An examination of the effects of copper in the polychaete *Hediste diversicolor*; focusing on how the effects of copper detected at the molecular level manifest at higher levels of biological organisation.

Being a thesis submitted in partial fulfilment of the requirements for a degree of Master of Science.

At the University of Hull

By

Ellen Bell (BSc).

March 2012.
Abstract.

"Resilience of many ecosystems is likely to be exceeded this century by a combination of climate change and pollution impacts among other drivers" (Lawrence 2009).

Copper, although it is an essential metal it can also be extremely harmful to aquatic, marine and estuarine organisms. But do the effects of copper detected at the molecular level manifest at higher levels of biological organisation? The test organism *Hediste diversicolor* was exposed to varying concentrations of copper chloride. Semi quantitative polymerase chain reaction (PCR) was used to detect changes in the mRNA gene regulation of acetylcholinesterase in response to copper exposure. These results were then compared to changes in the rate of burrowing in copper exposed *Hediste diversicolor*. The results of the study showed that at higher concentrations of copper, acetylcholinesterase mRNA was down regulated, while acclimatized worms were faster to burrow. However while a correlation between acetylcholinesterase and burrowing seems to exist further work with regards to quantitatively measuring mRNA regulation, actual tissue concentrations of copper uptake and enzymatic activity of acetylcholinesterase needs to be performed. There is also a need for further investigations into the burrowing behaviour of the worms due to a number of apparently confounding variables detected in the present study.
Acknowledgments.

I would like to pay special thanks to Professor Jeanette Rotchell, Dr Krysia Mazik and Professor Mike Elliott for their help, supervision and support throughout this MSc program.

I would also like to thank Dr Adelaide Lerebours and Emma Chapman for their assistance and guidance.
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List of commonly used abbreviations.

AChE- Acetylcholinesterase
ACH- Acetylcholine
ANCOVA- Analysis of co-variance
ANOVA- Analysis of variance
cDNA- Complementary DNA
GSH- Glutathione
GST- Glutathione S Transferase
mRNA- Messenger ribonucleic acid
MT- Metallothionein
PCR- Polymerase chain reaction
qPCR- Quantitative real time chain reaction
RNA- Ribonucleic acid
rRNA- Ribosomal ribonucleic acid
Chapter 1: Background.

1.1. Introduction

Aquatic environments are a common sink for a variety of different contaminants and many of these then subsequently accumulate in estuarine environments (Buffet et al., 2011). Estuaries are often the site of important urban and industrial activity (Durou et al. 2008). As a result many estuarine environments tend to have a mixture of contaminants in their sediments and water column (Durou et al., 2008). One of these contaminants is the trace metal copper. Although copper is an essential metal it can also be extremely harmful in aquatic, marine and estuarine organisms (Grant et al., 2010). It is used in antifouling paints on boats and ships and can thus enter the water column but it may also enter estuaries by leaching from old spoil heaps or from urban runoff (Bonnard et al., 2009; Galletly et al., 2007). Its effects on aquatic and marine life are well documented. These include protein and DNA damage, physiological abnormalities and behavioural changes all of which will be looked at in more detail at a later point (Geracitano et al., 2007; Poirier et al., 2006; Kalman et al., 2009). The main focus of this study will be in examining the potential for a recognisable link or correlation between the effects of copper at the molecular level and effects observed at higher levels of biological organisation. The test organism selected was the polychaete worm Hediste diversicolor. This species is a sediment dwelling marine worm which is widely dispersed across all European estuaries (Gillet et al., 2008). It is also often referred to as a keystone species due to its ecological importance as a food source, predator, filter feeder, and its role in bioturbation in estuarine environments (Lawrence and Soame 2009, Durou and Mouneyrac 2007, Kalman et al., 2009). It was for these reasons that H. diversicolor was selected as the test organism in this study.

This chapter will cover a short summary of Hediste diversicolor and a brief history of the site where individuals of this species were collected i.e. the Humber estuary. It will also summarise what is meant by a trace metal and the specific known effects of copper at the behavioural, physiological and molecular levels in Hediste diversicolor. Along with a short explanation of possible links between these levels and the costs of tolerance.
1.2. Aims and Hypotheses.

1.2.1. Aims.

1) To examine the potential effects of copper contamination at the molecular and behavioural level in *H. diversicolor*.

2) To find a link between impacts of copper at the molecular level and changes in behaviour in *H. diversicolor*.

3) To isolate and sequence a range of protein specific gene sequences that may later be used as a tool in molecular analyses with *H. diversicolor*.

1.2.2. Objectives.

1) Using PCR and qPCR the gene expression of a variety of proteins will be analysed. This will give an indication of whether a gene is being up or down regulated.

2) Using burrowing analyses it will be possible to monitor the burrowing speeds of *H. diversicolor* in contaminated and clean environments.

3) The results from the qPCR and burrowing analyses can then be compared to assess whether changes in one factor correlate with changes in another. For example changes in specific gene expression of a particular protein/enzyme may increase in one condition and in that same condition burrowing speeds may decrease.

1.2.3. Hypothesis.

The hypotheses (H₁) for this study are that;

1) Copper contamination will induce quantifiable changes in *H. diversicolor*, measured as mRNA expression or burrowing rate.

2) Copper induced changes at the molecular level will correlate with the behaviour of *H. diversicolor*.

The null hypothesis (H₀) for this study is that: Copper contamination will not induce any quantifiable changes in *H. diversicolor* either at the molecular or the behavioural level.
1.3. Trace metals in estuarine environments.

1.3.1. Overview of trace metals.

There are numerous pollutants that affect estuarine environments. These include trace metals, organochlorines (e.g. dichlorodiphenyltrichloroethane (DDT), gamma hexachlorocyclohexane (γHCH), alpha hexachlorocyclohexane (αHCH), polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), halogenated aromatic hydrocarbons and estrogenic chemicals (Durou et al., 2007b; Leaver et al., 2010). However this section will focus on trace metals, specifically copper. Before the effects of trace metal pollution can be described, it is important to understand what is meant by the term "trace metal". In the literature metals that have a detrimental impact on the environment are often referred to as either trace metal or heavy metal (Luoma and Rainbow 2008). From a physical viewpoint however, heavy metals are defined as 'a metal with a specific gravity of greater than 4 or 5,' while the term trace metal is used to describe any metal present at less than 0.01% concentration (Luoma and Rainbow 2008). However Luoma and Rainbow (2008) defined the term trace metal in a way which allows it to be used to refer to metals both essential and non essential that have a detrimental environmental effect. This definition will be adopted here as well. As such trace metals are defined as "all metals essential or not with ions that fall into the class B or borderline categories defined by Nieboer and Richardson (1980) with pragmatic additions of molybdenum (Mo) and selenium (Se)" (Luoma and Rainbow 2008). This definition is based on the work of Nieboer and Richardson (1980) who developed a categorization system for metal ions based on Lewis acid properties. Therefore to fully understand the definition adopted from Luoma and Rainbow (2008) the categorisation system by Nieboer and Richardson (1980) must also be understood. There are three categories that a metal may fall into using this system, class A, class B and borderline. Metals are classified according to their affinities for different elements. Class A metals are Lewis hard acids which are more ionic and will bind to different ligands with the following preference oxygen> nitrogen > sulphur. Class B metals are defined as Lewis soft acids which are more covalent and will bind ligands with the opposite preference to class A metals e.g. sulphur> nitrogen > oxygen. Meanwhile borderline metals hold intermediate properties (Luoma and Rainbow 2008). This definition means that all of the following metals are included as trace metals: antimony (Sb), arsenic (As), bismuth (Bi), cadmium (Cd), chromium (Cr), cobalt (Co), copper (Cu), gallium (Ga), gold (Au), indium (In), iridium (Ir), iron (Fe), lead (Pb), manganese (Mn), mercury (Hg), molybdenum (Mo), nickel (Ni), palladium (Pd), platinum (Pt), rhodium (Rh), selenium (Se), silver (Ag), tin (Sn), titanium (Ti), vanadium (V) and
zinc (Zn). Trace metals have a high affinity for fine grained suspended estuarine sediment; as a result trace metals once bound to sediments may be removed from the water column by deposition (Lee and Cundy 2001). Their deposition may also relate to the redox chemistry of the local sediment, for example in anoxic sediment a wide range of metals will form complexes and may only be released though oxidation mechanisms (Ducrotoy et al., 2011). However they may re enter the water column if the sediment is disturbed. This may be due to natural factors (e.g. erosion or bioturbation) or anthropogenic factors (e.g. dredging or land reclamation) (Lee and Cundy 2001).

Absorption of trace metals depends on their bioavailability in seawater and in food sources; although in the case of some marine organisms their trace metal content reflects the availability of metals generally found in the immediate environment (Nedwell, 1997). Once trace metals both, essential or non-essential, have entered the body of an organism, they bind to a protein for which they have some level of affinity (Luoma and Rainbow 2008). This binding can either be at the active site or on a different part of the protein. When a trace metal binds to the active site of a protein it will most likely block its catalytic activity and inhibit its normal metabolic function (Luoma and Rainbow 2008). However if a metal binds to a different part of the protein that is not the active site it may distort the overall shape of the protein which may in turn change the shape of the active site and make the protein either less efficient in its normal metabolic function or it may induce dysfunction in the protein (Luoma and Rainbow 2008). Once the effects at the molecular level reach a certain threshold, toxic effects may be seen at higher levels of organisation in the physiology, morphology or behaviour of the organism. As a result organisms have developed mechanisms for excreting or detoxifying metals that may enter their system. Detoxification mechanisms generally involve the organism in question producing proteins such as metallothionein which have a high affinity for trace metals. The affinity is so strong that it is unlikely that the metal will be discharged so therefore becomes biologically inactive. These mechanisms include metallothioneins and insoluble metalliferous granules. Damage to the organism only occurs when the rate of trace metal uptake in an organism is greater than its combined rate of excretion and detoxification (Luoma and Rainbow 2008).

The overall damage incurred by trace metals to environmental conditions is complex. Ecological risk from trace metal contaminated sediment or water is also dependent on metal mobility (Amiard et al., 2007). This metal mobility is in turn dependent on several other factors these include chemical factors, biological factors and physical factors (Table 1).
Table 1: Factors effecting metal mobility (adapted from Amiard et al., 2007)

<table>
<thead>
<tr>
<th>Chemical factors</th>
<th>Biological factors</th>
<th>Physical factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complication.</td>
<td>Accumulation.</td>
<td>Aggregation.</td>
</tr>
<tr>
<td>Filtration.</td>
<td>Filtration.</td>
<td></td>
</tr>
</tbody>
</table>

Another such variable factor that has an effect on how damaging trace metals are to the environment is their bioavailability. A bioavailable metal may be defined as “biologically available chemical states that can be taken up by an organism and can react with its metabolic machinery” (Han et al., 1996). Furthermore the net effect of trace metals on different species and their ecosystem may also depend on where in a specific environment the trace metals accumulate before organisms internalise them (Han et al., 1996). In estuarine ecosystems, although most trace metals enter the ecosystem through the water supply (via run off and urban discharges), concentrations of most metals seem to be significantly greater in the sediment (Han et al 1996). This occurs because while metals are in the water column they may be dispersed and diluted relatively easily; this is harder however once metals are bound to sediment. As a result it may be predicted that the effects of trace metals may be greater on sediment dwelling organisms such as H. diversicolor than on water dwelling organisms.

1.3.2. Copper as a pollutant.

Copper may be described as a trace metal under the definition of Luoma and Rainbow (2008). Copper, although in trace amounts, is an essential metal to all organisms it can also be very damaging in high concentrations in aquatic, marine and estuarine environments (Grant et al., 2010). In this study toxicity is used to refer to natural substances that may occur at unnatural concentrations. Under this definition of toxicity, copper is known to be one of the most toxic metals to marine invertebrates and as a result may cause significant changes in marine ecosystems (Galletly et al., 2007). Copper may enter an estuarine ecosystem via several pathways and its concentrations may be highly variable. These pathways include run off from mineral deposits, mining operations, industrial operations, corrosion of copper plumbing, leaching from wood preservatives, fertilizers, fungicides, algaecides, molluscicides, antifouling agents, urban runoff and sewage (Bonnard et al., 2009; Galletly et al., 2007). Some of these pathways will be investigated here in further detail. For example bacteriocide contain nano-sized copper oxide particles which have been shown to have a low dissolution rate and high
toxicity (Buffet et al., 2011). A further source of copper contamination is from mining operations; mining drainage can leach significant amounts of trace metals including copper and will continue to discharge trace metals even after the mine is abandoned. Old disused mines may flood and be eroded and there is a continuing release of trace metals from old spoil heaps (Burlinson and Lawrence 2007). Antifoulants are another large source of copper pollution. Since the gradual discontinuation of organotin (OT) antifoulants there has been an increase in the use of organic biocides and copper-based coatings for boats and ships. Both of these alternatives are cost effective and produce stable degenerative products however concerns have been raised regarding potential bioaccumulation in the environments exposed to them (Diers et al., 2006). It has been calculated that recreational vessels release 150µg/L of copper in leachate every two weeks or 11µg/L per day, while ships release 533µg/L of copper every two weeks or 38µg/L per day (Grant et al., 2010). After the initial application of copper-based antifouling paint, this rate of copper release can vary between 5-20µg/L per day for several months after application (Grant et al., 2010). Storm runoff can also release large amounts of copper into aquatic and marine environments, it has been estimated that copper carried in storm runoff may be between 14 to 740 µg/L (Grant et al., 2010).

1.4. The Humber Estuary.

1.4.1. Location, general geography and ecology.

The Humber estuary is situated on the North East coast of England. Its overall catchment area is thought to be 24 240Km$^2$, more than 20% of the total land area of England (Cave et al., 2003). Although it is fed by a wide range of rivers its main two freshwater sources are the rivers Trent and Ouse which provide an average fresh water flow of 250m$^3$.s$^{-1}$ (see Figure 1). It is a shallow estuary with a maximum tidal range of 7.2m (Cave et al., 2003).

Estuaries are areas with high levels of biological activity due to the mix of fresh and salt water (Durou et al 2008). It is because of the water mix found in estuaries that nutrient levels are so high. Saline water from the sea brings with it nutrients such as calcium, magnesium, sulphur and potassium, meanwhile fresh water supplies add nitrogen and phosphorous which are of limited availability in marine environments. These nutrients are necessary for plant growth, so when concentrations of the nutrients are increased plant growth increases which leads to the expansion of other trophic levels (Correll 1978).
Figure 1: showing the catchment area of the Humber and the main sites along the estuary (Thomson et al., 2004).

Estuaries such as the Humber are highly productive areas with high levels of biodiversity, the maintenance of which is vital in ecological quality of coastal areas (Gillet et al., 2008). The Humber estuary is a vital nursery for many fish (Cave et al., 2003) and all estuaries act as valuable feeding, rearing and migration zones for many marine species. However estuaries are highly changeable areas; salinity, pH, dissolved oxygen and redox potential are natural factors that change regularly due to the mix of fresh and saline water (Hong et al., 2011 Kalman et al., 2009). As a result some researchers argue that most estuarine species are already living near to their tolerance limit (Kalman et al., 2009). Any additional stresses imposed upon estuarine organisms from anthropogenic factors such as pollution are thought to have a greater impact, physically, behaviourally or biologically than would occur under normal circumstances (Kalman et al., 2009). This is a matter of some concern because the Humber estuary is also currently and historically an area of high industrial activity (Cave et al., 2003).
1.4.2. The Humber and industry.

