years after the spill, found that phagocytic activity of *C. gigas* haemocytes was decreased at 3 impacted sites, compared with a site not exposed to oil contamination. Grundy et al. (1996a) found that experimental exposure of *M. edulis* to anthracene, fluoranthene and phenanthrene led to decreased phagocytosis, both in vitro and in vivo. Adult mussels were exposed for 14 days to individual PAHs and a cocktail of all three, with exposure concentrations from 250ppb for a single PAH to 750ppb for the triple PAH cocktail. Grundy et al. (1996b)

also found that a 14 day exposure to the same triple PAH cocktail at the lower level of 500ppb still significantly inhibited phagocytosis, and exposure for 28 days led to a further reduction in the percentage of cells showing phagocytosis. *M. edulis* exposed to crude oil emulsion were shown to have a decreased number of circulating granular haemocytes (phagocytic cells) and their phagocytic activity was also reduced (McCormick-Ray, 1987). Exposure to a cocktail of 10 PAHs, including phenanthrene, at 50, 100 and 200ppm for 10 to 40 days, suppressed phagocytosis of yeast cells by *C. gigas* haemocytes (Jeong & Chu, 2005b) and caused dose-dependant lysosomal destabilisation. It is possible that the increased susceptibility of postlarval mussels to *V. tubiashii*, when co-exposed with phenanthrene, seen in the present study is a result of altered phagocytic capability.

It is believed that the effect of PAHs on phagocytic activity arises from the interactions of these lipophilic compounds with cell membranes (Grundy et al., 1996b; Dyrynda et al., 2000). The effect may be a direct result of membrane perturbation affecting the invagination of foreign matter or indeed it may be an indirect result of interference with lectin binding altering non-self recognition. Molluscan haemocytes have been shown to posses a number of binding sites for lectins (Pipe, 1990a). Lectins are carbohydrate binding proteins that, in the invertebrate immune system, may serve as opsonic factors (Yoshino, 1986) although not all molluscan species require opsonising molecules for effective phagocytosis (Wootton & Pipe, 2003). An opsonin is any molecule that acts as a binding enhancer for the process of phagocytosis. Wootton et al. (2003b) found in *M. edulis* exposed to 0.05ppm phenanthrene for 7 days that there was a concurrent decrease in binding of the lectin concanavalin A and also in the number of bacteria phagocytosed per haemocyte. The changes in lectin-binding capacity of haemocytes highlighted the ability of phenanthrene to interact with cellular membranes and it is thought that this general interaction with membranes is primarily responsible for phenanthrene induced modulations in the immune response of bivalves (Wootton et al., 2003b).

In conclusion, a number of authors have shown, from field and laboratory studies, that immune function can be altered by exposure to petroleum products and PAHs. Several studies have linked exposure to changes in specific immune parameters, particularly phagocytosis, degradative enzyme activity and lysosomal membrane perturbation.

These immune parameters are fundamental in preventing bacterial infections and their compromise may result in the increased susceptibility of postlarval *M. edulis* to the facultatively pathogenic bacterium *V. tubiashii*, seen in the present study when they are co-exposed to low doses of phenanthrene.

Chapter 8. General Discussion

8.1. General discussion

The aims of this thesis are given in the introduction (Chapter 1), briefly they are to assess immune function in developmental stages of the marine mussel, *Mytilus edulis*. Light and electron microscopy were used to examine haemocytes of larval, postlarval and adult mussels. The susceptibility of both larval and postlarval mussels to several common marine bacteria was examined. Three species of bacteria that have been linked with disease outbreaks were studied; these were *Vibrio tubiashii*, *Vibrio alginolyticus* and *Alteromonas haloplanktis*. Interactive effects of pollutants (copper and phenanthrene) and food availability on the susceptibility of postlarvae to bacteria was also examined.

