THE UNIVERSITY OF HULL

The Effects of Plastic Related Phthalates (DEHP) upon Ragworm, *Hediste Diversicolor*

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By

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ABSTRACT

The exposure of the general public to phthalates is widespread as well as quite variable. Many human consumer products consist of a range of phthalates as ingredients of plastic materials. These include building materials, clothing, household furniture, cosmetics, pharmaceuticals, medical equipment, dentures, nutritional supplements, food packaging materials, children toys, modeling clay, waxes, glow sticks, cleaning materials, lubricants, automobile parts and also some insecticides (Schettler, 2005).

Animals are exposed in their natural habitats to a variety of human activities including those related to dissolved chemicals that are released into aquatic systems and may impact their fitness and survival. The responses of animals to stress, generated by these chemicals, determines their biochemical and behavioural fitness. Phthalate esters are example of such chemicals. Di-2-ethylhexyl phthalate (DEHP) and *Hediste diversicolor* were used for the purpose of this study. *Hediste diversicolor* were exposed to various low concentrations of 0.05, 2, and 10 µg/L of DEHP in long term exposures (3 months), and to high concentrations of 100, and 500 µg/L DEHP in short term exposure studies (7 days). The study aims to provide evidence that the accumulation of DEHP in *H. diversicolor* results in changes to their behaviours, specifically feeding and burrowing activity, as well as changes in their biochemical responses to phthalate driven oxidative stress via catalase and superoxide dismutase enzymes. DEHP showed different levels of persistence in seawater depending on concentrations. DEHP accumulated significantly more in *H. diversicolor* and persisted also in the sediment, while it degraded quickly in seawater. Exposure to DEHP generated behavioural impairments in *H. diversicolor* in the form of a decrease in the feeding time and an increase in the burrowing time, a typical predator escape behaviour. In addition to this stressed worm also showed an increase in two biomarkers of oxidative-stress, CAT enzymes and stability in SOD enzymes. Field samples showed fluctuations of DEHP over the year and significant differences in DEHP levels between various locations in the Humber Estuary, but generally DEHP levels are significantly higher in *H. diversicolor* followed by the sediment and seawater.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

AChE	Acetylcholinesterase
A. marina	*Arenicola marina*
AP-1	Activator Protein-1
As	Arsenic
ATP	Adenosine Triphosphate
ATSDR	Agency for Toxic Substances and Disease Registry
BBP	Benzyl butyl phthalate
BCF	Bioconcentration Factor
BOD	Biological Oxygen Demand
BOP	Butyl Octyl Phthalate
BzBP	Benzyl Butyl Phthalate
c AMP	cyclic AMP
CAT	Catalase
Cd	Cadmium
CH_4	Methane
CHO	Chinese Hamster Ovary
c GMP	cyclic GMP
Co	Cobalt
CO_2	Carbon Dioxide
CoA	Acetyl-coenzyme A
Cu	Copper
CuZnSOD	Copper Zinc Superoxide-Dismutase
Cr	Chromium
DAG	diacylglycerol
DBP	Di-butylphthalate
DDE	Dichloroethylene
DDT	Dichloro-Diphenyl-Trichloroethane
DEHP	Di-(2-Ethylhexyl) Phthalate
DEP	Diethyl Phthalate
DiBP	Di-*i*-Butyl Phthalate
DiDP	Di-*i*-Decyl Phthalate
DiNP	Di-*i*-Nonyl Phthalate
DMP	Di-Methyl Phtalate
DNA	Deoxyribonucleic Acid
DNMT	DNA methyl transferases
DNOP	Dioctyl Phthalate
D. magna	*Daphnia magna*
DOP	Dioctyl Phthalate
EC	Electron Capture
EDCs	Endocrine-Disrupting Compounds
ER	Endoplasmic Reticulum
ERA	Environmental risk assessment
ETC	Electron-Trans- Port Chain
Fe	Iron
FI	Flame Ionization
FOXO	Forkhead Homeobox Type O
FSH	Follicle-stimulating Hormone
GC	Gas Chromatography