The Humber estuary has acted as a major industrial point since the 18th century, when it became a major trade route (Cave et al., 2003). However the Humber catchment area has a history of smelting that dates back to the Romans (Lee and Cundy 2001). In the industrial age it also supported the iron, steel and textile factories. In post-industrial years, however, the old iron, steel and textile industries declined and chemical, petrochemical and power industries increased (Cave et al., 2003). In 2006 there were 1.5 million people living and working within the boundaries of the Humber flood plain and 11 million people living in the Humber catchment area (Boyes and Elliott 2006). There are currently 44 major plants including power stations running in the Humber catchment area, these all use the local water supply as coolant and release effluent (see Figure 2 for major points of effluent release) into the Humber or one of its sources (Cave et al., 2003). Up to the 1970s the Humber estuary also housed two of the country’s major fishing ports of Hull and Grimsby (Figure 1) which have since become less nationally significant; in 2003 Humber ports receive 13% of the UK’s seaborne trade (Cave et al., 2003).

![Figure 2: showing some of the major points of industrial discharge along on the Humber (from Elliott and Boyes 2002).](image)

1.4.3. Trace metals in the Humber.

Historically high levels of zinc and lead due to mining run off have been recorded in the Humber, from sediment dating back as far as 1250 AD as well as high levels of other metals because of more recent industrialization. However as previously mentioned the main sources
of industry in the Humber catchment are chemical, petrochemical and power plants. Each coal fired Power Station in the tidal reach of the Ouse catchment, can on an annual basis release up to a tonne of copper, cadmium and lead into the atmosphere and a further tonne of copper into the river (Cave et al., 2003). One of the largest tin smelters in Europe (Capper Pass) was situated on the North bank of the estuary and discharged effluent directly into the Humber until it closed in 1991 (Cave et al 2003). In 1997 the Power Station at Drax released 72 tonnes of trace metals (not including cadmium and mercury) into the local natural water sauce (Cave et al., 2003). Between 1985 and 1992 an estimated 420kg of Cu, 540kg of Pb and 2670kg of Zn entered the Humber every day (Lee and Cundy 2001). Furthermore the partial residence time of most metals is estimated at 18 years so all metals added to the Humber catchment may be expected to remain present for 18 years (Cave et al., 2003). The majority of these metals become sequestered within the sediments (Moreira et al., 2006). Aquatic sediment have been shown to act as a source, sink and cycling centre for many forms of contaminant including metals (Moreira et al., 2006). The metals which have been found to have the greatest load in the Humber and its sources are arsenic, mercury, copper, cadmium, nickel and lead. Furthermore any natural inputs of dissolved and particulate matter will have been perturbed by anthropogenic activities this includes metals (Cave et al., 2003). In 1991/1992 levels of various heavy metals in the subsurface seawater from the mouth of a number of British estuaries were measured.

Table 2: Showing dissolved metal concentrations (µg/L) in subsurface sea water from British estuary mouths. Table adapted from Luoma and Rainbow 2008.

<table>
<thead>
<tr>
<th></th>
<th>Copper</th>
<th>Zink</th>
<th>Manganese</th>
<th>Nickel</th>
<th>Cadmium</th>
<th>Mercury</th>
<th>Lead</th>
</tr>
</thead>
<tbody>
<tr>
<td>Humber</td>
<td>1.8</td>
<td>5.1</td>
<td>6.8</td>
<td>1.6</td>
<td>0.076</td>
<td>0.0014</td>
<td>0.024</td>
</tr>
<tr>
<td>Tweed</td>
<td>0.58</td>
<td>0.58</td>
<td>5.0</td>
<td>0.48</td>
<td>0.012</td>
<td>0.0044</td>
<td>0.096</td>
</tr>
<tr>
<td>Tyne</td>
<td>0.43</td>
<td>1.2</td>
<td>2.1</td>
<td>0.36</td>
<td>0.018</td>
<td>0.0042</td>
<td>0.086</td>
</tr>
<tr>
<td>Wear</td>
<td>0.46</td>
<td>1.3</td>
<td>2.5</td>
<td>0.51</td>
<td>0.025</td>
<td>0.0034</td>
<td>0.230</td>
</tr>
<tr>
<td>Tees</td>
<td>1.6</td>
<td>2.6</td>
<td>13</td>
<td>0.3</td>
<td>0.020</td>
<td>0.0045</td>
<td>0.096</td>
</tr>
<tr>
<td>Mersey</td>
<td>1.7</td>
<td>3.9</td>
<td>11</td>
<td>1.2</td>
<td>0.030</td>
<td>0.0021</td>
<td>0.130</td>
</tr>
<tr>
<td>Dee</td>
<td>1.4</td>
<td>2.4</td>
<td>5.5</td>
<td>0.87</td>
<td>0.018</td>
<td>0.0023</td>
<td>0.170</td>
</tr>
</tbody>
</table>

The Humber when compared to other estuaries had higher copper values (Table 2). This evidence from the literature serves to demonstrate the potential levels of trace metal
pollution that can be expected in the Humber and as is shown trace metal levels can be expected to be high.

1.5. **Hediste diversicolor.**

1.5.1. Physiology, behaviour and lifestyle.

*Hediste diversicolor* (O.F. Müller 1776) is a marine polychaete worm of the phylum Annelida, class Polychaeta, order Aciculate, family Nereididae. It was formerly also known as *Nereis diversicolor*. *H. diversicolor* is found in marine brackish waters and is present in all European estuaries (Gillet et al., 2008). Because they live in estuarine habitats they have to be able to tolerate large variations in temperature and salinity and survive hypoxia (Scaps 2002). *H. diversicolor* can live in very variable density ranges from 3700 to 60 000 individuals per square metre with a skewed gender ratio favouring females (Durou and Mouneyrac 2007, Möller 1985).

*H. diversicolor* are characterised by several morphological features including an eversible proboscis with small paragnaths on oral and macillary rings, a subtriangular prostomium with four small eyes, two large palps, two short frontal antennae, peristomium and four pairs of tentacular cirri (Figure 3) (Scaps 2002). Full maturity takes one to two years although some authors have reported a 3 year maturation period (Olive and Garwood 1981; Scaps 2002). Because *H. diversicolor* are semelparous the conclusion to this maturation process is a onetime spawning event which is also the termination of the individual worm’s life. There are generally thought to be up to two spawning events per year (Durou and Mouneyrac 2007). Because these spawning events are terminal, *Hediste diversicolor* as a population, coordinate their maturation process. Thus the final stages of maturation are rapid and occur in accordance to environmental cues such as temperature, light hours and tidal patterns, as well as physiological (endocrine) cues and pheromones (Scaps 2002).

*H. diversicolor* live in y or u shaped burrows (Dales 1950, Durou and Mouneyrac 2007). They are extremely territorial and will defend their burrows against intruders of their own species. Contact with each other on the sediment surface leads to retreat of both parties (Scaps 2002).

*H. diversicolor* adopt several methods of feeding including omnivory, active predation, suspension and deposit feeding (Kalman et al., 2009; Riisgaard and Kamermans, 2001 as cited in Lawrence and Soame 2009). However there are two main methods of food collection that they adopt. The first of these is that they leave their burrow and move along the sediment surface looking for food, catching, ingesting and digesting it immediately. Alternatively they
are able to gather food via mucous secretions (Scaps 2002). Individuals produce a mucous net which is set across the worms burrow the worm then creates a current of water to move through the net by undulating its body. Suspended food particles are caught in the net and may are eaten by the worm (Riisgard 1991). It is thought that these two methods of feeding are interchangeable. The second method is a suspension feeding while the first is that of a predator or scavenger. Riisgard (1991) showed that *H. diversicolor* is preferentially a suspension feeder and uses the second feeding method before resorting to the first feeding method. He observed that *H. diversicolor* only resort to the first feeding method when there are not a sufficient number of algal cells present in suspension (Riisgard 1991).

Figure 3: Anatomical diagram of *H. diversicolor* (adapted from Scaps 2002).
1.5.2. Role in estuarine ecosystems.

*H. diversicolor* is generally considered to be a keystone species (Durou and Mouneyrac 2007). They can live at extremely high densities and as such form a major constituent of the sediment (Berthet et al., 2003). They have several important roles within ecosystems. One of these roles is as a vital food source for wading birds, flatfish and crustaceans (Durou and Mouneyrac 2007, Berthet et al., 2003). In the summer *H. diversicolor* are the primary food source for the black headed gull. Female oystercatchers favour *H. diversicolor* all year round. In winter months *H. diversicolor, Nephthys hombergi, and Scolopus armiger* make up 99% of prey for the bartailed godwit (Lawrence and Soame 2009). *H. diversicolor* larvae also act as a food source for large crabs, shrimp and small fish such as gobies (Scaps 2002). However because of its close relationship with contaminated sediment and its role as a food source for many other species it also acts in the transport, bioaccumulation and biomagnification of contaminants up the food chain (Kalman et al., 2009).

As well as being a food source *H. diversicolor* also has an impact upon its environment in gathering food. Although they are omnivorous they also engage in active predation, filter and suspension feeding. The effects of active predation act to regulate benthic populations (Lawrence and Soame 2009). Their efficiency as suspension and filter feeders is also very high. It has been estimated that within fjords they can collectively daily filter the entire water mass three times and that they can reduce the phytoplankton biomass by 50% within 5 hours (Lawrence and Soame 2009).

A third role of *H. diversicolor* within ecosystems is that they have a high impact on sediment via bioturbation (Kalman et al., 2009). Bioturbation is effectively the mixing and displacement of sediment particles and is closely related to the burrowing behaviour of *H. diversicolor* (Bonnard et al., 2009). Bioturbation also creates oxygenated zones in anoxic sediment promoting microbial growth (Scaps 2002). Because of the high potential density of *H. diversicolor* per square metre (37-3700 individuals) they are able to have a significant impact on sediment and its composition (Kalman et al., 2009). This includes biogeochemical-cycling and distribution of both nutrients and contaminants and has a significant effect on metal speciation (Durou et al., 2008).

1.5.3. *H.diversicolor* as a bio-indicator species.

One form of bio-monitoring uses bio-indicator species as a way of monitoring on levels of pollution in a particular environment. This includes quantifying of contaminant concentrations
in species known to accumulate chemicals in their tissues (Durou et al., 2007a). *H. diversicolor* is considered a good bio-indicator species because it accumulates contaminants from sediment in its tissues they are also relatively sedentary so can give a good indication of the local environmental conditions. Therefore by quantifying levels of contaminant present in *H. diversicolor* it is possible to monitor levels of contamination in marine environments (Durou et al., 2007a). They also act as a good species for investigating contaminant effects because they are easy to keep in laboratory conditions and can be collected in large numbers. They have been used as an effective model species since the 1950s (Scaps 2002).

1.6. Trace metal contamination and *H. diversicolor*.

This section will focus on the effects of trace metals at different levels of biological organisation in *H. diversicolor* with specific emphasis on the effects of copper. However some of the information here is linked to mixed contaminants present in estuarine waters and not to a specific contaminant.

1.6.1. Effects of trace metals at the molecular level.

Most chemicals, including metals, affect the biochemistry of the organism via both genotoxic and metabolically toxic mechanisms (Depledge 1989). However copper is only harmful if the rate of intake is greater than the organism’s ability to excrete or detoxify it (Grant et al., 2010). In marine invertebrates such as *Hediste diversicolor*, trace metals are generally taken up across apical cell membranes of surface epithelial cells via facilitated diffusion through transporter proteins. The larvae of *H. diversicolor* are particularly sensitive to low levels of copper in their environment (Ozoh 1994). Studies have shown that these larvae are good accumulators of copper, cadmium and lead (Ozoh 1994). Copper can also interact directly with proteins and with DNA (Geracitano et al., 2002). In the organism *Laeonereis culveri* (*acuta*) (*Polychaeta*) copper induced damage to DNA included double and single strand breaks (Geracitano et al., 2002). Effects from anthropogenic contamination including trace metals have also been observed to impact on the body concentrations of glycogen, lipids and proteins (Durou et al., 2007b). *H. diversicolor* were assessed from the Seine estuary (a highly contaminated site) and compared to *H. diversicolor* from the Authie estuary (a relatively clean site), both located in France (Durou et al., 2007b). *H. diversicolor* from the Authie estuary had significantly higher levels of glycogen, lipids and proteins (Durou et al., 2007b). However this study did not focus on specific contaminants but the effects of the general contaminant chemistry of the Seine estuary so although these effects may be copper-induced they may also be induced by other
contaminants or via synergistic effect of a contaminant mix. Enzymes are also frequently assessed in *H. diversicolor* from contaminated sites. These include acetylcholinesterase (AChE), glutathione S transferase (GST), superoxide dismutase (SOD), catalase (CAT) and metallothionein (MT), amylase (Am) and carboxymethylcellulase (CMCase) (Moreira et al., 2006; Poirier et al., 2006; Kalman et al., 2009). AChE is a neurotransmitter and may play a role in movement and coordination (Bonnard et al., 2009). GST plays a role in cellular protection against oxidative stress along with SOD and CAT (Durou et al., 2007a). MT is a cysteine-rich protein which plays a specific role in metal detoxification (Poirier et al., 2006). Both Am and CMCase are digestive enzymes the depletion of which may impact on the organism’s ability to feed effectively (Kalman et al., 2009).

Oxidative stress may occur in the presence of copper and iron among other contaminants (Durou et al., 2007a). In individuals of *H. diversicolor* subjected to oxidative stress, CAT increases to act in breaking down hydrogen peroxide into hydrogen and oxygen and, as such, works as an antioxidant. This prevents peroxidation of membrane lipids, thereby protecting the organism from physiological damage (Durou et al., 2007a). Oxidation may also have deleterious effects on *H. diversicolor* respiratory pigment haemoglobin. Oxidation of haemoglobin produces metahaemoglobin which has no physiological function. In this circumstance, CAT, SOD and GST may have significant beneficial antioxidant functions in protecting the organism against metahaemoglobin build up (Geracitano et al., 2002). Levels of CAT, SOD and GST have all been measured in the annelid worm *Laeonereis culveri (acuta)*, following exposure to copper (Geracitano et al., 2002). CAT and SOD both increased, even at lower copper concentration elevations (31.25µg/l) while GST only increased at the higher concentrations of copper (62.50µg/l) (Geracitano et al., 2002). In *H. diversicolor* GST and CAT have been found to increase in activity in worms exposed to cadmium. In this study although GST and CAT did increase in lower cadmium concentrations again it was only at the highest cadmium concentration that GST and CAT increased significantly (Banni et al., 2009). Metallothionein (MT) is involved in the detoxification of trace metals (Poirier et al., 2006) and can be predicted to be induced by metal exposure (Durou et al., 2007a). Metals are bound to MT proteins which are then broken down by lysosomes (Mouneyrac et al., 2003). They are therefore viewed as homeostatic proteins (Nunez-Nogueira et al., 2010). In cadmium exposure experiments with the terrestrial annelid worm *Eisenia fetida* inductions of the MT gene have been noticed within the first 14 hours of exposure (Brulle et al., 2007). MT also has different levels of affinity for different metals in-vitro studies have shown that there is a hierarchical sequence Hg>Cu>Ag>Bi>Cd>Pb>Zn>Co (Poirier et al., 2006).
Metals are also known to cause adverse effects on the neurotransmitter AChE. AChE may be inhibited by some pesticides, carbamates, organophosphorus compounds and some trace metals (Durou et al., 2007a). AChE activity has been noted as being twice as high in copper-exposed *H. diversicolor* (Bonnard et al., 2009). After initial exposure however AChE appeared to be inhibited but this was followed by a rapid increase in AChE to compensate for previous inactivity (Bonnard et al., 2009). When *H. diversicolor* was exposed to cadmium (a toxic non-essential trace metal) AChE activity increased at lower cadmium concentrations but decreased at higher concentrations (Banni et al., 2009). This implies that AChE may be resilient at lower trace metal concentrations but not at higher ones. Finally Am and CMCase have been found to be elevated in *H. diversicolor* from the Seine estuary in France which is highly contaminated (Kalman et al., 2009). However because of the diversity of contamination in the Seine estuary it is not known whether this effect is due to trace metal contamination or another form of contamination.