The emergence of *Vibrio* spp. as etiological agents of disease in cultured bivalves will be, undoubtedly, one of the major problems in the next few years (Pallaird et al., 2004). Symptoms of vibriosis in bivalve larvae include: abnormal swimming behaviour, reduced feeding and general inactivity, due to proliferation of bacteria throughout the soft tissues (Gosling, 2003). Gross signs of vibriosis, preceding the high, sudden mortalities, include initial settling of larvae in cultures and general decrease in larval motility, followed by decreased growth and velar abnormalities (Sindermann, 1988). Vibrio bacteria proliferate throughout the larval tissues causing lysis and necrosis. Larvae probably die as a result of starvation (Ford & Tripp, 1996) as the velum is often the first site of vibriosis infection. The disease (vibriosis) effects are enhanced by the secretion of water soluble toxins (Sindermann, 1988). Nottage and Birkbeck (1987a) reported the production of a proteinase that is toxic to bivalves by *V. alginolyticus* NCIMB 1339, the same strain used in the present studies.

Bacterial diseases affect bivalves differently according to their developmental stage. In general, adult bivalves do not suffer high mortality when experimentally challenged with bacteria that are pathogenic to larvae. Trochophore and D-shell larval *M. edulis* were exposed to *V. tubiashii* and *V. alginolyticus*. *V. tubiashii* was found to be more

virulent to mussel larvae than *V. alginolyticus*. The experiments demonstrated that not only does mortality alter with the species of bacteria the larvae are exposed to, but the number of bacteria present also has a significant effect on virulence. Both bacteria species studied demonstrated dose-dependant larval responses, with mortality levels being lower at lower bacterial concentrations. The bacteria levels used in these experiments are environmentally realistic, Nogami and Maeda (1992) state that the stable maximum density of bacteria in the environment seems to be about 10^6 cells/ml, corresponding to the highest concentration used in the present studies.

The *Vibrio* species studied appear to be more virulent to trochophores than D-shells. This is consistent with published studies which report that pathogenicity decreases as age increases, with *Vibrios* being more pathogenic to larvae than adults. Susceptibility to bacteria also alters with age within the larval stages of mussels, the older the larvae are the less sensitive they are (Nottage & Birkbeck, 1986a). This is a general trend in sensitivity to both bacteria and pollutants, with higher sensitivity being seen at earlier life stages. For example, larval and juvenile stages of marine invertebrates are more sensitive to PAHs and petroleum than adults (Pelletier et al., 1997). It is also worth noting at this point that pathogenicity of bacteria may alter with the strain studied, hence it is important that no direct comparisons are made between studies unless the bacteria are from the same source. *V. alginolyticus* has been widely reported as pathogenic to bivalve larvae, however, a strain studied by Sugumar et al. (1998) did not produce any larval mortalities in *C. gigas* larvae.

In the present study differences in larval health were noted between 2 experiments, run at different times of year, exposing D-shells to *V. tubiashii*. These differences were seen in the control cultures and were thought to be due to differences in the health of the larvae as a result of the quality of eggs and sperm spawned. The level of live animals in controls, was much higher in the 5 d experiment (94%) compared with the 48h experiment (69%). The 48h exposure was carried out using larvae from gametes spawned towards the end of spawning period for the population of mussels used. The 5 day experiment was carried out with gametes from the start of the spawning season the following year. Vitellogenesis, in mussel populations in southwest Britain, takes place from November to January (Bayne et al., 1975), therefore oocytes spawned in early in the year could contain more stored yolk than the later spawning oocytes. In the early

stages of larval development, there is a complete dependence on the stored energy reserves acquired from the adults during vitellogenesis (Bayne et al., 1975).

In aquaculture, the mass loss of juvenile stage animals is considered a more insidious problem than the losses of larval stage animals because of difficulties in monitoring and enumerating chronic losses once individuals are transferred to the growing beds. Consequently it was of additional interest to study the susceptibility of postlarvae to potentially pathogenic bacteria. Stress can also affect disease susceptibility, and therefore further experiments were run to see whether the absence of food or presence of pollutants acted as a stressor altering the susceptibility of postlarvae to bacterial infection.