GPX	Glutathione Peroxidase
GSH	Glutathione
GSI	Gonadal Somatic Index
H^+	Hydrogen ion
H. diversicolor	*Hediste diversicolor*
HIF-1a	Hypoxia Inducible Factor-1a
Hg	Mercury
H_2O	Water
H_2O_2	Hydrogen Peroxide
HOMO	Highest of the Occupied Molecular Orbitals
HPLC	High Performance liquid Chromatography
HPVC	High Production Volume Chemicals
HRP	Horseradish Peroxidase
IP_3	·inositol 1,4,5-trisphosphate
L-Arg	L-Arginine
LH	luteinizing Hormone
LLE	liquid–liquid Extraction
LOD	limit of Detection
LOOH	Lipid Hydroperoxides
MBP	Mono-butyl Phthalate
MBzP	Monobenzyl Phthalate
MEHHP	Mono-(2ethyl-5-hydroxyhexyl)-Phthalate
MEHP	Mono-(2-ethylhexyl) Phthalate
MEOHP	Mono-(2-ethyl-5-oxohexyl)-Phthalate
MEP	Mono-Ethyl Phthalate
Mn	Manganese
MnSOD	Manganese Superoxide-Dismutase
MS	Mass Spectrometry
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Oxidase
NF-κB	Nuclear Factor Kappa B
NHE	Normal Hydrogen Electrode
Ni	Nickel
NO	Nitric Oxide
·NO	Primary Nitric Oxide
NOS	Nitric Oxide Synthase
Nrf2	Nuclear Factor E2-Related Factor 2
NS	Nitrosative Stress
N. virens	*Nereis virens*
O_2	Oxygen
$O_2^{·-}$	Superoxide anion
$O_2{-}$	Superoxide Radical Anion
$^1O_2$	Singlet Oxygen
·OH	· Hydroxyl radical
·OOH	Peroxyl Radical
$ONOO^-$	Peroxynitrite
OS	Oxidative Stress
p53	Tumor Suppressor 53
PA	Polyamide
PAEs	Phthalates/Phthalic Acid Esters
Pb	lead
PBDEs	Polybrominated Diphenyl Ethers
PCBs	Polychlorinated Biphenyls

PDEs	Phthalate Diesters
PE	polyethylene
PET	Terephthalate
PMEs	phthalate Monoesters
POD	Peroxidase
POPs	Persistent Organic Pollutants
PP	Polypropylene
PPARs	Peroxisome Proliferator Activators
PPARγ	Peroxisome Proliferator-Activated Receptor Gamma
PS	Polystyrene
PU	Polyurethane
PVA	Polyvinyl Alcohol
PVC	Polyvinyl Chloride
RNS	Reactive Nitrogen Species
RO$^{\cdot}$	Alkoxyl Radical
ROS	Reactive Oxygen Species
Se	Selenium
SO$_4^{\cdot}{-}$	Sulfate Radical
SOD	Superoxide Dismutase
SPE	Solid-phase Extraction
SPSS	Statistical Package for the Social Sciences
STWs	Sewage Treatment Works
TA	Terephthalic Acid
TCA	Tricarboxylic Acid
TMZ	Turbidity Maximum Zone
USEPA	U.S. Environmental Protection Agency
UV	Ultraviolet
WCOT	Wall-Coated Open-Tubular
XOx	Xanthine Oxidase
Zn	Zinc

CHAPTER 1: INTRODUCTION

1.1. PLASTIC DEBRIS

Since the 1950's, there has been an increasing abundance and ubiquity of plastic debris. Although the problem is widely recognized, it is growing fast, and even if stopped now, still it will persist for many centuries. Plastic debris accumulation reports spread rapidly and appeared in influenced taxa in various geographies and bathymetries of affected sites; and affected countries began to monitor their beaches and start clean-up operations (Barnes et al., 2009).

However, despite the magnitude of the problem, plastic accumulation assessment has lagged behind especially on the seabed (Goldberg, 1994). Dumping of rubbish at sea continued to escalate, even though it was prohibited in 1990 under the International Convention on the Prevention of Marine Pollution (MARPOL 73/78) with a view to reducing plastic debris thrown overboard from ships due to the continuous increase in global use of plastics (Barnes et al., 2009).