1.6.2. Effects of trace metals at the physiological level.

Physiology is defined as "that part of biology dealing with functions and activities of organisms, as opposed to their structure" (Lawrence 2008). In this section the physiological effects of copper on *H. diversicolor* will be assessed.

Trace metals have been observed to accumulate in *Hediste diversicolor* following exposure in their natural environment (Berthet et al., 2003). Copper granules for example have been observed accumulating in epidermal cells, the nephridia and epithelial cells (Nedwell 1997). This may be related to the fact that sediment bound metals have a greater impact due to *H. diversicolor*’s close relationship with sediment. It has been suggested that an accurate estimation of the level of damage to *H. diversicolor* may be obtained by calculating the concentration of a given metal in the sediment (Berthet et al., 2003). Copper ion channels allow the passage of both copper and cadmium (Kalman et al., 2010). *H. diversicolor* being a deposit feeder among other things it may ingest twice its own body weight per day in metal containing sediment from its surroundings (Kalman et al., 2010). Copper appears in tissue samples from *H. diversicolor* in the form of granular deposits but at the cellular level it mainly occurs as membrane bound structures (Nedwell, 1997).

The two most studied physiological effects of trace metals upon the physiology of *H. diversicolor* are on its growth and reproduction. This is because *H. diversicolor* is considered a keystone species in estuarine environments and potential effects on growth and reproduction may in turn affect population and ecosystem structure (Lawrence et al., 2009). Growth, repair
and reproductive mechanisms may be directly affected by feeding depression. Feeding depression may occur due to many contaminants including trace metals due to either avoidance behaviour or alterations to physiological mechanisms such as inhibition of digestive enzymes (Kalman et al., 2009). An inverse relationship has been detected between the weight of individual *H. diversicolor* and metal concentrations in their immediate environment (Poirier et al., 2006). Reproduction has also been studied in contaminated waters. Individual *H. diversicolor* from the Seine and Authie estuary (France) were compared (Durou et al., 2007b). The Seine estuary is known to be highly polluted with a mixture of contaminants while the Authie is relatively clean (Durou et al., 2007b). Oocyte numbers in individual *H. diversicolor* from the Authie estuary were significantly higher than in individuals from the Seine (Durou et al., 2007b). Furthermore, female *H. diversicolor* from the Seine reached sexual maturity prematurely and there were significantly fewer sexually mature individuals from the Seine (Durou et al., 2007b). However because the Seine estuary contains mixed contaminants these effects observed may not necessarily be induced by copper or even trace metals. A separate study using *Styela plicata* (Asciidiacea) observed that the number of successfully hatched larvae decreased with increases in copper concentrations (Galletly et al., 2007). This section has described a number of different physiological traits associated with copper contamination the most ecologically important being those that affect growth and reproduction.

### 1.6.3. Effects of trace metals at the behavioural level.

In order to describe the effects of copper at the behavioural level it is first important to clarify what is meant by the term behaviour. Behaviour has been defined as "the relationship of animals to their environment and to other animals" (Lawrence 2008). This section will therefore assess the effects copper pollution may have on the relationship *H. diversicolor* has with its environment and with other animals. The most regularly used reliably quantifiable behavioural trait observed in *H. diversicolor* is its burrowing behaviour. Burrowing behaviour is often used to measure *H. diversicolor* reactions to copper or other contaminants (Kalman et al., 2009, Bonnard et al., 2009). Upon exposure to copper, *H. diversicolor* has been described as being hypoactive which implies the inhibition of behavioural or locomotive activity (Bonnard et al., 2009). Burrowing has been observed as being severely impaired when *H. diversicolor* specimens from relatively clean environments are exposed to soluble copper (Buffet et al., 2003). A reduction in burrowing behaviour leaves the worm more vulnerable to predator attack and wave action thereby reducing its fitness and chances of survival (Kalman et al., 2009). However these impacts on burrowing decrease as
tolerance develops in the worms. For example Bonnard et al. (2009) observed that after one day worms in the negative control with no copper exposure were significantly quicker to burrow than the worms in conditions with 25, 50, 100, and 150 µg Cu\(^{-1}\). However after a second day there was no significant difference between worms kept in the negative control condition and worms in the environmentally relevant 25µg Cu\(^{-1}\) exposure condition. But worms in the negative control were still significantly faster at burrowing than worms kept in the 50, 100 and 150 µgCu\(^{-1}\) exposure conditions. Bonnard hypothesised that this demonstrated the development of tolerance within copper-exposed populations of *H. diversicolor*.

A secondary behavioural characteristic observed in *H. diversicolor*, but less frequently referred to in the literature, is feeding behaviour which is measured by observing the quantities of food ingested. This characteristic links the behavioural level and the physiological level. The act of feeding is classified as a behavioural trait whereas digestion and processing of the ingested material is described as a physiological trait (section 1.5.2). Feeding depression has been observed in *H. diversicolor* in response to contaminated environments (Kalman et al., 2009). Kalman et al. (2009) took *H. diversicolor* from the highly contaminated Loir estuary and the relatively clean Bay of Bourgneuf (both in France). *H. diversicolor* from both areas were then offered a food source and allowed to feed at will. *H. diversicolor* from the Loir consumed less food. This result was considered to be due to feeding depression as an avoidance mechanism in response to the contaminants present. It is thought that this feeding depression occurs due to avoidance behaviour in reaction to contamination (Kalman et al., 2009).

1.6.4. Tolerance and its costs.

Environmental pressures are one of the strongest selective forces; they can influence behaviour, physiology and morphology (Pease et al., 2010). These environmental pressures can be either natural e.g. temperature and salinity, or anthropogenic e.g. contaminants. Which can result in reductions in survival, growth and fecundity (Pease et al., 2010). In dealing with pressures from contamination, there are two strategies adopted by organisms (Galletly et al., 2007). The first of these strategies is avoidance. However many aquatic, marine and estuarine species cannot move sufficiently fast or far too adequately avoid what can be rapid environmental changes. As such a secondary strategy is adaption, by which organisms can increase resistance to environmental change; an example of this is the use of proteins such as metallothionein to render metals biologically inactive. However in developing resistance there is a fitness compromise (Galletly et al., 2007). There are several ways in which tolerance may
be developed in *H.diversicolor*: decrease the absorption of the pollutant, increase secretion of the pollutant or storage of pollutant in a non toxic physiochemical form e.g MT (Berthet et al., 2003). As a result of energy being invested in these processes, energy is not being invested in other processes such as growth and reproduction. Tolerance can be very costly in energy and this may mean that there are further consequences at higher levels of biological organization (Durou et al., 2007b). There is a genetic element to metal tolerance shown in that first filial generations (F1 generations) are able demonstrate aspects of tolerance without elevated copper concentrations (Pook et al., 2009). Specific mechanisms for copper tolerance in *H.diversicolor* include: over production of a gene product, underproduction of a gene product, alteration of metal target or receptors, changes in gene regulation and increases in the production of detoxifying enzymes or other proteins (Pook et al., 2009). However protein synthesis and turnover is one of the most resource intensive processes in the cellular energy budget (Pook et al., 2009).

Such a large drain on energy and resources means that other aspects of the organism’s energy budget must have energy restrictions, such as growth, repair, metabolism and reproduction (Durou et al., 2008). An example of this has been shown in that samples of *H. diversicolor* taken from the Seine estuary are smaller in size and had fewer oocytes where as samples of *H. diversicolor* taken from the Authie estuary were larger, had more oocytes, a greater female to male ratio and a greater fecundity (Durou et al., 2008). An estimated 70% of the energy budget is invested in reproduction in *H. diversicolor* from cleaner environments; for worms from contaminated waters this budget must be divided between growth, maintenance, reproduction and tolerance (Durou et al., 2008, Pook 2009). Further examples of tolerance can be demonstrated *in vitro*. In studies where worms have been exposed to different concentrations of copper, control worms were only better at burrowing for one day; after the second day of the experiment worms from lower copper concentrations were as efficient at burrowing as the controls (Bonnard et al., 2009). Tolerance also remains with individuals even after they have been acclimatized to uncontaminated conditions again emphasizing the genetic element of tolerance (Burlinson and Lawrence 2007). The genetic element of tolerance is thought to evolve over generations in conjunction with Darwinian selection (Nedwell, 1997). This means that tolerant genotypes in contaminated waters will have an advantage over non-tolerant genotypes and as a result are more likely to survive to reproduce and spread their genome to the next generation. However due to the costs associated with tolerance this will only happen in contaminated waters. This results in populations of *H. diversicolor* from
contaminated waters being able to tolerate higher concentrations of copper than other populations (Nedwell, 1997).

1.6.5. Connecting levels of biological organisation.

Throughout this chapter, possible connections have been drawn between different levels of biological organisation. In section 1.6.4, the tradeoffs between being tolerant to contaminants and efficient growth and reproduction were shown. The tolerance mechanisms were all at the molecular level yet the costs of being tolerant were shown at the physiological level and above. Various authors have already highlighted the potential relationship between AChE and body movement. Bonnard et al. (2009) described how AChE activity was twice as high in worms exposed to copper but that the worms were observed as being hypoactive. In this case, there is a possible correlation between levels of AChE activity and worm behaviour thereby linking the molecular level with the behavioural level of biological organisation. Links have been hypothesised between feeding of *H. diversicolor* and the levels of activity and concentration of digestive enzymes such as Am and CMCase (Kalman et al., 2009). Further work examining tolerance and its costs showed that most mechanisms for tolerance occur at the molecular level but the costs of tolerance occur at the physiological level in reductions of oocyte number and smaller size (Pook et al., 2009). All impacts thus far discussed are aspects of the individual level of biological organisation, which extends to the population, community and ecosystem. Although this is not a point of specific interest to this research it is important to remember that because *H. diversicolor* is a keystone species has a close relationship with sediment due to its burrowing characteristics (which acts as a sink for many contaminants including metals). It also acts as an important food source for wading birds and flat fish; it may act in the transmission of pollutants up the food chain (Berthet et al., 2003). Furthermore, it has been observed that any feeding depression that may occur as a result of contaminants may also be translated into reductions in detritus processing and organic matter decomposition in the immediate environment (Moreira et al., 2006). It is therefore important to fully understand how the effects of copper or any pollutant at different levels of biological organisation impact on each other.
1.7. A description of potential behavioural analyses.

There are a number of potential behavioural traits that may be measured in *H. diversicolor*. Here the most commonly used and most quantifiable of these were selected, namely feeding behaviour and burrowing behaviour (Kalman et al., 2009, Bonnard et al., 2009).

1.7.1. Feeding behaviour experimental analyses.

Feeding behaviour is quantified by measuring the amount of food ingested over a set time scale (Kalman et al., 2009, Pook 2009). Two different examples of quantifying feeding behaviour will be shown here. The first of these methods was used by Kalman et al (2009). Kalmans method included counting 20 *H. diversicolor* worms into plastic beakers with 30ml of saline water and adding 100 individual frozen larvae of *Artemia salina*. *H. diversicolor* were then allowed to feed. After a set period of time *H. diversicolor* were washed to remove any further traces of the larvae and the remaining larvae were counted. In using this method feeding rate could be expressed as the number of larvae consumed per hour (Kalman et al 2009). A second method for measuring feeding behaviour was employed by Pook (2009). Pook’s method worked similarly to that of Kalman whereas Pook used 48 individual *H. diversicolor* altogether and kept them in individual containers with a length of silicone tubing which was to act as a surrogate burrow (there being no sediment present) and saline water. Individuals were then offered a pre-weighed dry fish food pellet and allowed to feed. After this length of time the remains of the fish pellet were collected washed and dried before being reweighed. The difference in weight of the fish pellet would show the amount of food individual *H. diversicolor* had consumed over a twelve hour period (Pook 2009).

1.7.2. Burrowing behaviour experimental analyses.

The other commonly used behavioural aspect in *H. diversicolor* is burrowing. There are many variations in measuring burrowing behaviour but all follow the same general theme. The main method was developed by Bonnard et al (2009) but variations on this method have been adopted by Kalman et al (2009) and Buffet et al (2011) among other authors. Bonnard’s method involved placing individual *H. diversicolor* into 100ml containers containing 5cm of artificial sediment (in this case sand gathered from Fontainebleau that was subsequently acid washed) and either artificial sea water or sea water from the organisms’ natural habitat. *H. diversicolor* were then placed on top of the sediment and their positions recorded every two minutes. The time when an organism was completely buried was the result recorded for
analysis (Bonnard et al 2009). Kalman et al (2009) used the same method but instead of using artificial sediment used sediment from the collection site of the *H. diversicolor*. Buffet et al (2011) also used a similar technique based on Bonnard’s method. However Buffet also used natural homogenized sediment and used different time intervals to record the positions of *H. diversicolor* (Buffet et al 2011).

**1.8. A description of potential physiological analyses.**

Although physiological analyses were not experimentally assessed due to lack of time it is important to examine the range of experimental analyses available for completeness and to highlight areas of further study. There are a range of physiological analyses that are regularly used with *H. diversicolor*; these include measuring ingestion against egestion, scope for growth, biometric measurements such as length and weight and reproductive capacity. Each of these measurements will be summarised and briefly analysed in this section.

**1.8.1. Ingestion vs egestion.**

The various methods for measuring ingestion are described in 2.2.1 where they are used as a method for analysing feeding behaviour. However these methods may also be utilised for measuring the rate of ingestion if individual organisms are weighed after feeding trials (Kalman et al 2009). Excretion may be measured by filtering drying and weighing faeces after a set time period (Kalman et al 2009).

**1.8.2. Scope for growth.**

Scope for growth is a measure of the balance of energy in an animal. It can otherwise be described as the difference between energy intake and energy costs through maintenance and excretion. In *Hediste diversicolor* energy intake may be measured using the ingestion methods described in part 1.8.1. Maintenance values may be obtained by measuring *H. diversicolors* respiration in terms changes in oxygen saturation of *H. diversicolors* water (Pook 2009). Scope for growth may then be calculated using the following equation:

\[
\text{Scope for growth} = A - (R + U)
\]

Where A is energy absorbed from diet, R is maintenance requirements expressed in respirometry measurements and U is energy lost through respiration (typically <5% of the total energy budget) (Pook 2009).
1.8.3. Biometric measurements.

There are a number of biometric measurements that may be taken to analyse the physiology of *H. diversicolor*. These include weight, length and paragnath patterns. Both dry and wet weights may be taken from *H. diversicolor* to compare mass between conditions. Length can be useful however *H. diversicolor* are physiologically fragile and can easily lose tail segments without suffering long term injury. This can cause reliability issues when using length or weight as an analytical tool. For this reason partial length analysis (or L3 analysis) was developed. The sum of the first three segments (i.e. the peristomium, prostomium and first chaetiger) make up the L3 measurement which may then be used as a reliable alternative to length and weight analysis. Total jaw length may also be measured which provides a second reliable biometric measurement to be used instead of length and weight (Durou et al. 2008). The other biometric method that may be utilized for *H. diversicolor* is the paragnath pattern. This simply implies counting the number of paragnaths (teeth) in each pharyngeal zone using a binocular microscope (Maltagliati et al. 2006).