Postlarvae collected from the field for use in exposure experiments were shown to have an indigenous bacteria flora. The introduction of the spat to the nutrient rich 5% Marine Broth media then led to a proliferation of these indigenous bacteria. It was therefore necessary to eliminate these indigenous bacteria by pre-treatment with Provasoli's antibiotic mixture before the postlarvae were used in bacterial exposure experiments.

Postlarvae were exposed to the same strains of bacteria used in the larval exposure studies and were shown to be less susceptible to the bacteria than the larval stages. The results obtained indicated that there are differences in virulence to postlarval mussels between the bacterial species investigated. Postlarvae exposed to bacteria in all experiments often produced large quantities of mucus, although they remained alive at the end of the exposure period. The use of mucus to isolate and protect an animal from its environment is common to marine molluscs (Davies & Hawkins, 1998). Mucus forms a line of defence in many marine invertebrates (Astley & Ratcliffe, 1989). Excess mucus production by bivalves after exposure to pollutants, such as heavy metals and hydrocarbons, has been reported previously (Davies & Hawkins, 1998).

As with the larval studies, seasonal effects were seen. These effects were thought to be linked to the nutritional status of the postlarvae brought in from the field. None of the seasonal effects seen in the postlarval studies carried out appear to result from differences in reproductive cycle as the postlarvae were not sexually mature. The earliest gonad development and gamete storage in *M. edulis* occurs at 12-15mm shell

length (Toro et al., 2002) and the postlarvae used in the exposure experiments were 0.5-3mm shell length. In addition histological sections showed no gamete development. In the food availability studies there was a much higher mortality of postlarvae exposed to the high *V. tubiashii* levels in the winter compared with the spring. Higher mortality was seen in the winter experiment, it is thought that as winter turned to the spring there was more food available in the environment so the animals that were brought in had more reserves and were therefore better able to cope with food deprivation. Previous nutritional history has been shown to have an effect on subsequent exposure to stressors (Beaumont & Toro 1996; de Pablo et al., 2000). Thus tolerance to food deprivation is closely related to previous food levels. Compromised nutritional status may be correlated with the onset of, or with increased susceptibility to, diseases (Elston 1984). In order to eliminate any possible effects of previous nutritional status on the relative susceptibilities of postlarvae to the 3 species of bacteria studied, an experiment was conducted with postlarvae for all 3 experiments being collected from the field at the same time. This experiment demonstrated that there were strain specific effects of bacteria, with the level of normal postlarvae at the end of the exposure period being related to the bacterial strain they were exposed to.

In the absence of food, the presence of *V. tubiashii* or *V. alginolyticus* was generally deleterious, as seen in previous exposure experiments. In contrast, high doses (10^6 bacteria ml^{-1}) of *A. haloplanktis* significantly increased the number of normal postlarvae at the end of this experiment. Bacteria may provide a substantial part of the carbon and nitrogen requirement of some bivalves, especially in the absence of other food sources. It is therefore possible that high levels of *A. haloplanktis* are of nutritional benefit to postlarvae in the absence of phytoplankton. The lower level of *A. haloplanktis* (10^4 cells ml^{-1}) did not significantly increase the level of normal postlarvae: it is possible that the lower level of dosing was not nutritionally rich enough to make a difference. This demonstrates that potentially pathogenic bacteria may be effectively used as a food source in the absence of other food but this is dependant on the virulence of the bacterial strain.