To assess the problem of plastic debris accurately and meaningfully, long-term and large-scale monitoring is a basic requirement throughout ranges of debris in many countries and environments, with special emphasis on the sea floor (Ryan et al., 2009; Thompson et al., 2009). It should be noted that many types of natural marine debris (e.g. pumice) continue to accumulate and float on global ocean surfaces, which is augmented by the spread of floating shells, fruits, seeds and wood. Besides, human activities such as travel on water are consistently accompanied by ocean surface littering. However, the biggest challenge in ocean surface pollution came with mass production of plastics and transportation of plastic items globally. Macroplastics (>200 mm), microplastics (>5 mm) and Nanoplastics (<100 nm) accumulation on the ocean surface have increased tremendously during the last four decades (Thompson et al., 2004; Barnes, 2005; Koelmans et al., 2015).

The value of plastics lies in their cheap, light and durable properties, there multiple uses as well as their 'throw-away' property. Although plastic degrades in ultraviolet light, the sea water haline and cooling effects mean that such degradation will take long periods of exposure (Gregory, 1999). On the other hand, as marine organisms foul relatively quickly, their debris may become protected from UV light. The persistence of plastic debris was proved by an example of an albatross that was found to have swallowed a piece of plastic that it took from a plane that exploded 60

years earlier, about 9600 kilometres away from where the albatross was found (Weiss et al., 2006). Large amounts of plastic debris are found on the sea surface in the form of plastic bags, shoes, gloves, children's toys etc. that littered the sea shores after being washed off from ships' containers (Weiss et al., 2006). Other sources of plastics debris come directly from landfill sites that are used to dump plastic items after use, and the seashore was often the place of choice for such landfills (Derraik, 2002; Barnes, 2005). Ivar do Sul and Costa (2007) conducted a study on the seashores of Central and South America where they found huge amounts of marine debris and organisms living in huge build-up of plastic debris, whilst in other places fish and other bigger sea animals created their own mega-environments on plastic debris.

1.1.1. NATURE OF PLASTIC DEBRIS

Plastics belong to the class of synthetic organic polymers, and although their beginnings have not yet crossed the century mark (Gorman, 1993) the United States of America alone, was by 1988 producing more than 30 million tons of plastics per year (O'Hara et al., 1988). The reasons behind their popularity lies in their versatile uses that make them ideal for many container and receptacle uses, in all walks of life (Hansen, 1990; Laist, 1987). They are lighter, more durable, stronger and cheaper than all other comparable materials (Laist, 1987), which makes them most suitable for producing a very broad range of goods and products.

A recent comprehensive report about the world production of plastics showed that in 2013 production exceeded 299 million tons (EPRO et al., 2015). This high production came in many forms including natural and modified natural polymers, thermoplastics, and, recently, due to growing environmental concerns, plastics that are biodegradable (Shah et al., 2008; Reddy et al., 2013).

Coupled with the resistance of many such plastic materials to degradation, this ultimately resulted in their build up in the environment resulting in many detrimental ecotoxicological consequences, especially their harmful impact on the aquatic environment (Fischer et al., 2015; Duis & Coors, 2016; Rocha-Santos & Duarte, 2015; Katsnelson, 2015; Eerkes-Medrano et al., 2015).

Fergusson (1974), who was a member of the British Plastics Federation Council as well as a Plastics Institute Fellow, said that 'plastics litter is a very small proportion of all litter and causes no harm to the environment except as an eyesore'. Such a comment not only shows how the harmful and drastic environmental effects of plastic debris were long ignored, but also point to the fact that the plastics industry also failed

to forecast the boom of plastics production. However, plastic's abundance in marine life and vast expanses of sea has made the rapid accumulation of plastic debris a no longer negligible potential hazard (Laist, 1987).