1.8.4. Reproduction.

Reproduction is a physiological trait often examined in the literature with reference to *H. diversicolor*. Reproduction tends to be measured in this species by counting the number of oocytes in mature females so the potential for female reproductive output can be measured (Durou et al., 2008).

1.9. A description of potential molecular analyses.

Molecular techniques allow an investigation of the biological impacts of heavy metal exposure at the sub-cellular, molecular level and can act as an early warning of higher level detrimental biological effects (Sarkar et al., 2006). The genes selected for investigation here were acetylcholine esterase (AChE), metallothionein (MT) and glutathione S transferase (GST). These three proteins have been selected because they hold key roles in either the normal function or metal tolerance of *H. diversicolor* (Berthet et al., 2003Durou et al., 2007a, Kalman et al., 2009.).

1.9.1. AChE analysis.

The main acetylcholinesterase assay has been used previously to examine the activity of the protein. A number of papers in the literature have demonstrated the effective use of Ellman’s
(1961) method adapted by Galgani and Bocquene (1991) on *Hediste diversicolor* (Kalman et al 2009). Ellman's method is based on the following equations:

$$\text{Enzyme}$$

\[
\text{Acetylthiocholine} \rightarrow \text{Thiocholine} + \text{Acetate}
\]

\[
\text{Thiocholine} + \text{Dithiobinitrobenzonate} \rightarrow \text{Yellow colour.}
\]

So, using these reactions and a photometer the absorbance can be used to indicate the activity of acetylcholine esterase (Ellman et al 1961). This method was later developed for use with microplate readers by Galgani and Bocquene (1991). The later method works by homogenizing tissue in Tris buffer and centrifuging before filtering the supernatant. The filtered supernatant could then be used in the assay to examine acetylcholinesterase activity. This technique was shown to work successfully on *H. diversicolor* by Kalman et al (2009).

1.9.2. GST analysis.

By far the most frequently used GST assay used in the literature was developed by Habig et al (1974); this measures the activity of GST. This method as with Ellman's (1961) method has since been developed for use on microplate readers and is used to measure GST activity in tissue. One of glutathione S transferase’s functions is to catalyse the oxidation of glutathione (GSH). Habig spectrophotometric method worked by measuring the changes in absorbance of a Cary 15 dual beam spectrometer in samples where GSH was being oxidised. This method was then adapted for microplate readers and has been successfully used with the following protocol.

1.9.3. Metallothionein analysis.

There are several methods for examining concentrations of MT proteins in organisms, for example ELISA (enzyme linked immuno sorbent assays) and differential pulse polarography (Pedersen et al 2008). To date, only the differential pulse polargraphy has been used for *H. diversicolor* (Berthet et al., 2003). It will be the latter of these methods that is examined here because it has already been standardised for *H. diversicolor*. Pooled *H. diversicolor* were homogenized in TRIS buffer and NaCl solution. Soluble and insoluble fractions are separated by centrifugation, following this; the heat stable thiolic compounds including MTs are separated by centrifugation of the soluble fraction after heat treatment. The heat treated cytosol was then fractionated using gel chromatography. From the gel fractions quantities of MT can be
analysed using differential pulse polarography which analyses the absorbance of the samples (Berthet et al 2003; Pedersen et al 2008). In this way the amount of MT or metallothionein-like proteins (MTLPs) can be analysed in *H. diversicolor* tissue samples.

1.9.4. **Quantitative real time polymerase chain reaction (qPCR).**

Quantitative real time polymerase chain reaction (qPCR) has not been a common method used with *H. diversicolor* yet it has the potential to be a very valid method for the development and detection biomarkers. This is because it detects changes in mRNA which is the construction scaffold for protein synthesis; changes in mRNA concentration would be one of the earliest indicators of changes to protein concentrations. The qPCR is a modified version of the PCR system. The PCR works by amplifying a target gene by using DNA polymerase to separate double stranded DNA (under denaturation conditions) then allowing predesigned primers to attach (under annealing conditions) to target sites and with the use of free nucleotides reproduce the opposite strand of the gene (under elongation conditions). This process is then repeated a number of times so that one double stranded gene becomes two which becomes 4 which becomes 8 etc. Using this method a specific gene may be selectively amplified. qPCR uses this method but in the reaction mix a specialised dye is added which binds to double stranded DNA. The qPCR machine then scans the samples throughout the amplification process so that the amount of DNA and rate of DNA amplification may be measured. From these data it is then possible to extrapolate whether a specific gene coding for a specific protein was up or down-regulated in an organism.

1.10. **Summary.**

This chapter has covered a range of points however the important ones will be highlighted here.

1) *H. diversicolor* is a keystone species and as such is an important member of estuarine communities.

2) Copper contamination has an impact on *H. diversicolor* feeding and burrowing behaviour.

3) Copper contamination reduces size and fecundity and therefore fitness in populations of *H. diversicolor*.

4) *H. diversicolor* has a range of protection mechanisms against copper contamination including antioxidants such as GST, CAT and SOD but also metal detoxifying proteins such as MT.
5) Copper contamination can impact on levels and activity of the neurotransmitter AChE and digestive enzymes such as Am and CMCase in *H. diversicolor*.

6) There are a wide range of methodological analyses available for use on *Hediste diversicolor* including molecular, physiological and behavioural analyses.
Chapter 2: Molecular biomarker development for trace metal-induced biological effects.

2.1. Introduction.

This chapter relates to the analysis of the heavy metal-exposed organisms using molecular biological techniques and the sub-cellular level of biological organisation. When an organism is exposed to contaminants such as heavy metals, many effects are triggered at the molecular level of organisation, including potential changes in gene expression and changes in enzyme activities (Bonnard et al., 2009, Brulle et al., 2007). It is possible to exploit these early changes of biological effect as early warning biomarkers. This chapter indicates proteins and their encoding genes chosen for analysis, namely acetylcholinesterase (AChE), glutathione S-transferase (GST) and metallothionein (MT). It also includes a description of each protein of interest including their structure and function, an in depth description of the methods used, a results section displaying all the results obtained from this study and finally a section discussing those results.

2.1.1. Rationale for the proteins targeted and methodology selected.

AChE, MT and GST were selected for the following reasons. AChE plays a key role in neurotransmission in the synaptic cleft and it may be affected by metals (Silverthorn., 2007; Bonnard et al., 2009). Although hypothesised, no effects on burrowing behaviour in *H. diversicolor* have been associated with AChE (Kalman et al., 2009; Bonnard et al., 2009). Bonnard et al. (2009) attempted but could not demonstrate effects between AChE and burrowing though they did observe that when exposed to Cu AChE activity increased and burrowing speed decreased in *H. diversicolor*. Kalman et al. (2009), however, showed that *H. diversicolor* from the contaminated Loire estuary (France) had a reduced level of AChE activity while burrowing ability remained equal between worms from contaminated and cleaner waters. Thus the potential link between AChE levels and burrowing has yet to be demonstrated. MT or metallothionein-like proteins (MTLPs) have been observed being induced in the laboratory and in the field by metals in a range of annelid worms (Durou et al., 2007a). However some debate their relevance to *H. diversicolor*. MT has not been characterised in *H. diversicolor* although metallothionein-like proteins (MTLPs) have been observed. Poirier et al. (2006) found no significant relationship between MTLP concentrations and metal exposure. However MT or MTLPs have been observed binding to metals in *H. diversicolor* and acting in
the metal detoxification process (Berthet et al., 2003, Mouneyrac et al., 2003). GST also has a role in the defence of _H. diversicolor_ exposed to chemical stress. In the multi-polluted Seine estuary (France) the activity of GST in _H. diversicolor_ was shown to be elevated (Durou et al., 2007a). Changes in the activity or concentration of MT and GST may have impacts at the behavioural level due to the cost of tolerance but they have thus far been overlooked or unobserved.

Molecular level techniques allow the earliest possible detection of a biological impact. The qPCR technique quantifies changes in mRNA expression and this study aims to apply it to the AChE, GST and MT gene sequences for _H. diversicolor_. The technique also has an advantage as it can also be used for all three proteins in parallel (unlike protein–level analyses which would require an individual technique for each protein). This way all three proteins can be quantified using the same methodology and calibrated using an internal reference gene. qPCR is a commonly used and highly sensitive technique for measuring differences in mRNA expression. However to use methods like the qPCR a specific base pair sequence is required for each gene of interest. Currently the GenBank database holds no known sequences for _H. diversicolor_. As a result specific base pair sequences had to be isolated and characterised. This process will be the main focus of this chapter.

2.1.3. The role of Acetylcholinesterase (AChE)

AChE was first identified in _H. diversicolor_ in 1996 (Scraps et al., 1996). AChE has a key role in the breakdown of the neurotransmitter acetylcholine into acetic acid and choline in the synaptic cleft of cholinergic synapses during the transmission of a signal across cholinergic synapses (Sarkar et al., 2006, Moreira et al., 2006). When a nervous impulse is started in the form of an action potential it travels down neurones but the action potential alone cannot cross breaks in the neurone (synapses). To overcome this problem the pre-synaptic cleft is induced to release the neurotransmitter acetylcholine which travels to the post synaptic cleft and restarts the action potential. AChE then breaks down the acetylcholine so that the post synaptic cleft is not continually firing new action potentials (Silverthorn., 2007).

The crystal structure of AChE was originally identified in 1991 in _Torpedo californica_ and has since been found to have a high level of structural conservation across many species (Silman and Sussman 2008). AChE has a high specific activity and functions at a very high rate (Divir et al., 2010). It is a homodimer and contains two polypeptides with identical amino acid sequences (Divir et al., 2010). It is structurally composed of 12 central β sheets which are
surrounded by 14 α helices (Divir et al., 2010). It also has a deep narrow gorge (20 Å long) lined with 14 highly conserved aromatic residues which lead to its active site (Divir et al., 2010). AChE therefore has an important role in the correct transmission of nerve impulses. In the species of interest, *H. diversicolor*, it has also been theoretically linked with alterations in burrowing behaviour (Kalman et al., 2009; Bonnard et al., 2009). It has also been reported that a number of pesticides, carbamates, organophosphorous compounds have inhibitory effects on AChE while some metals also affect its activity (Scaps et al. 1997 in Bonnard et al., 2009; Durou et al., 2007a). Durou et al. (2007a) found that AChE from *H. diversicolor* from the multi-polluted Seine estuary (France) had a reduced rate of activity when compared to worms from the relatively clean Authie estuary. However although Bonnard et al. (2009) expected Cu to have inhibitory effects on AChE due to previous studies they actually found that activity of AChE increased in *H. diversicolor* exposed to copper.

2.1.4. The role of Glutathione S Transferase (GST).

GST is a phase II catalyst that acts in metabolising lipophilic organic contaminants and plays a role in cellular protection against oxidative stress caused by various pollutants (Durou et al., 2007a). One of the main roles of GST is to catalyse the conjugation of glutathione (GSH) with compact metals (Moreira et al., 2006). Although it also conjugates GSH with other xenobiotics including a range of carcinogens and environmental pollutants (Downs 2005).

There are three phases in this particular pathway of detoxification, phases I and II involve the conversion of lipophilic and non-polar substances into a water soluble metabolite. Once water soluble the metabolite is less toxic and can be eliminated from the cell in phase III of the detoxification process (Sheehan et al., 2001). The specific role of phase II enzymes like GST is to catalyse the conjugation of lipophilic and non-polar xenobiotics to an endogenous water soluble substrate such as reduced GSH. Such conjugation is a major phase II detoxification reaction across many different species (Sheehan et al., 2001). GSTs themselves are dimeric, mainly cytosolic and have an extensive range of ligand binding properties. Although GSTs are a complex superfamily they all have the same basic structure. GSTs all have two distinct domains, the N terminal domain and the C terminal domain (Sheehan et al., 2001). The N terminal domain consists of four β sheets and three flanking α helices (Sheehan et al., 2001). The arrangement of these structures is similar to the thioredoxin fold which is commonly found in several protein families that have evolved to bind to cysteine on GSH (Sheehan et al., 2001). A linker sequence connects the N terminal to the C terminal. The C terminal of the protein consists of five α helices although the number of helices varies greatly between classes
of GST (Sheehan et al., 2001). The C terminal domain contains most of the residues that interact with the hydrophobic second substrate. It has been hypothesised that the variety in C terminal domains between different GST classes may be responsible for substrate specificity (Sheehan et al., 2001).

In *Hediste diversicolor* GST is known to have a role in antioxidant defence (Durou et al., 2007a). It has also been observed working with higher rates of activity in *H. diversicolor* from the Seine, France (which is generally considered to be pollutant rich), than in worms from the Authie, France (which is relatively clean) (Durou et al., 2007a). GST has also been observed increasing in activity in *H. diversicolor* exposed to copper oxide nano-particles (Buffet et al., 2011). As a result it has been recommended by several authors as a potentially useful biomarker of cellular defence (Durou et al., 2007a, Buffet et al., 2011).

### 2.1.5. The role of Metallothionein (MT).

MTs are a group of cysteine rich and low molecular weight proteins. They were discovered over four decades ago and may be characterised by their selective metal binding affinities (Hardivillier et al., 2006). MTs play an important role in the homeostasis of essential metals such as zinc and copper and also detoxify non-essential trace metals such as cadmium (Berthet et al., 2003, Brulle et al., 2007). MTs also play a role in protecting cells against alkylating agents, oxygen radicals and ionizing radiation (Hardivillier et al., 2006). MTs work in the regulation of essential metals and detoxification of non-essential metals by simply binding to metal ions so that they cannot bind to other proteins and in doing so neutralizing their effects (Berthet et al., 2003; Carpene et al., 2007). MTs provide a reservoir for copper and zinc ions in the form of macromolecules; however they can also restore the functions of proteins that have been inactivated due to metal binding (Florianczyk 2007). It has also been observed that the high expression of MT genes blocks the processes behind cell apoptosis (controlled cell death), while low expression of MT genes appears to increase rates of apoptosis (Florianczyk 2007). MTs are degraded during digestion and it is assumed that they probably release their metal ions at this point (Berthet et al., 2003).

As previously mentioned MTs are cysteine-rich; they may have as many as twenty cysteine residues (30% of the amino acid content), and the amino acid sequence is a highly conserved single chain (Florianczyk 2007). The crystal structure of the protein appears to be shaped like a dumbbell. It consists of a polypeptide backbone which is wrapped around a thiolate core forming a two domain scaffold. The α and β domains are separated by a linker region and each domain binds metals of different affinities (Liang et al., 2009). Thionein (unbound MT’s) are
synthesised at the ribosomal level and are known for being structurally stable (Carpene et al., 2007). Synthesis of thioneins can be triggered by heavy metal exposure among other factors (Florianczyk 2007). Although MTs have not been characterised in H. diversicolor, proteins that share a similar structure and function have been identified. These are generally referred to as Metallothionein-like proteins (MTLPs) (Poirier et al., 2006 and Mouneyrac et al., 2003). These MTLPs have been observed binding to metals in H. diversicolor (Mouneyrac et al., 2003). In H. diversicolor collected from the Backwater estuary (UK) the pool of Cd, Cu and Zn bound to MTLP represented 22%, 17% and 13% respectively of the whole (Mouneyrac et al., 2003). These data indicate that in H. diversicolor metallothionein-like proteins have relatively small metal binding role. However it may also be argued that MTs have a high turnover rate so there can be expected to be little constancy in levels of bound MTLP (Mouneyrac et al., 2003).

2.2. Specific aims and objectives.

2.2.1. Aims.

The aim was to isolate and characterise gene sequences for AChE, GST and MT in H. diversicolor with the intention of using these in a qPCR (chapter 4).