Lethal and sublethal effects of copper have been noted in *M. edulis* larvae and postlarvae (Hoare & Davenport, 1994). Co-exposure experiments were carried out to study, indirectly, whether low copper would alter immune function by assessing

susceptibility of postlarvae to a facultatively pathogenic bacterium. Postlarval mussels possess metallothioneins (MT) and are therefore capable of sequestering the copper. The initial hypothesis investigated was that the immune function of postlarvae would be deleteriously affected by exposure to copper and that this would have an impact on their susceptibility to *V. tubiashii.* Clear seasonal effects were again seen in these experiments, individuals used in the experiments run in the early summer were shown to have a significantly higher survival rate than those in experiments run in the autumn. In addition to differences relating to the nutritional status of the postlarvae the observed seasonal effects may also be a result of changes in MT levels in the postlarvae. There have been many reports of temporal changes in MT levels in *Mytilus*, MT concentrations tend to peak in mid summer, May to July, (Viarengo et al., 1987; Serra et al., 1999; Geffard et al., 2005). In contrast to exposure to *V. tubiashii* alone, there was no deleterious effect as a result of co-exposure to copper and *V. tubiashii.* The results showed that the presence of copper appeared to increase postlarval survival, with a significant benefit conferred by co-exposure to copper and *V. tubiashii.*

It appeared that there was some interaction between the copper, *V. tubiashii* and the postlarvae that reduced the pathogenicity of this bacterium to the postlarvae. Elevated copper concentrations induce natural estuarine microbial communities to produce copper-complexing ligands which dramatically reduce the free Cu^{2+} ion concentrations in the water column (Dryrden et al., 2004). Gordon et al. (2000) have demonstrated that marine *Vibrio* bacteria produce copper-binding ligands, the concentration of which increased as the copper concentration was increased. The pathogenicity of *Vibrio* bacteria is, at least in part, mediated by the production of extracellular proteinases (Nottage & Birkbeck, 1987a; Lee et al., 1996; Gómez-León et al., 2005). The reduction in pathogenicity seen when postlarvae are co-exposed to copper and *V. tubiashii* may be a result of the bacteria synthesising copper-binding proteins in preference to the extracellular proteinases that induce vibriosis. In conclusion, exposure to copper increases the survival of postlarvae when they are co-exposed with *V. tubiashii.*

The presence of phenanthrene, however, was shown to increase the susceptibility of postlarval mussels to *V. tubiashii.* Exposure to phenanthrene alone, at 0.2ppm for 7 days, did not lead to increased postlarval mortality; however, co-exposure to the potentially pathogenic bacterium *V. tubiashii* led to a significant decrease in the number

of normal postlarvae at the end of the experiment. Immunomodulation associated with hydrocarbon exposure has been demonstrated in bivalve molluscs by several researchers (Coles et al., 1994; Krishnakumar et al., 1997; Gaganire et al., 2006). The decreased survival rates of postlarvae co-exposed to phenanthrene and *V. tubiashii* in the present study imply that phenanthrene affects some aspects of juvenile mussel immune function. Several authors have previously demonstrated that the alteration of haemocyte parameters in bivalves exposed to pollutants could be associated with an increase in disease susceptibility. The most widely recorded effect of PAH exposure on immune function is suppression of phagocytosis. It is believed that the effect of PAHs on phagocytic activity arises from the interactions of these lipophilic compounds with cell membranes (Dyrynda et al., 2000; Grundy et al., 1996b). Several studies have linked exposure to changes in specific immune parameters, particularly phagocytosis, degradative enzyme activity and lysosomal membrane perturbation. These immune parameters are fundamental in preventing bacterial infections and their compromise may result in postlarval *M. edulis* being more susceptible to the facultatively pathogenic bacterium *V. tubiashii* when exposed to low doses of phenanthrene. The results obtained in the present study suggest that the immunosuppression may lead to increased mussel mortalities if the exposure to PAHs occurs at the same time as exposure to elevated levels of pathogenic bacteria.