1.1.2. CHEMICAL INGREDIENTS OF PLASTICS

The main framework of plastic, the polymer, is composed of separate monomers, which have been polymerized. Additives, such as pigments and fillers, flame retardants, and stabilizers, are added to induce specific characteristics into the plastic, for instance strength, colour, and flexibility (Lithner et al., 2011; OECD 2004). During the chemical process, including usage and the disposal of waste, however, these chemicals, together with by-products, some extremely dangerous, are released (Teuten et al., 2009; Lithner et al., 2011; Oehlmann et al., 2009; Halden, 2010; Papaleo et al., 2011). It has been calculated that >50 % of plastics produced are toxic, according to the European Union and the United Nations, as reflected in their frameworks on by-products, additives, and monomer constituents (Lithner et al. 2011).

The lengthy monomer chains constituting the framework are, because of the large size of their molecules, deemed biochemically inactive (Teuten et al., 2009; Lithner et al., 2011). However, some monomers may still be toxic to human and animal health (Xu et al., 2004; Halden, 2010; Lithner et al., 2011). When polycarbonate is produced, bisphenol A may cause disturbances to the endocrine system (Crain et al., 2007; Oehlmann et al., 2009; Halden, 2010), while production of polyvinyl chloride (PVC) uses polyvinyl chloride monomer and styrene, which can result in genetic mutations and carcinogens (Xu et al., 2004; Papaleo et al., 2011; Lithner et al., 2011) causing these chemicals to be cited as noxious by the U.S. Environmental Protection Agency (USEPA) and Agency for Toxic Substances and Disease Registry (ATSDR).

Upon exposure, catalysts and by-products, initiators, and surfactants are also seen as toxic (Lithner et al., 2011). For example, endocrine interference in molluscs is caused by tributyltin Oehlmann et al., 1996), and fish development is disrupted by copper chloride Anderson et al., 1991) which are used as catalysts (Lithner et al., 2011); while methanol, and cyclohexane, included in the manufacture of 1.2-dichlorobenzene (Gowariker et al., 2003; Braun et al., 2005; Lithner et al., 2011) are known as carcinogenic (Lynge et al., 1997).

UV-stabilizers, flame-retardants, antioxidants, and plasticizers are all used as additives. Some 50% of the total weight of PVC plastics may be comprised of phthalates (Bauer & Herrmann, 1997). Some 73% of additives by volume, globally, is

required by PVC. This is not the case for other plastic types; however, polypropylene and polyethylene follow with 10% by volume, with styrenics trailing at 5%. This illustrates that additives are not evenly spread over all types of plastic (Lithner et al., 2011). Many additives are either known as toxic or thought to be hazardous, such as lead heat stabilizers and PBDEs, or polybrominated diphenyl ethers (flame retardants), and phthalate plasticizers (Oehlmann et al., 2009; Halden, 2010; Lithner et al., 2011).

Harmful by-products are also produced during manufacturing such as phthalate formation during the manufacturing cycle of polystyrene (Kwon & Castaldi, 2008; Zabaniotou & Kassidi, 2003). Residuals of these by-products are sometimes difficult to remove, and are subsequently carried over to make a constituent part of the plastic products, they then join the many chemicals that make up the contaminants cocktail that results from the accumulation of plastic debris in seawater. For this reason, the polymer type should be taken into consideration when dealing with hazards resulting from the build up of plastic debris (Bergmann et al., 2015).

1.1.3. SOURCES OF PLASTICS THAT ENTER THE MARINE ENVIRONMENT

Among the plastics that enter the marine environment, some with a clear function can be easily identified and traced, with confidence, to certain industrial and consumer sectors points of origin such as effluent treatment, fishing, shipping and tourism. Fishing net pieces are evident examples of plastics that can be traced back to the fishing industry and cotton bud sticks factories as specific sectors due to wrong disposal processes (Veiga et al., 2016).