2.2.2. Objectives

Using molecular techniques such as PCR and cloning techniques gene sequences may be isolated from H. diversicolor tissue and characterised using gene sequencing techniques.

2.3. Methodology.

2.3.1. Animals.

The polychaete worms (H. diversicolor) were collected by hand from mudflats at Paull (Humber Estuary (53.723191,-0.235109), and transported back to the laboratory for behavioural analysis in sediment from the collection site (Chapter 3). After behavioural analysis, whole worms were wrapped in tin foil and snap frozen in liquid nitrogen. The samples were then stored at -80°C until molecular analysis.

2.3.2. Total RNA isolation and purification from H. diversicolor tissue.

Total RNA was extracted from the tissue using reagents and the manufacturer’s protocol for ‘RNA isolation high pure RNA tissue kit’ (Roche, Burgess Hill, UK). Approximately 20mg of
tissue from the anterior end of the worm was first disrupted using a hand held homogeniser in 400μl of lysis buffer (containing 4.5M guanidine-HCl, 100 mM) and centrifuged at room temperature for two minutes at 14,000rpm. The supernatant was then transferred into a clean tube. 200μl absolute ethanol was added to provide appropriate binding conditions and the sample was then applied to a silica-gel based column, spun for 30 seconds at 14,000rpm and the flow-through liquid discarded. To avoid genomic DNA contamination, DNase digestion was performed by adding 10μl of DNase I working solution and 90μl DNase Incubation buffer (containing 1M NaCl, 20mM tris-HCl and 10mM MnCl₂) and left at room temperature for 15 minutes. The column was washed several times with two ethanol-based buffers (wash buffer 1 contained 5M guanidine-HCl and 20mM tris-HCl and wash buffer 2 contained 20mM NaCl and 2mM of tris-HCl) to eliminate the contaminants and the flow-through liquid discarded. The column was transferred into a clean tube and eluted by centrifugation for 1 minute at 8000rpm with 100μl elution buffer (containing nuclease free double distilled water). The sample was stored at -20°C until further processing.

2.3.3. First strand synthesis of cDNA for RT-PCR.

The Superscript VILO cDNA synthesis kit from Invitrogen Life Technologies (Paisley, UK) was used to synthesize first-strand cDNA from total RNA. RNA concentration was first analysed using a Qubit fluorometer (Invitrogen Life Technologies). This was to ensure that the concentration of cDNA per sample were equal. A master mix was made up containing 199μl of QuantIt RNA buffer and 1μl of QuantIt RNA reagent for every sample tube used. 190μl of this master mix was then added to two standard tubes along with 10μl of with either standard 1 or standard 2 (pre diluted RNA standards). For the other samples 199μl of master mix was added to each tube along with 1μl of RNA preparation. The QuantIt fluorometer was then calibrated using the standard solutions and the concentration of the RNA samples calculated.

For each sample 4μl of 5 x Vilo reaction mix (random primers, MgCl₂, dNTPs) and 2μl of 10 x superscript enzyme mix (SuperScript ™III RT, RNaseOUT™ recombinant ribonuclease inhibitor, proprietary helper protein) was used. RNA was then added in varying amounts depending on how concentrated it was to standardise the amount of starting template. The solution was then made up to a total reaction volume of 20μl with diethylpyrocarbonate (DEPC) treated water. Samples were then incubated at 25°C for 10 minutes, 42°C for 60 minutes and 85°C for 5 minutes. Samples were stored at -20°C.
2.3.4. Design of degenerate oligonucleotide primers.

Oligonucleotide degenerate primer sequences were designed using sequences from other species available in GenBank (shown in Table 3) and by aligning them using Clustal Software in order to identify areas of conservation (Figure 4). Degenerate primers were designed for the genes of interest, AChE, MT and GST, as well as for the reference gene 18S rRNA. The primers designed and utilised are listed in Table 4.

Table 3: the species sequences used to design degenerate primers.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Species</th>
<th>GenBank Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S rRNA</td>
<td>Nereis limbata</td>
<td>U36270</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>Nereis succinea</td>
<td>AY210447</td>
</tr>
<tr>
<td>18s rRNA</td>
<td>Nereis pelagica</td>
<td>AF474279</td>
</tr>
<tr>
<td>AChE</td>
<td>Sitobian avenea</td>
<td>AAU11286</td>
</tr>
<tr>
<td>AChE</td>
<td>Camponotus floridanus</td>
<td>EFN69188</td>
</tr>
<tr>
<td>AChE</td>
<td>Xenopus laevis</td>
<td>NP_001121332</td>
</tr>
<tr>
<td>AChE</td>
<td>Cyprinus carpio</td>
<td>BAH11081</td>
</tr>
<tr>
<td>AChE</td>
<td>Rattus norvegicus</td>
<td>AAH94521</td>
</tr>
<tr>
<td>AChE</td>
<td>Caenorhabditis elegans</td>
<td>AAC14016</td>
</tr>
<tr>
<td>MT</td>
<td>Eisenia fetida</td>
<td>GU177855</td>
</tr>
<tr>
<td>MT</td>
<td>Lumbricus rubellus</td>
<td>AJ005822</td>
</tr>
<tr>
<td>GST</td>
<td>Cipangopaludina cathayensis</td>
<td>ACJ03598</td>
</tr>
<tr>
<td>GST</td>
<td>Cristaria plicata</td>
<td>ADM88875</td>
</tr>
<tr>
<td>GST</td>
<td>Ruditapes philippinarum</td>
<td>ACM16805</td>
</tr>
<tr>
<td>GST</td>
<td>Ictalurus furcatus</td>
<td>ADO28330</td>
</tr>
<tr>
<td>GST</td>
<td>Mus musculus</td>
<td>NP_861461</td>
</tr>
<tr>
<td>GST</td>
<td>Eisenia fetida</td>
<td>ADV57678</td>
</tr>
</tbody>
</table>
Table 4: forward and reverse primer sequences used in the isolation of 18S rRNA, AChE, MT, and GST.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Forward</strong></td>
<td></td>
</tr>
<tr>
<td>18s</td>
<td>GCGRGTATCATCGACACAAG</td>
</tr>
<tr>
<td>AChE</td>
<td>GAGATGGGGAATCTCATAC</td>
</tr>
<tr>
<td>MT</td>
<td>GTGCAATCGGRCGTCGC</td>
</tr>
<tr>
<td>GST</td>
<td>CAATCATAAGCTTGCGGCTCC</td>
</tr>
<tr>
<td><strong>Reverse</strong></td>
<td></td>
</tr>
<tr>
<td>18s</td>
<td>CTTGGAATGGTGATGCGCGCTTT</td>
</tr>
<tr>
<td>AChE</td>
<td>ACTGTCTCCTAAAGAAAAACGCCGAC</td>
</tr>
<tr>
<td>MT</td>
<td>GTACAGGAGGAGAACAAGCTCTCTAGGAAAAACGCCGAC</td>
</tr>
<tr>
<td>GST</td>
<td>TTGTGAATTCTAGGCGCGCTCT</td>
</tr>
</tbody>
</table>

Figure 4: nucleotide alignment of 18S rRNA sequences in the species *Nereis succinea, Nereis limbata* and *Nereis pelagica*. Asterisks denote homology. Highlighted areas indicate the chosen primer sites.
2.3.5. PCR analysis of target and reference sequences.

All the reactions were carefully prepared using autoclaved tubes and autoclaved disposable pipette tips in order to avoid contamination of the samples with foreign DNA. The reagents used were aliquoted to prevent degradation by repetitive thawing/freezing cycles. Oligonucleotide primers employed in the reaction were synthesized by Invitrogen Life Technologies and supplied in lyophilised form. In the laboratory, the primers were re-suspended in molecular grade deionised water to a concentration of 50 μM.

Standard PCRs were performed in a final reaction volume of 50µl. This consisted of 5µl of 10 X buffer (100mM Tris-HCL, 500mM KCL, 15mM MgCl₂) (Fisher scientific, Loughborough, UK), 1µl of PCR grade nucleotide mix (containing dATP, dCTP, dGTP and dTTP at 10 mM each from ROCHE), 2µl of forward and reverse primer (50 μM), 0.5 µl of Taq DNA polymerase (5U/µl) (Fisher Scientific), 2µl cDNA and 37.5µl dH₂O.

Amplifications were carried out using a Techne Thermal Cycler equipped with a heated lid. All reactions were initially denatured at 95°C for 2 minutes then for 35 cycles of 94°C for 30 seconds (denaturation), 50°C for 30 seconds (annealing) and 72°C for 30 seconds (elongation). There was then a final elongation step at 72°C for 5 minutes following this, samples were held at 4°C. Positive and negative controls were set up alongside each set of PCR reactions. Negative controls consisted of all components of the PCR reaction excluding the template DNA while the positive control was the reference gene 18S rRNA. The former control was to ensure that there was no contamination, while the latter was to ensure that the reaction was working and that the template cDNA is not damaged.

The conditions listed above were selected because after several trial runs they were found to optimize DNA amplification.

2.3.6. Agarose gel electrophoresis of DNA.

1.5% agarose gels were prepared using 1.2g agarose (Fisher Scientific) dissolved in 80ml of 1X TBE electrophoresis buffer (Tris-borate, EDTA buffer concentrate, 0.89M Tris base, 0.89M Boric acid and 0.02M EDTA) (Fisher Scientific). This mixture was then heated in a microwave oven. The solution was allowed to cool to approximately 60°C and 8µl of ethidium bromide (10mg/ml) (Invitrogen Life Technologies) was added and mixed thoroughly. The agarose was then poured into the holding tray ensuring that the teeth of the Teflon comb were immersed and allowed to set for approximately 30 minutes at room temperature prior to removal of the comb and submerging into the electrophoresis buffer in the tank filled with 1X TBE
electrophoresis buffer (Tris-borate EDTA buffer concentrate) (Fisher scientific). 15µl of PCR products was first mixed with 2µl of 6X blue loading dye (New England Biolabs, Hitchin, UK) and then loaded into the wells of the gel. A 100bp molecular weight ladder (New England Biolabs) was used in order to size the PCR fragments. A current of 70V was then applied to the gel and stopped when the dye had migrated an appropriate distance through the gel. Gels were examined on a UV transluminator (Syngene, Cambridge, UK) and photographed using Genesnap software (Syngene, Cambridge, UK).

2.3.7. Isolation of DNA fragments from agarose gel slices.

The gel containing the DNA fragments of interest was cut and removed on a UV transluminator using a clean scalpel. The gel slice was placed into a pre-weighted clean 1.5 ml plastic tube and processed according to the NucleoSpin Extract II PCR clean-up and Gel Extraction protocol (Macherey-Nagel, Loughborough, UK). 200µl NT buffer (containing guanidine thiocyanate) was added to each 100mg of agarose gel and samples were incubated for 10 minutes at 50°C. Tubes were disrupted periodically to dissolve the gel slice. Following this the dissolved gel was added to NucleoSpin Extract II Columns silica-gel membrane and centrifuge for 1 minute at 10,000rpm. 700µl of NT3 buffer (containing chaotropic salt) were added to the column and centrifuged 1 minute at 10,000rpm in order to remove any trace of agarose. The column was subsequently centrifuged for 2 minute at 10,000rpm to eliminate any trace of NT3 buffer that might interfere with downstream application and then placed into a clean 1.5ml tube. To elute the DNA, 30µl NE buffer (5 mM Tris-HCl, pH 8.5) was applied to the centre of the membrane, left for 1 minute and centrifuged 1 minute at 10,000 rpm. The sample was stored at -20°C.

2.3.8. Cloning PCR-generated fragments of DNA.

The cloning technique allowed the separation of specific DNA fragments from a PCR mixture and the production of them in large quantities. To achieve this, the DNA cloning methods using bacterial plasmids were adopted. This process was completed using the TOPO TA cloning kit for sequencing (Invitrogen Life Technologies). It was first necessary to insert DNA fragments into plasmids. 4µl of PCR product or isolated DNA fragments from part 2.3.7. were added to each plasmid reaction along with 1µl of salt solution (1.2M NaCl, 0.6 M MgCl₂), and 1µl of TOPO vector (10ng/µl of plasmid vector, 50% glycerol, 50 mM Tris HCl, 1 mM EDTA, 2 mM DTT, 0.1% triton X 100, 100µg/ml bovine serum albumin (BSA) and 30µM phenol red). This mixture was gently mixed and centrifuged and incubated overnight at 15°C.
Plasmids were then inserted into chemically competent *E. coli* (One shot® reagents, Invitrogen Life Technologies). *E. coli* were allowed to thaw on ice, when the cells started to thaw, 2µl of pre-prepared plasmid was added to each vial and mixed gently. Cells were then incubated for 30 minutes on ice. The cells were heat shocked for 30 seconds at 42°C. Cells were once again placed on ice for 2 minutes. 250µl of S.O.C medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM HCL, 10 mM MgCl₂, 10 mM MgSO₄ and 20 mM glucose) at room temperature was added to each vial. Vials were capped and put in a shaking incubator for 1 hour at 37°C and 225rpm. The contents of each vial were then spread onto agar plates (2.5g yeast extract, 5g bacto-tryptone, 2.5g NaCl, 10g agar and 500ml H₂O). Two plates were used per vial one plate was spread with 25µl of *E.coli* and +200µl of *E.coli* was spread on the other. These plates were allowed to incubate overnight at 30°C. Plates were taken out the next morning and white colonies were picked using a sterile pipette tip and aseptically added to 10ml LB broth (H₂O, bacto tryptone, yeast extract, NaCl 58.44 g/M) 1 colony was added to every 10 ml vial of LB broth and incubated overnight in a shaking incubator at 37°C and 200rpm. Overnight cultures were stored at 4°C.

### 2.3.9. Extraction and purification of plasmid DNA.

Plasmid DNA was extracted from *E. coli* using the Wizard® plus SV minipreps (Promega, Southampton, UK). 3ml of the overnight cultures from section 2.3.8. were pelleted by centrifugation at 8000rpm for 5 minutes. The pellet was then re-suspended via vortex in 250µl of re-suspension solution. 250µl of cell lysis solution was then added to each sample and inverted 4 times before adding 10µl of alkaline protease solution and again inverting the tubes four times. This mixture was then allowed to incubate for 5 minutes at room temperature. Following this 350µl of neutralization solution was added and again tubes were inverted 4 times. The samples were then centrifuged at top speed for 10 minutes. A spin column was inserted into a collection tube and the supernatant from the last centrifugation step was added to the top chamber. This was centrifuged at top speed for 1 minute and the flow through liquid was disposed of. The column and collection tube were paired again and 750µl of wash solution (containing ethanol) was added to the top chamber, the flow through was again discarded and collection tube and spin column were again paired. 250µl of the wash solution was added to the top chamber and centrifuged again for 2 minutes at 14,680rpm. Finally the spin column was added to a 1.5ml micro-centrifuge tube and 100µl of nuclease free water was
added to the top chamber. This was then centrifuged again at top speed for 1 minute. The flow through liquid (which contained plasmid DNA) was then stored at -20°C.

The plasmid DNA from this step was used in a PCR process using the M13F and M13R primers provided with the TOPO TA cloning kit for sequencing (Invitrogen Life Technologies) from section 2.3.8. The PCR products were then run on a gel as described in section 2.3.6. Any solution that provided bands of the right weight were then sent for sequencing.