Clearly knowledge of the ontogeny of the bivalve immune system is important as mussel larvae appear to be susceptible to vibrio infections. Preliminary studies on the immune ontogeny of antimicrobial proteins have revealed that not all immune capacities are present through all the life stages (Mitta et al., 2000), in this sense mussel larvae do not possess a mature fully developed immune system. Tirapé et al. (2007) studied expression patterns of immune-related genes during ontogenesis in the oyster *C. gigas*, showing variation in expression levels depending on developmental stage and gene studied. The increased susceptibility of mussel larvae, relative to adults, to vibrios may be a result of an immature immune system. Although larvae are thought to possess phagocytic cells, it may be that these cells cannot kill the bacteria in the same way as adult haemocytes. Both phagocytosis and the generation of superoxide anion have been demonstrated in both whole larvae and disaggregated cells, but phagocytic capabilities are lower in larvae compared with adults (Dyrynda, 1995). The ontogeny of immune function may go some way to explaining why larval mussels are more susceptible to

bacterial challenge than adults, but clearly more work is needed before the development of the immune system of *M. edulis* is fully understood.

D-shell larvae were analysed using electron microscopy to look for evidence of haemocytes in situ. At this early developmental stage the larvae consist mainly of undifferentiated cells which are densely packed. No putative blood cells were identified, although the developing digestive gland and gut were clearly visible. Many of the larval cells contain large numbers of mitochondria indicating that they require substantial amounts of energy, possibly because they are undergoing differentiation and development.

M. edulis haemocyte ontogeny was further investigated with the analysis of juvenile (postlarval) and adult haemocyte samples using both light and electron microscopy. Adult haemocytes fall into 3 categories: agranular hyalinocytes; granulocytes containing small granules; and granulocytes containing large granules (Pipe, 1990). All 3 of these adult haemocyte types were seen in haemolymph samples taken from postlarval mussels. However, in addition, a previously unreported haemocyte was seen in the postlarval haemolymph at both light and electron microscope level. When differentially stained this cell type was seen to be basophilic in nature, containing a few (less than 4) eosinophilic staining granules. Largely agranular cells containing a small number of granules were also seen in postlarval haemocyte samples examined using electron microscopy. The percentage of these previously unreported haemocytes in haemolymph samples decreased as the size of the postlarvae increased. In contrast, the percentage of eosinophilic cells seen in the smallest postlarvae examined was low, but increased with increasing size of the animals examined. The putative new blood cell described in these small postlarvae may be a precursor to the eosinophilic cells seen in adults.

There are 2 main schools of thought on the ontogeny of mussel blood cells, the discovery of the new putative blood cell does not fit with the 2 distinct cell line theory of Cheng (1981), as it appears to be an agranular cell with eosinophilic granules and thus may represent a precursor to the mature eosinophilic cell. Instead it would support the model put forward by Mix (1976), stating that haemocytes originate from one common stem cell with agranular hyalinocytes being the precursor cells, maturing into a

continuous series of morphologically and functionally related cells, Bachère et al. (2004) also concur with this theory of blood cell ontogeny.

In summary it was found that larval *M. edulis* were more susceptible to the bacteria examined than postlarvae, but the susceptibility of postlarvae to bacteria altered with the introduction of stressors. Food deprivation and co-exposure to phenanthrene increased the susceptibility of postlarvae to *V. tubiashii*. However, co-exposure to copper decreased the susceptibility of the postlarvae to *V. tubiashii*. There were shown to be strong seasonal effects that further altered the susceptibility of postlarvae to bacteria, these effects were thought to be due to nutritional status of the postlarvae as opposed to reproductive effects as the postlarvae were reproductively immature. The ontogeny of *M. edulis* haemocytes was examined. Blood cells were not detected in electron micrographs of D-shell larvae but were identified in both in situ in postlarval sections processed for electron microscopy as well as in haemolymph samples taken from postlarvae. A novel blood cell type was described in postlarvae, lending support to the model initially put forward by Mix (1976) stating that haemocytes derive from a single cell line.

Chapter 9. Bibliography.

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