However, many plastics that enter the marine environment cannot be directly traced back to a particular point of origin, or method or pathway of release, but to several possible points of origin and method and pathway of entry, and even geographic sources. Among these are plastic drinks bottles left by tourists on local beaches, thrown overboard from ships, or improperly disposed on land near the shores and then washed into the seawater by storm runoff. They can also enter the marine environment from rivers and streams, and as they can float, they are easily transported into other areas by water currents and winds. Thus, they may originate from several points of origin via different pathways. In   terms of shape, size and state, they may include fragments of larger plastic pieces, liquid and solid state cosmetics and abrasive additives and other products; fibers released from textiles and drained into the seawater; spillage of raw

material powders and pellets; or similar items in transit scheduled to be processed into common plastic items (Veiga et al., 2016).

Moreover, microplastic particles that make up ingredients in cleaning agents and cosmetic products can enter the marine environment released by sewage discharges directly to the sea or via rivers (Napper et al., 2015). Microplastics also make up ingredients of abrasives in shot blasting industrial processes, and can be discharged into the marine environment. Another point of origin of microplastic particles is synthetic fibers discharged from textile factories and washed into the sea. They may also be carried to the aquatic environment through sewage disposal systems (Browne et al., 2011). By virtue of tiny sizes, microplastics are not easily removed from the sewage by means of sewage treatment and thus find their way to marine environments. Even in cases where particles are removable by sewage treatment, they can still enter the aquatic environment if sewage sludge is dumped at the sea or disposed of onto the land and then carried by runoff to the sea (Zubris& Richards, 2005). Plastic pellets (the ones called "mermaids tears" or nurdles) and powders (e.g. the ones used in roto-moulding) are also carried to the marine environment as a result of loss during transport and handling. Another direct source of small plastic particles is shredded plastic waste which is released into seawater during waste processing, recycling or disposal (Veiga et al., 2016).

Finally, determining the point sources of microplastic particles is extremely difficult after having entered the open marine environment. However, assessment of the concentrations and types of potential points of origin, such as roadside storm drains and sewage outlets, may be used to assess them as actual point sources and then assess the pathways through which they enter the marine environment (Veiga et al., 2016).

1.1.4. THE THREATS OF PLASTIC DEBRIS TO MARINE BIOTA

According to Andrady and Neal (2009), the amount of plastic produced every year is growing by 5% per annum ending up forming part of the anthropogenic debris clogging the marine environment and having an adverse effect on the organisms living it (Laist, 1997; Katsanevakis, 2008; Derraik, 2002; Shomura & Yoshida, 1985). The situation has been deteriorating to the extent that it is now considered a global challenge (UNEP, 2011). Plastic fragments sometimes become less visible as they are scattered in seawater in the form of small particles but they do not actually disappear ('plastic soup') (Andrady, 2015). Plastic fragmentation may result from abiotic factors (Andrady, 2011) or from the digestion processes of marine animals (Van Franeker et al., 2011). As the

particles become smaller, they become more available to animals at the base of the food chain. The possible dangers that can result from this ingestion are what make this problem one that needs serious attention. The consequences for the humans who will eventually consume food coming from the marine environment also needs to be evaluated (UNEP, 2011; Koch & Calafat, 2009; Galloway, 2015).

Plastic pollution that has considerable impact on marine organisms results when wildlife becomes entangled in marine debris, such as jumbles of discarded fishing gear and fishing lines (Baulch & Perry, 2014; Laist, 1997). In such cases, entangled biota become unable to move, breathe or feed. Besides, many marine animals mistake the plastic parts for food and feed on it (Laist, 1997; Day et al., 1985). Even where plastic material does not necessarily kill these marine organisms, it can still have undesirable effects such as decreasing their fitness and tempering with their reproduction and their survival (McCauley & Bjorndal, 1999; Van Franeker 1985; Bjorndal et al. ,1994).

Even though most of the attention has been placed on mammals, birds, and turtles in the past, the problems caused by entanglement on other organisms such as invertebrates and fish are also starting to attract attention. Kiessling et al. (2015) identify another problem that can be caused by ingestion and entanglement; the horizontal migration of these organisms to ecosystems or vertically towards the seabed.