2.3.10. Sequencing.

Promising solutions from section 2.3.9. were analysed using the Qubit fluorimeter to assess the concentration of DNA. This process for this was the same as described in 2.3.3. with the exceptions that a Quant It ds DNA Br buffer, Quant it ds DNA Br reagent and Quant it ds DNA Br standards 1 and 2 were used instead of an RNA buffer, RNA reagent and RNA standards 1 and 2. It was then possible to calculate how much solution should be used to give a final concentration of 6ng/µl in a total volume of 5µl. According to these concentrations the appropriate amount of DNA was added to a 1.5ml micro centrifuge tube along with enough M13F primer to give 5mM in 5µl. Water was then added to this solution to bring the final volume up to 10µl. These tubes were packaged and sent for sequencing at Macrogen Europe (Amsterdam, Netherlands).

2.4. Results.

2.4.1. mRNA concentration.

Good quality messenger RNA was collected from *H. diversicolor* at yields that are considered to be acceptable. mRNA yields from individual *H. diversicolor* ranged from 4.1ng/µl and 49.2ng/µl.

2.4.2. PCR bands.

Figures 5 and 6 demonstrate the range of PCR products obtained using *H. diversicolor* cDNA and the various primer sets. Bands of PCR products were obtained for 18s AChE, MT and GST (Figures 5 and 6). Appropriate bands were selected by comparing the molecular weight relative to the ladder run in parallel on these gels with the expected molecular weight based on the primer design. In this case the expected molecular weights were as follows: 18S was 252bp, MT was 105bp, AChE was 128bp and GST was 147bp. The gels show that the 18S fragment was approximately 300bp, which was considered sufficiently close a match to the expected weight.
to send the fragment away for commercial sequencing. MT primers produced two bands: one at 400bp and one at 100bp. Out of these two values only the smaller was considered potentially viable so were sent for sequencing. AChE primers also produced two bands one was 350bp and one was 200bp. Once again only the smaller of these values was sent for sequencing because it was closer to the predicted weight for the AChE band. GST primers produced bands weighing 300bp and 100bp and the 100bp fragment was sent for sequencing again because it was the smaller in size.

Figure 5: An image of a 1% agarose gel taken under UV light, showing potential bands representing partial PCR products for 18s and MT. The bands labelled 18s and MT were found using the corresponding primers.

Figure 6: An image of a 1% agarose gel taken under UV light, showing potential bands for 18s, AChE and GST. Bands labelled 18s, AChE and GST were found using the corresponding primers.
2.4.3. Sequencing.

PCR products of an approximately correct size for the target genes/primer sets were sent to Macrogen for sequencing as described in part 2.3.10. As the positive control, 18S gene, had not yet been identified in *H. diversicolor* that also had to be sequenced. After sequencing the resulting base pair sequence was analysed using the BLAST tool on the NCBI website ([http://www.ncbi.nlm.nih.gov/genbank/](http://www.ncbi.nlm.nih.gov/genbank/)). This analysis showed that both 18s and AChE had sufficiently high homology with other sequences for the same gene in other species to allow a confident assumption that the sequences found were those of AChE and 18S (see figures 7 and 8). Sequences for MT and GST were not found in the time allowed.

![Nucleotide sequence fragment found in *H. diversicolor* for AChE](image)

**Figure 7:** The nucleotide sequence fragment found in *H. diversicolor* for AChE.

**Figure 8:** The nucleotide sequence fragment found in *H. diversicolor* for 18s.

2.4.4. *H. diversicolor* AChE gene characterisation.

Using Clustal analysis the degree of homology between AChE gene sequences of several species and with that isolated herein could be analysed (figure 6).

![Sequence alignment of the AChE cDNA fragment from *H. diversicolor* with AChE sequences from *Carassius auratus* and *Loligo opalescens*. Asterisks mark areas of homology](image)

**Figure 9:** Sequence alignment of the AChE cDNA fragment from *H. diversicolor* with AChE sequences from *Carassius auratus* and *Loligo opalescens*. Asterisks mark areas of homology.
By analysing homologous areas of the AChE genes shown in figure 6 it is possible to calculate that the AChE gene fragment from *H. diversicolor* was 70% homologous with the equivalent fragments from *C. auratus* (GenBank Accession number: AY847745) and *L. opalescens* (AA065384.1).

### 2.5. Discussion.

This chapter identified the nucleotide sequences in *H. diversicolor* for AChE, GST and MT, which could then be used in a qPCR with cDNA from *H. diversicolor* which had been exposed to varying levels of copper contamination. Initially the appearance of bands in the gels after PCR had been carried out indicated that there were nucleotide sequences of the correct weight that responded to the primers used. However the cloning and sequencing processes showed that these nucleotide sequences did not correspond to previously found MT or GST sequences. However the sequence found with AChE primers in *H. diversicolor* held sufficient homology with known sequences on GenBank to be identified as part of the AChE gene. When comparing the AChE sequence with the corresponding fragments of AChE sequences in other species there is a 70% homology. AChE has already been reported as having regions that are highly conserved across species (Divir et al., 2010). The results from the clustal analysis (part 2.4.4) show that the sequence for AChE in *H. diversicolor* also has large areas of conservation with AChE sequences from other species.

AChE has been recommended for use as a biomarker in the literature because it is known to be inhibited by organophosphates, carbamates and some metals (Kalman et al., 2009 and Bonnard et al., 2009). *H. diversicolor* has also been recommended as a bio-indicator due to its role as a food supply and its potential to act in bioaccumulation and biomagnification (Scaps and Borot 2000). As a result it may prove useful to know the partial sequence for AChE in *H. diversicolor* as this allows new methods of monitoring. An example of this is the use of the qPCR. Knowing the partial sequence for AChE in *H. diversicolor* will make the adaptation of qPCR more readily available for bio-monitoring using *H. diversicolor*. Quantitative PCR could be used in this instance to measure the up or down regulation of AChE in *H. diversicolor* exposed to a range of different contaminants including metals such as copper.
2.6. Summary.

The main concepts of this chapter include:

1) The successful isolation and characterisation of part of the AChE mRNA sequence.
2) The AChE mRNA fragment may be used in quantitative methods such as qPCR to determine if the gene is up or down regulated in individual H. diversicolor exposed to Cu.
Chapter 3: Sub-lethal behavioural response to trace metal exposure.

3.1. Introduction.

This chapter focuses on a sub-lethal behavioural response in *H. diversicolor* to heavy metal exposure, using burrowing behaviour as the behavioural response.

3.1.1. Justification of behavioural analysis and methodology.

Burrowing is an important behavioural process for *H. diversicolor*. It is an essential part of the lifestyle of *H. diversicolor* and plays an important part in their survival strategies. From a physiological point of view the burrowing behaviour of *H. diversicolor* is a series of chain reactions based on instinctive reflexes (Bonnard et al., 2009). Because of its dependence on reflexes it has been hypothesised that AChE (see chapter 2) may be associated with burrowing behaviour (Bonnard et al., 2009). Effective burrowing is essential to *H. diversicolor* because it protects them from both predation and wave action (Kalman et al 2009). As such the ability to burrow effectively increases an individual’s fitness and on a larger scale effective burrowing across the population means a higher survival rate and probability of reproductive success. Burrowing rates and efficiency can also have impacts at the community and ecosystem levels because not only do *H. diversicolor* via their burrowing assist bioturbation but they are also keystone species and the success of their own populations may have important impacts on the community as a whole (Lawrence and Soame 2009, Scaps 2002). Copper is known to reduce burrowing ability and speed in *H. diversicolor*, as previously described in section 1.6.3 (Bonnard et al., 2009, Buffet et al., 2011). Burrowing behaviour is easy to quantify while adding a minimum amount of experimentally induced stress to the organism. The term experimentally induced stress in this context refers to stress caused to the test animal by experimental procedures rather than the test variable. It is important to minimise experimentally-induced stress because if not minimised the behavioural results obtained may be caused by the experimental environment rather than contaminant exposure.

The method used to measure burrowing behaviour was developed to cause minimum levels of experimental stress to the organism so that the stress caused by copper contamination could be observed. Natural homogenised sediment was used instead of artificial sediment for this reason and temperature, light dark cycles and salinity were set to mirror the natural environment as closely as possible. Exposure concentrations of copper chloride were selected...
via a literature review comparing the concentrations authors had used in mesocosms and had detected in other estuaries. The results from this literature review are shown in table 5 which indicates a wide range of copper concentrations both in the environment and used in mesocosms. A range of copper concentrations published in the literature (table 5) were used to determine a set of exposure concentrations for the exposure experiment. These were 0 (control), 300, 600 and 1200µg/L of CuCl₂ 2H₂O. The concentrations were also based largely on the work of Pook (2009) and were selected from both the resistant and non resistant exposures used in his study. This range was selected because the *H. diversicolor* sampled from the Humber could be expected to have some level of resistance to copper already due to exposure in their natural environment (Nedwell 1997). However because the level of resistance, if any, is unknown, exposures were selected to contend with all scenarios for all resistance levels.

Table 5: Summarising the concentrations of copper in natural environments and the copper concentrations previously used in exposure trials.

<table>
<thead>
<tr>
<th>Test organism</th>
<th>Natural Cu exposure.</th>
<th>Mesocosom Cu exposure</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Mussel (Mytilus edulis)</em>, Oyster (Crassostrea gigas), Ragworm (<em>H. diversicolor</em>).</td>
<td>Mean estuarine sediment contamination: 45.3 mg kg⁻¹</td>
<td></td>
<td>Amiard et al., 2007.</td>
</tr>
<tr>
<td><em>H. diversicolor</em></td>
<td>Mean sediment contamination: Bay of Somme: 0.64 µg g⁻¹d.w</td>
<td></td>
<td>Berthet et al., 2003.</td>
</tr>
<tr>
<td></td>
<td>Blackwater estuary: 53 µg g⁻¹d.w</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Seine estuary: 48 µg g⁻¹d.w</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Boulogne harbour: 153 µg g⁻¹d.w</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Restronguet creek: 4413 µg g⁻¹d.w</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>H. diversicolor and S. plana</em></td>
<td>0, 25, 50, 100, 150 µg CuL⁻¹. Concentrations based on sub-acute and natural environmental concentrations</td>
<td></td>
<td>Bonnard et al., 2009.</td>
</tr>
<tr>
<td><em>H. diversicolor</em></td>
<td>Acute condition 0.5-4mg L⁻¹ Chronic condition 1mg L⁻¹ of Cu after 6 days conditions raised to 2mg L⁻¹ after 62 days Cu was finally increased to 4mg L⁻¹ to increase mortality. After 81 days Cu was increased to 8mg L⁻¹.</td>
<td></td>
<td>Burlinson and Lawrence 2007.</td>
</tr>
<tr>
<td><em>Laeonereis acuta</em></td>
<td>Chronic exposure lasted 14 days and two concentrations of Cu were used. These were 31.25 µg</td>
<td></td>
<td>Geracitano et al., 2002.</td>
</tr>
</tbody>
</table>
3.2. Specific Aims, Objectives and Hypotheses.

3.2.1. Aims.

1) To examine the potential changes in the burrowing behaviour of *H. diversicolor* under different levels of copper exposure.

2) To explore potential changes in burrowing behaviour which may occur between pre- and post-contaminant acclimatized worms.

3.2.2. Objectives.

Using a range of copper contaminant concentrations, the speed in which *H. diversicolor* burrowed into the sediment was observed so that comparisons could be made between contaminant concentrations. Burrowing behaviour was also observed between worms before they had had time to acclimatize to the contamination and worms after they had acclimated.

3.2.3. Hypotheses.

The hypothesis (Hₐ) for the experiment detecting sub lethal behavioural responses was: there will be a difference in burrowing speed of *H. diversicolor* exposed to different copper concentrations.

The null hypothesis (Hₒ) was that there would be no difference in the burrowing speed of *H. diversicolor* exposed to different copper concentrations.
3.3. Methodology.

3.3.1. Field methods and storage.

Individual *H. diversicolor* were collected from the Humber estuary at Paull, a site situated halfway along the Humber estuary on the north bank (53.7231, -0.235109) in May 2011. This site was chosen because it was known that *H. diversicolor* were abundant and relatively unaffected by industry compared to other areas of the Humber (Mazik 2004). *H. diversicolor* were collected at low water by hand from a point approximately 6 metres from the bank. They were transported in sealable buckets containing sediment from their natural habitat. Additional buckets of sediment were also collected to be used in creating a mesocosm environment. *H. diversicolor* were kept for six days before being used in burrowing trials and were stored in a cold room set to 11°C in their native sediment with a small amount of artificial sea water at a salinity of 15 covering the sediment; this water was changed daily.

3.3.2. Creating the mesocosm environment.

As many naturally occurring variables from the *H. diversicolor*’s natural environment were replicated in the experimental mesocosm as is reasonably possible. Artificial seawater was made using tap water that had been aerated and dechlorinated and instant ocean sea salt. Tap water was kept in the cold room for 24 hours at 11°C with air stones; this allowed it to aerate and dechlorinate. After 24 hours instant ocean sea salt was added until the water had a salinity of 15, this salinity was selected because it matched the salinity of the water at Paull. A salinity refractometer was used to measure salinity.

Sediment from the collection site of *H. diversicolor* was homogenized by forcing it through a 1mm sieve to remove any larger particles and any additional large biota which may already be present in the sediment. This sediment was then split into 12 smaller containers so that the base of the container had a 5cm layer of homogenized sediment. This sediment was then left to settle overnight at 11°C. Artificial saline water was also made up but was also intentionally contaminated with CuCl$_2$·2H$_2$O at the following concentrations 0µg/L, 300µg/L, 600µg/L and 1200µg/L. These concentrations were chosen after examining both naturally occurring copper contamination and copper contamination previously used in mesocosm environments in the literature (see table 5). 120ml of this contaminated saline solution was then added to the sediment lined bucket so that for each concentration of contaminated saline water there were three repeat conditions. Contaminated water was changed on a daily basis. The set up of conditions and replicates may be seen in figure 10.
3.3.3. Burrowing trials.

Two sets of burrowing trials were run in the contaminated mesocosm described section 3.3.2. The first of these was an acclimatisation trial in which un-acclimatized worms were exposed to copper contaminated water and their burrowing behaviour observed. Worms were acclimated so that behaviour could be observed and compared between pre- and post-exposed worms. The second trial examined burrowing behaviour in worms that had been acclimatised.

In the acclimatisation trial worms were picked from the sediment rinsed in saline water and placed in one of the containers. Ten worms were added to each container and the number that had completely burrowed was counted after 15, 45, 75, 135, 255, 375 and 495 minutes. These worms were monitored over the next four days at 4 hour intervals to determine if they emerged from the sediment. After seven days worms were considered to be acclimatised to the copper contaminated water and could therefore be submitted for the second burrowing trial.

Worms from the first burrowing trial were once again picked from the sediment, rinsed with saline water and placed in a second contaminated mesocosm which was set up again as
described in part 3.2.3. The previously described methodology for measuring burrowing speed in worms was then repeated using the same individuals as before. Data were analysed using a combination of analysis of variance, analysis of covariance and Probit analysis. All statistical analyses were performed using the SPSS statistical software. Differences were considered significant if p<0.05.

3.4. Results.

3.4.1. Data from pre acclimatization trials.

The results from burrowing trials in pre-acclimatized worms show that the majority of *H. diversicolor* had completely submerged themselves in the first 15 minutes regardless of the concentration of copper they were exposed to (Figure 11). Control *H. diversicolor* were the fastest to submerge themselves, yet all *H. diversicolor* across all conditions had completely submerged themselves by 375 minutes. Some of the worms in the 600µl condition do not follow the general trend shown in figure 11. However this is a small minority and may be considered an anomaly. The large error bars indicate a high degree of variation early on in the experiment however they reduced in size as the trials proceeded. Across all repeats and conditions one worm was not recovered at the end of the trial and was presumed dead.

A two way analysis of variance (ANOVA) was performed on the data from pre acclimatization trials. ANOVA was used to test for differences caused by exposure and time on the numbers of *H. diversicolor* submerged. The ANOVA showed that time was the only variable to cause a significant difference in the number of *H. diversicolor* burrowed (f =5.126, df =6, p= <0.001). Exposure was shown to have no significant effect on the number of *H. diversicolor* burrowed in each condition (f =1.598, df =3, p = 0.2). When assessing the combined impact of time and exposure in an ANOVA the results showed that there was no significant impact on burrowing (f =0.498, df=18, p=0.948). An analysis of covariance (ANCOVA) was then performed. This test was used to remove any statistical influence from time as a variant. The ANCOVA showed that there was no significant effect from exposure on *H. diversicolor* in this trial (f =1.702, df =3, p =0.173).
Figure 11: Burrowing rates in groups of pre-acclimatized *H. diversicolor* from different copper exposure conditions at the various time points. Error bars demonstrate a 95% confidence limit.

Following this a probit analysis was performed and plotted so that the ET-50 (relating to the time variable) and EC-50 (relating to the concentration variable) values could be examined. Both ET-50 and EC-50 values indicate the level at which 50% of individuals responded to either time or concentration. For example ET-50 indicates the time at which 50% of individuals would respond at each concentration, and EC-50 indicates the concentration at which 50% of individuals would respond at each time point. In the pre-acclimatization trial ET-50 increased with concentration (Figure 12) and EC-50 values increased as time increased (Figure 13). Given that the response measured was time taken to burrow these results can be interpreted in the following way. ET-50 values indicate that the higher the concentration the longer it takes for individuals to burrow, while EC-50 values show that the longer individuals are exposed the higher the contaminant concentration must be for individuals to burrow. However these figures were developed by back extrapolating the existing data and can therefore not be reliably used. This was because when the first data points were gathered over 50% of the worms had already burrowed so the only way EC-50 and ET-50 values could be calculated was to back extrapolate the data.
Figure 12: changes of ET-50 values over an increasing contaminant concentration range in the pre acclimatization trial.

Figure 13: changes in EC-50 values with an increasing time range in the pre acclimatization trial.

3.4.2. Data from post acclimatization trials.

In post acclimatization trials *H. diversicolor* exposed to higher concentrations of copper were faster to burrow. The control worms were the slowest to burrow. At no time point did 100
percent of the worms burrow (Figure 14). Error bars are wide which would indicate a high degree of variation among replicates. Following this trial between 40 and 50 percent of the worms were recovered the rest were presumed dead.

Once again, ANOVA tests were performed on the data from post acclimatization trials. This analysis showed that individually both time and exposure had a significant impact on the burrowing of *H. diversicolor* at a 0.05 significance level (time ($f = 98.238, df = 6, p > 0.01$) exposure ($f = 36.104, df = 3, p > 0.01$)). However when looking at the combined impact of time and exposure there was no significant impact on burrowing ($f = 1.690, df = 18, p = 0.069$). An analysis of covariance (ANCOVA) was then performed to determine whether the effects of exposure were significant if the effects of time were removed. The results from the ANCOVA showed that exposure had a significant effect on burrowing even after the effects of time had been removed ($f = 18.241, df = 3, p > 0.01$).

![Figure 14: Burrowing rates in groups of post acclimatized *H. diversicolor* from different copper exposures at various time points. Error bars demonstrate a 95% confidence limit.](image)

Once again a probit analysis was performed for this trial. In this case the ET-50 values decreased with increasing concentration (Figure 15) the response measured were time taken to burrow. This would indicate that the higher the contaminant concentration the faster the response time (burrowing speed). EC-50 values also decreased with increasing time (Figure 16). This shows that the longer the worms were exposed the smaller the contaminant
concentration needed to be before they responded (burrowed). These figures may be considered more reliable than those calculated for the pre acclimatization trials because they were not back extrapolated.

Figure 15: changes of ET-50 values over an increasing contaminant concentration range in the post acclimatization trial.

Figure 16: changes in EC-50 values with an increasing time range in the post acclimatization trial.
3.4.3. Comparing pre and post acclimatization trials.

When comparing pre- and post-acclimatization trials it is apparent that *H. diversicolor* in all conditions were slower to completely submerge themselves (Figure 17). However while in pre-acclimatization trials control worms were fastest to burrow and worms from higher concentrations were slower. In post acclimatization trials the trend is reversed. Post-acclimatization trials showed that worms in higher concentrations were fastest to burrow and control worms were slowest. There is also a high difference in mortality between the two trials, only one death was recorded in the pre-acclimatization trials where as 50 to 60 percent of worms across all repeats and conditions died in the post acclimatization trials. ANOVA tests were also performed to assess for differences at each time point between pre and post acclimatization trials. At every individual time point there was a significant difference between number of worms burrowed from pre and post acclimatization trials ($p=>0.01$).

![Figure 17: comparing data from pre and post acclimatization trials in terms of the percentage of worms burrowed at each time point from each exposure.](chart.png)
3.5. Discussion.

3.5.1. Overview and analysis of results.

The pre-acclimatization trials showed no significant effect of copper exposure on burrowing rate. Most worms had completely burrowed in the first 15 minutes of the trial. However although not significantly different from other conditions, worms in the 1200µl/L copper condition were slower to burrow. These data show that copper had no significant effect on the burrowing behaviour of *H. diversicolor* and the only significant variable in this trial was time. The probit analysis for these data was considered unreliable because it was extrapolated. It shall therefore not be discussed in any further detail here. If this trial was to be repeated burrowing would be observed at earlier and smaller time intervals because most worms had already burrowed by the 15 minute time interval.

In contrast post-acclimatization data showed a significant effect for copper exposure on burrowing rate. Worms exposed to high concentrations of copper (1200µl/L) were significantly faster to burrow then worms from other conditions. Although it is of note that only the water was contaminated not the sediment. It may be that having sensed the copper the worms burrowed in an attempt to escape contamination. These data could therefore indicate an escape response. However post-acclimatized worms from all conditions were significantly slower to burrow then they had been in the pre-acclimatization trial; surprisingly control worms were the slowest to burrow. This along with the unprecedented rate of mortality observed in the post acclimatization trial would indicate the presence of a confounding variable; control worms were expected to have burrowed at the same speed both before and after acclimatization and mortality rates should have been equal between the two trials. One theory for the cause of this is the effect of experimentally induced stress. Although measures were taken to match the mesocosm environment to the worm’s natural environment, the mesocosm will never be a true replication of the natural environment. Several variables from the natural environment were not replicated in the mesocosm. Tidal movements were not simulated and pH, light brightness and water oxygen levels were not monitored in the mesocosm environment. These variables would be monitored should the test be repeated. Test worms were also not provided with additional food because it was thought that the sediment they were in would contain enough biota for them to feed on. When the post acclimatization trial was performed worms had been kept in the laboratory for ten days and had also been removed from their sediment three times. This may have created stress that was unrelated to copper exposure and could relate to the slow burrowing times indicated in
the control worms. However, despite the behaviour in the control worms, copper does seem to have had an effect in the exposure conditions. If the changes in behaviour seen in the post-acclimatization trial were entirely due to confounding environmental variables then burrowing speeds across all conditions would not be significantly different, as in the pre-acclimatization trial, all worms would have been exposed to the same confounding variables. The post-acclimatization probit analysis also supports this as both ET-50 and EC-50 values decrease with either concentration or time (Figures 15 and 16 respectively). The ET-50 values indicate that the higher the contaminant concentration the faster the response time, while the EC-50 values would suggest that the longer the worms were exposed to copper the smaller the copper concentration would have to be for 50% of them to respond. So effectively the higher the contaminant concentration the lower the tolerated exposure time and the longer the worms were exposed the less copper they could tolerate.

It has been observed previously that *H. diversicolor* exposed to copper are slower to burrow then worms in copper-clean uncontaminated environments (Bonnard et al., 2009). However the literature also suggests that tolerance will develop in *H. diversicolor* exposed to smaller copper concentrations and that they would, given time, burrow as effectively as unexposed worms (Bonnard et al., 2009). In the present study the reverse has happened. It seems likely that *H. diversicolor* in higher exposures burrowed faster due to an escape response. They sense toxicants in the water and burrow in the hope of escaping it. This only protect them for a short while unless the toxicant had a high mobility and low half life, *H. diversicolor* have to irrigate their burrows and this would draw contaminant into it unless it had already dispersed. Only the artificial sea water was spiked, not the sediment, so it may have been that in high exposures *H. diversicolor* burrowed immediately to shelter from the contamination in the sediment. However this does not explain why even worms in the negative control were significantly slower to burrow in the post-acclimatization trial when compared to the pre-acclimatization trial. It may be that the *H. diversicolor* were handled too much in setting up the experiment or that there were confounding variables associated with keeping the worms in laboratory conditions. This would have been the same for worms across all conditions but may explain the uniform increase in burrowing time observed in the post-acclimatization trial. Worms at higher exposures would have been handled to the same extent but may also be actively trying to escape copper contamination, while control worms would not. It may be of interest in the future to spike the sediment with copper as well as the water to observe the effect of a fully contaminated environment on *H. diversicolor*. It may also be useful to examine
the potentially confounding effects of laboratory environments on test organisms in a quantitative manor.

3.5.2. Conclusions.

1) Copper has a significant impact on the burrowing speeds of acclimatized *H. diversicolor* but not pre acclimatized *H. diversicolor*.

2) Laboratory procedures appear to have significant confounding effects on the behavioural responses of *H. diversicolor*.

3) Higher copper doses appear to initiate an escape response in *H. diversicolor* which make them burrow at faster rates than *H. diversicolor* exposed to lower doses.
Chapter 4: Development of a semi-quantitative PCR assay of AChE mRNA expression

4.1. Introduction.

The work featured in chapters 2 and 3 described the partial cDNA sequence for AChE and 18s rRNA and the generation of experimentally copper exposed *H. diversicolor* tissue. This chapter extends the work from chapters 2 and 3. A semi quantitative PCR assay was developed to analyse AChE mRNA expression (relative to an internal control gene, 18S rRNA) in worm tissue from different copper exposures.

An aim of this study was to use fully quantitative PCR techniques to detect variations in gene expression in copper exposed worms. However, complications with primers, equipment and time restrictions meant that an alternative semi-quantitative PCR approach was adopted. This approach can be used to compare levels of gene expression in organisms from different conditions but data are not fully quantitative so cannot be statistically tested; it provides information regarding possible trends only. Semi-quantitative PCR involves amplifying a reference gene (such as 18s rRNA used herein) and the gene of interest (in this case AChE) in the same PCR reaction. The theory being that the gene regulation of the reference gene should change only minimally regardless of the experimental conditioning of the organism yet the gene of interest varies. Changes can then be detected using gel imaging of PCR products and described qualitatively.

4.2. Specific aims objectives and hypotheses.

4.2.1. Aims.

1) To explore the potential changes in AChE mRNA expression in copper exposed *H. diversicolor*.

2) To gather data relating to AChE gene expression that may allow access to a potential relationship between burrowing and AChE.

4.2.2. Objectives.

In order to meet the aims set out for this chapter a semi-quantitative PCR was used. This would allow AChE mRNA expression to be subjectively measured in worms from different copper exposures and compared to control worm AChE expression.
4.2.3. Hypotheses.

The null hypothesis \( (H_0) \) for this section is:
There will be no difference in AChE mRNA expression in worms from different contaminant exposures.

The alternative hypothesis \( (H_A) \) for this section is:
There will be a difference in AChE mRNA expression in worms from different contaminant exposures.

4.3. Methodology.

4.3.1. Animals.

Two \( H. \) diversicolor organisms from each replicate condition in the behavioural trials were snap frozen in liquid nitrogen and their mRNA isolated and purified. cDNA was then made with the mRNA template using methods described in section 2.3.2 and 2.3.3.

4.3.2. Specific primer design.

Specific primers were designed based on the gene fragment for AChE and 18s shown in figures 7 and 8 in section 2.4.3. Primers were designed so that they had a high GC content (over 50%) and were more then 20 base pairs long, which are characteristics of optimally designed primers (Table 6). As in chapter 2 the primers were synthesised by Invitrogen Life Technologies (Paisley, UK) and re-suspended in molecular grade deionised water to a concentration of 50 μM.

Table 6: Specific primers designed for use in the semi quantitative PCR.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence(5’–3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
<td></td>
</tr>
<tr>
<td>Specific AChE</td>
<td>AACCCCACCACAAGAGTCAG</td>
</tr>
<tr>
<td>Specific 18s</td>
<td>GCTCCCCTCTCGGAATCGAACC</td>
</tr>
<tr>
<td>Reverse</td>
<td></td>
</tr>
<tr>
<td>Specific AChE</td>
<td>CTGTAGAAGCCTCCCCCATA</td>
</tr>
<tr>
<td>Specific 18s</td>
<td>CGGCCCCTCCTCCACTCTTGG</td>
</tr>
</tbody>
</table>

4.3.3. Semi quantitative PCR.

Again all the reactions were prepared using autoclaved tubes and autoclaved disposable pipette tips in order to avoid contamination of the samples with foreign DNA. Each reaction was performed in a final reaction volume of 50µl. This consisted of 5µl of 10x cDNA PCR
reaction buffer (400mM Tricine-KOH, 150mM KOAc, 30mM Mg(OAc)$_2$, 37.5µg/ml bovine serum albumin) (Clontech, Saint-Germain-en-Laye, France), 1µl of PCR grade nucleotide mix (containing dATP, dCTP, dGTP and dTTP at 10 mM each) (ROCHE, Burgess Hill, UK), 1 µl of forward and reverse primer for both 18s and AChE (50 µM), 1µl of 50X Advantage cDNA polymerase mix (containing Klen Taq-1DNA polymerase, Taq start antibody (1.1µg/µl), glycerol, 40mM Tris-HCL, 50mM KCL, 25mM (NH$_4$)$_2$ SO$_4$, 0.1mM EDTA, 5.0mM β-mercaptoethanol and thesit) (Clontech), 2µl of cDNA and 37µl of dH$_2$O. A negative control was also set up adjusted to these reactions which contained all the above ingredients except cDNA, instead an additional 2µl of dH$_2$O was added.

Amplifications were again carried out using a Techne Thermal Cycler. All reactions were denatured at 95°C for 2 minutes and then went through 35 cycles of 94°C for 30 seconds, 50°C for 30 seconds and 72°C for 30 seconds. Then there was a final elongation step at 73°C for 5 minutes the samples were then held at 4°C and stored at -20°C.

The conditions above, along with the new DNA taq polymerase and buffer, were selected after several trials because they optimize DNA amplification.

4.3.4. Agarose gel electrophoresis of DNA

A 0.8% agarose gel was prepared using 100ml of 1X TBE electrophoresis buffer (Tris-borate, EDTA buffer concentrate, 0.89M Tris base, 0.89M Boric acid and 0.02M EDTA) (Fisher Scientific Loughborough, UK), 0.8g of agarose (Fisher Scientific) and 8µl of sybr®safe (Invitrogen Life Technologies). The agarose was then poured into the holding tray ensuring that the teeth of the Teflon comb were immersed and allowed to set for approximate 30 minutes at room temperature prior to removal of the comb and submerging into the electrophoresis buffer in the tank filled with 1X TBE electrophoresis buffer (Tris-borate EDTA buffer concentrate) (Fisher scientific). Once again 15µl of PCR product was mixed with 2µl of loading dye (New England Biolabs, Hitchin, UK) and then loaded into the gel. 5µl of 100bp molecular weight ladder (New England Biolabs) was also added. A current of 70V was then applied to the gel. Gels were examined on a UV transluminator (Syngene, Cambridge, UK) and photographed using Genesnap software (Syngene).
4.4. Results.