Regulatory agencies have identified many of the ingredients used in the manufacture of plastics as hazardous (Lithner et al., 2011; Browne et al., 2013; Rochman, 2013; USEPA, 2013; European Commission, 2014). There is increasing evidence supporting this concern, such as Lithner et al. (2011) observation that the polymerization reactions which are part of the process of manufacturing plastic are not usually completed, which leaves some residual materials that can easily be washed off the plastic. Furthermore, additives are usually loosely attached to the matrices of polymers, and often make up the major chemical pollutants that leak from plastic debris (Lithner et al., 2011; Engler, 2012).

Some plastics, such as, PVC, polycarbonate, high-impact polystyrene and polyurethane foam are composed of polymers made from monomers that have been classified as hazardous. These substances are considered to have the potential to cause cancer and also interfere with the process of reproduction (Lithner et al., 2011). Other monomers that have a negative effect on the aquatic environment include the phthalate plasticizer Benzyl butyl phthalate (BBP), m-phenylenediamine, 1, 4-dichlorobenzene, and p-phenylenediamine (Lithner et al., 2011). Not all monomers are hazardous; examples include polypropylene and polyethylene. Even with this being said, these

monomers contain some harmful additives. Some of those additives identified by Halden (2010) and Lithner et al., (2011) include flame retardants, lead heat stabilizers, polyfluorinated compounds, phthalate plasticizers, and triclosan. One of these additives is phthalates, which Grün and Blumberg (2007) report have been indicated to have an effect on the pathways used for signalling nuclear hormone receptors. Kim et al. (2002) add that this additive can also cause disruptions to the endocrine system. The dangers associated with brominated flame retardants identified by Darnerud (2003) and de Wit (2002) include a reduction in spawning success in fish, neurobehavioral development disorders in mice, and teratogenicity and thyroid hormone alterations in rats.

Other additives contain hazardous degradation products. Examples include nonylphenol, which is a known degradation product of nonylphenol ethoxylates. This is a surfactant that can cause the disruption of the endocrine in fish (Gray & Metcalfe, 1997; Seki et al., 2003; Kawahata et al., 2004) and in polychaetes (Ayoola et al., 201; García-Alonso et al., 2011).

When we try to understand the manner in which plastic debris can impact marine organisms, it is imperative that we gauge its effects at standard ambient concentrations and under standard environmental conditions of exposure (Rochman and Boxall, 2014). This view is supported by Crain et al., (2007) and Oehlmann et al., (2009) who noted that even though evidence of toxicity tends to occur at levels higher than ones that generally exist in the environment in some chemicals such as bisphenol A and phthalates. These unwanted effects can occur at concentrations which are environmentally relevant.

1.1.5. THE DEGRADATION OF PLASTIC AND PATHWAYS

When plastic degrades, it goes through a physical or chemical change in polymer triggered by factors in the environment. Examples of these factors include biological activity, light, and effects of chemicals, heat, and moisture. When the process involves making changes in the properties of the polymer, through a biological, chemical, or physical reaction that produces bond succession and results in chemical changes, the process is classified as polymer degradation. The changes related to degradation include changes that can be seen in the material properties of the plastic such as delamination, crazing, alteration in the electrical and optical characteristics, phase separation, and discolouration. Pospisil and Nespurek (1997) note that these changes can result in the creation of new functional groups, bond scission, or

transformation in the chemical structure. The degradation can be classified as either biological, photo or thermal.

There are various types of additives and polymers that can be combined when manufacturing objects that have specific characteristics and properties. Some of the well-known polymers include polyvinyl alcohol (PVA), polyethylene (PE), terephthalate (PET), polypropylene (PP), and polyamide (PA). When any of these polymers find their way into the ocean, their impact on the environment depends on a number of conditions. Some of them were identified by Wright et al. (2013b), such as the ability of the material to float, the position it takes within the column of the water, and its capacity to interact with the biota. Table 1.1 graphically represents the idea of polymer densities. Those polymers that are denser than sea water, such as PVC, will sink towards the seabed, while those lighter than the water such as PE and PP, will remain floating within the water column. Ye and Andrady (1991), and Lobelle and Cunliffe (2011) reason that processes such as the colonization and biofouling of the organisms that reside on the plastic surface make the particles heavier, which then makes it easy for them to sink to the bottom sediments. Other factors that can change the density of objects and thus their distribution include fragmentation and degradation coupled with the leaching of additives. The rate of degradation of plastic material depends on a number of variables including the type of the polymer, temperature, amount of oxygen available, the chemical additives available and other environmental factors. Andrady (2011) acknowledges that when compared to beaches, which tend to have higher temperatures than that of the water, the weathering of plastic happens at a slower rate. The plastic contends with the actions of the waves, abrasion, and grinding by the sand, leading to fragmentation and embrittlement. The results of fragmentation are the so-called microplastics (Barnes et al., 2009). Microplastics is too small to be seen by the naked eye and form powdery fragments in the water.