4.4.1. PCR bands.

The semi quantitative PCR shows that the larger and more prominent band (approximately 200bp) is 18s and the smaller band beneath is AChE (approximately 300bp) (figure 18). The AChE band gets fainter in worms exposed to higher concentrations of copper. This suggests that AChE is down-regulated in worms exposed to higher degrees of copper contamination. However, the reference gene is also reduced at the highest levels of copper exposure (1200µg/L). In a quantitative PCR any effect on 18s would be subtracted from effects on AChE because it probably occurred due to differences in enzyme activity in the reaction rather than copper exposure. There is also a small amount of contamination which in the form of two faint bands can be seen in the negative control (Figure 18). However because the bands do not continue through all the wells and time was limited the PCR was not run again to remove the contamination.

Figure 18: An image of a 0.8% agarose gel taken under UV light showing changes in 18s and AChE regulation at different copper exposures. The larger of the two bands shown is 18s and the smaller AChE. The cDNA of two worms from each repeat exposure condition were used.
4.5. Discussion.

The results (figure 18) indicate that there is a possible reduction in AChE mRNA expression (relative to an internal reference gene) in *H. diversicolor* exposed to higher concentrations of copper. This may suggest that the AChE gene is being switched off in worms exposed to copper. If this was the case, it would mean that there is less AChE protein being produced, less AChE would in turn mean that acetyl choline (ACh) is not being broken down in the post synaptic cleft as effectively (Silverthorn., 2007). With ACh not being broken down a nerve impulse will be constantly firing in the post synaptic cleft which could be seen as constant but potentially disorganised movement in the worm (Lehtonen et al., 2003). However the semi quantitative PCR also showed a decrease in the 18s gene in worms exposed to higher concentrations of copper. 18s is a reference gene and is unlikely to be effected by environmental changes. Therefore changes in 18s regulation shown in the gel are probably caused by changes in taq polymerase activity in the PCR reaction. Due to the semi quantitative nature of the test it is impossible to measure statistical significance without relying entirely on subjective techniques. Hence it is difficult to measure the statistical difference between 18s and AChE expression across worms from different exposures. However when subjectively analysing figure 18, it would appear that the AChE band is reduced to a greater extent than the 18s across worms from different exposures. It can therefore be accepted that copper exposure reduces AChE regulation in *H. diversicolor*. Despite this apparent positive conclusion to the effects of copper on AChE further work is necessary to add confirmation. Due to lack of time this study did not examine the enzymatic activity of AChE or actual AChE protein concentration in *H. diversicolor* from different copper exposures. AChE has been previously reported to be a highly active enzyme (Divir et al., 2010). Activity has also been reported to fluctuate in accordance with ambient contamination (Scaps et al., 1997, Bonnard et al., 2009; Durou et al., 2007a). Bonnard et al. (2009) found that AChE activity significantly increased in *H. diversicolor* exposed to copper. It may therefore transpire that although the AChE gene is down-regulated in worms exposed to higher copper concentrations the activity of AChE increases to maintain a homeostatic balance. However the interpretation of the results from Bonnard et al. (2009) is disputed. Kalman et al. (2009) and Durou et al. (2007a) found that AChE activity was decreased in *H. diversicolor* from multi-polluted water bodies. Evidentially the data from these studies are not directly comparable with those shown here because both Durou et al. (2007a) and Kalman et al. (2009) were observing multiple pollutants and not specifically copper. However Frasco et al. (2005) studied the effects of a number of metals (including copper) on AChE activity in vitro. They found that copper significantly inhibited the activity of AChE in vitro. As a result because
of the contrary data surrounding AChE activity it is difficult to draw any definite conclusions regarding the regulation and activity of AChE in the presence of copper in *H. diversicolor*. This study indicates that down-regulation of AChE translation which may lead to a reduction in AChE concentration in the organism. However other studies have indicated increases in the enzymatic activity of AChE which are not addressed here. This means that the results found by this study are not representative of the entire relationship between copper and AChE.

4.6. Conclusions.

1) The AChE mRNA appears to be down-regulated in worms exposed to high concentrations of copper.
2) However the reduction of mRNA does not necessarily mean that there is less net AChE activity throughout the worms because Bonnard et al. (2009) indicated an increase in enzyme activity.
3) Further analysis with regards to AChE protein concentration and activity should be made alongside optimization of qPCR techniques to determine the true effects of copper on AChE.
Chapter 5: General discussion.

5.1. Overview of results.

The main aim of this study is to establish potential connections between the biological effects of copper exposure at two different levels of biological organisation: molecular and whole organisms as shown by a behavioural response. This chapter synthesises the work and also includes a critical appraisal of the research behind this study as well as highlighting areas that require further work.

An AChE mRNA gene fragment has been isolated and sequenced; so that a semi-quantitative PCR could be carried out. The semi quantitative PCR showed that the gene coding for AChE was down-regulated at higher copper doses (1200µg/l). The effects of copper exposure on the burrowing behaviour of worms were also observed. The post-acclimatized worms in higher copper exposures (1200µg/l) were faster to burrow. A summary of the toxicity results (Figure 19), shows that the greatest percentages of burrowed worms were found at higher copper doses and later time intervals.

![Figure 19: The percentage of worms burrowed at different times and contaminant concentrations.](image)

As AChE mRNA expression was only assessed using a semi-quantitative analysis it is not possible to statistically determine whether a quantitative relationship between AChE and burrowing exists. However by comparing the data obtained here it would appear that at higher...
copper doses (1200µg/l) AChE is down-regulated and burrowing is faster. Given the chemical pathways in which AChE is involved, a correlation between the two is logical as follows. AChE breaks down the neurotransmitter acetylcholine in the synaptic cleft therefore preventing acetylcholine from binding to its post synaptic receptor and in doing so stopping the post synaptic neurone from continually being activated (Silverthorn., 2007, Lehtonen et al., 2003). This activity has been demonstrated in many organisms, including worms (Scaps et al., 1996, Bonnard et al., 2009). If AChE concentration is then reduced, as the data suggest, acetylcholine would not be cleaved so effectively and neural transmissions may fire with greater frequency. This may in turn lead to a greater rate of burrowing.

This study is the first to use *H. diversicolor* as a test organism to examine AChE mRNA expression. Other studies have, been conducted and use Ellman’s (1961) method which may be used to analyse the potential enzymatic activity of AChE. Several authors including Bonnard et al. (2009), Durou et al (2007a) and Kalman et al. (2009) have demonstrated the effective use of Ellmans (1961) method on *H. diversicolor*. While both Durou et al. (2007a) and Kalman et al (2009) studied the activity of AChE in multi-polluted waters (Seine and Loire respectively) and relatively clean waters (Authie and Bay of Bourgneuf respectively), both found that the activity of AChE was significantly reduced in *H. diversicolor* collected from the multi-polluted waters when compared to individuals from relatively clean waters. Therefore using data from the current study and the results found by Durou et al. (2007a) and Kalman et al. (2009) it appears that while AChE mRNA is down regulated, the enzymatic activity of AChE is also inhibited. Hence the AChE function in the organism is decreased; this would in turn lead to higher acetylcholine levels and theoretically more neuronal activity. However because these other studies were looking at multi-polluted ecosystems, it is difficult to directly compare the data from this study which focuses principally on copper and the data of Durou et al. (2007a) and Kalman et al. (2009). Bonnard et al. (2009) used Ellman’s (1961) method of analysis to examine AChE but were attempting to determine the specific effects of copper. Bonnard et al. (2009) observed that the enzymatic activity of AChE was significantly increased in *H. diversicolor* exposed to copper when compared to control worms. This is in contrast to the results produced in this study using mRNA expression in which down-regulation is observed. The results of Bonnard et al. (2009) may thus demonstrate an increase in the enzymatic activity, possibly due to homeostatic mechanisms. If mRNA coding for AChE is blocked or inhibited then there would be a reduction in AChE, however a potential way for the organism to rectify this issue is to increase the enzymatic activity of the AChE present and maintain homeostatic equilibrium (Figure 20). As AChE gene regulation was not measured quantitatively it is not possible to
reliably compare the present data set with those of Bonnard et al. (2009) to determine whether the increase in AChE activity is sufficient to nullify the decrease in AChE mRNA regulation. The data pertaining to AChE activity in the presence of metals across the literature are inconclusive. Bonnard et al. (2009) found evidence of an increase in AChE activity in copper contaminated conditions. However data from in vivo and in vitro studies indicate a different outcome for AChE activity in metal exposures. Frasco et al. (2005) found that in vitro copper inhibited AChE activity. Celso et al. (2006) in contrast, found that in the mussel Perna perna, copper had no significant effect on AChE activity. Because of the high level of uncertainty over changes in the activity of AChE, and because of criticisms made of AChE analysis using Ellmans (1996) method in the presence of trace metals (Frasco et al 2005) it is difficult to relate the potential outcomes of these molecular data to the burrowing behaviour observed in this study. The burrowing data may also be compared to data from a number of other studies. Kalman et al. (2009) observed that H. diversicolor from relatively clean natural environments were slower to burrow when exposed to sediment collected from multi-contaminated marine sites. However as the worms in that study were exposed to multiple pollutants not specifically copper it is not valid to make an accurate comparison between the data from this study and the data from Kalman et al. (2009). However Bonnard et al. (2009) found that H. diversicolor exposed to higher copper doses (50, 100, 150 µg Cu 1⁻¹) were slower to burrow than control individuals. This also contrasts with the present study although the copper concentrations used by Bonnard et al. (2009) were lower than those used in the present study. It therefore seems likely that in the present study the high copper concentrations present in the water initiated an escape response in H. diversicolor which meant they rapidly burrowed into the comparatively clean sediment.

When combining the data found in this study with those indicated by other studies in the literature it is possible to extrapolate four potential pathways the copper, AChE and burrowing relationship could follow, these are shown in a conceptual modal (Figure 20). If copper was released into a surface waters this study indicates that in H. diversicolor there would be a decrease in AChE mRNA expression. There are no other studies to date that contradict this. However there is some confusion in the literature as to changes in the enzymatic activity of AChE in copper exposed organisms. If AChE increases in activity as Bonnard et al. (2009) suggest it may nullify the effects of the reduction in AChE mRNA expression and maintain homeostatic equilibrium or if activity increases sufficiently there will be a decrease in neuronal activity. This may then lead to a decrease in burrowing which have adverse effects on the organism leading to it suffering further contaminant exposure and increasing the risk to the
organism of predation and wave damage. Alternatively if the enzymatic activity of AChE decreases as data from Frasco et al. (2005) and Kalman et al. (2009) appear to indicate there will be an increase in neuronal activity. However this may not necessarily lead to organised locomotion. There is evidence to suggest that continual neuronal stimulation will lead to tetanus, paralysis and eventual death (Lehtonen et al., 2003). However logically a decrease in the enzymatic activity of AChE may result in faster burrowing behaviour which would lead to a temporary escape from water borne contamination.
Figure 20, A conceptual model showing the way in which the results found here link with those from Bonnard et al. (2009), Frasco et al. (2005) Kalman et al. (2009) and Lehtonen et al. (2003). Question marks indicate theoretical pathways that need further research to confirm.
5.2. Critique and further work.

There are a number of potential issues relating to the molecular analysis. The first of these refers to the semi quantitative nature of the PCR used to measure AChE mRNA regulation, as this test is not fully quantitative it cannot be statistically tested. As a result all analyses can only be obtained subjectively. There are also issues with the breadth of this study as actual concentrations and enzymatic activity of AChE were not measured. There is conflicting evidence with regards to the enzymatic activity of AChE in copper exposed organisms so to fully understand the impact of copper on AChE it is would be necessary to use an assay such as that developed by Ellman to examine the enzymatic activity. However the use of Ellman’s method in the presence of metals has been criticised because of potential interference from metals binding to buffers and reaction agents which obscures the final results (Frasco et al., 2005).

Given more time, the results from the molecular work could also be extended by applying quantitative PCR techniques to the AChE sequence, as well as the other genes of interest (such as those coding for metallothionein and GST). This would provide fully quantifiable and statistically testable data which could be compared to results from burrowing trials. It would also be beneficial to continue the isolation and sequencing work with MT and GST and observe any fluctuations gene expression using qPCR techniques. Because of their protective roles against trace metal contamination it would be valuable to examine the gene expression relating to MT and GST in comparison to environmental contaminant thresholds. Finally it would also be of value to examine potential changes in enzymatic activity of AChE and GST using either Ellmans (1961) method or Habig’s (1974) method. Once performed a quantitative real time PCR could be used in conjunction with Ellman’s (1961) or Habig’s (1974) technique to show the full behavioural profile of AChE in copper exposed _H. diversicolor_. These techniques when combined could show rates of production and activity of AChE.

The burrowing trials can be improved on a number of points. Worms in the control conditions were expected to show no changes in burrowing behaviour between pre- and post-acclimatization because they were not exposed to copper. However they were significantly slower to burrow post acclimatization, as were worms in all conditions when compared with their pre acclimatized counterparts. The differences observed between pre- and post-acclimatisation trials may be a due to toxic effects accumulating in the worms or because toxicity takes a specific period of time before manifesting as a recognisable behavioural trait. However it could also be due to stress induced from handling and artificial housing environments. Despite this confounding factor there is still a significant trend demonstrating
that worms in higher copper exposures were faster to burrow than those in lower exposures. This is thought to demonstrate an escape response because only the water in the mesocosm was contaminated and the sediment would have been comparatively clean. Although other variables are affecting the worms behaviour there is still a measurable copper-induced effect on the burrowing speed of *H. diversicolor*.

There are several aspects of the burrowing trials that could be improved and/or extended. In the behavioural analysis only the water was contaminated rather than the sediment whereas in the *H. diversicolor* natural environmental, contaminants would be present in the sediment as well as the water. In order to artificially contaminate sediment, acid-washed sand would have to be used instead of natural sediment. However this in turn would increase uncertainty because it would be an additional artificial variant in the mesocosm environment which may produce experimentally-induced stress in the test organisms. If acid-washed sand were to be used, a control in natural sediment should be run at the same time so that changes caused by sediment or sand may be evaluated. Further areas of study could also involve examining potential effects on the physiology and morphology of *H. diversicolor*. Chapter 1 describes a range of physiological and morphological observations and experiments including size and weight measurements, scope for growth and reproduction tests. These may if examined have a valid bearing on the study and further its scope to include effects at molecular, physiological and behavioural levels and when effects at one of these levels manifests at another.

### 5.3. Final conclusions and recommendations.

- The effects of copper at the molecular and behavioural levels in *H. diversicolor*, to establish a link between these two levels in *H. diversicolor* and to isolate a range of protein specific gene sequences in *H. diversicolor*.
- AChE mRNA expression decreases at higher copper exposures and burrowing speed increases.
- There was correlation between AChE mRNA expression and burrowing behaviour in *H. diversicolor*.
- A protein specific gene sequence for AChE was also successfully isolated over the duration of this study.
- This thesis has provided a starting point for further molecular analysis in *H. diversicolor* and has found evidence for a potential connection between the molecular and behavioural levels of organisation.

Following this the later recommendations can be made:-
• For confirmation, of the correlation found further research must be done. This study, although it demonstrates a reduction in mRNA expression for AChE, it is not in a quantitative format.

• Therefore the study does not examine AChE enzymatic activity. Both of these issues should be addressed before confirmation of a link between burrowing and AChE can be established.

• The behavioural burrowing analysis could also be improved to address the issue that control worms were affected by a confounding variable, although it is not clear what this variable is.
References.


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