Table 1.1: Density range of common environmentally relevant polymers (Avio, 2016).

MATRIX	DENSITY (g/cm^3)
Distilled water	1
Sea water	1.025
Polyethylene (PE)	0.93- 0.98
Polypropylene (PP)	0.89- 0.91
Polystyrene (PS)	1.04- 1.11
Polyvinylchloride (PVC)	1.20- 1.45
Polyamide (PA)	1.13- 1.5
Polyethylene terephthalate (PET)	1.38- 1.39
Polyvinyl Alcohol (PVA)	1.19- 1.35

According to Gu et al. (2000a), the degradation process of both natural and synthetic plastic is aided by microorganisms, which include fungi and bacteria. Such biodegradation depends on the properties of these microorganisms and is active under the different conditions of the soil, as degradation is caused by microorganisms that vary in nature, with each having their different optimal growth conditions. Glass and Swift (1989) noted that polymers, particularly in plastic form, are substrates for heterotrophic microorganisms.

There are different factors that have an influence on the process of biodegradation of plastic. These include the characteristics of class of organisms, the type of pre-treatment and the polymer. The polymer characteristics responsible for degradation that were identified by Gu et al. (2000b) and Artham and Doble (2008) include mobility, additives, tacticity, substituents that exist in its structure, crystallinity and molecular weight.

The degradation process starts with the polymer being changed into monomers, which are later mineralized. The reason why the majority of polymers need to be depolymerised to monomers of smaller molecular weight and only then they are taken up and biodegraded in biological cells, is that they are initially too big to make their way through cellular membranes.

According to Swift (1997), there are a number of biological and physical forces that can lead to the initial breakdown of a polymer. Examples of these physical forces include the likes of freezing, thawing, heating, cooling, or moisture and drying. According to Kamal and Huang (1992), these different conditions cause chemical damage, which could include the cracking of the polymer material.

The degradation process can also be a result of the growth of fungi, which can result in small-scale pressure, which later leads to blowing up. This happens when polymer solids are infiltrated by the fungi (Griffin, 1980). Jun et al. (1994) and Toncheva et al. (1996) identify some synthetic polymers, like poly (caprolactone) that require microbial enzymes to be depolymerised. This process is then followed by the monomers being absorbed into the cells of the microbial where the biodegradation process takes place (Goldberg, 1995). Göpferich, (1997) identified the Abiotic hydrolysis as the most fundamental reaction that must take place for the environmental degradation of synthetic polymers to proceed. Examples of these polymers include, silicones or polydimethylsiloxanes (Xu et al., 1998), poly (ethylene terephthalate) (Heidary and Gordon, 1994), polycarboxylates (Winursito and Matsumura, 1996), and

polylactic acids and the copolymers thereof (Hiltunen et al., 1997; Nakayama et al., 1996).

It has generally been observed that the greater the molecular weight of the polymer, the more difficult is its degradation process by the microorganisms (Shah et al., 2008). It is the opposite when it comes to dimers, monomers, and oligomers of polymer repeating units as they can be mineralized and degrade easily. High molecular weights lead to considerable decreases in solubility, making microbial attack on these plastics difficult, as bacteria favour substrates that can be assimilated via the bacterial cellular membrane and subsequently degraded further by cellular enzymes. According to Doi (1990) and Gu et al. (2000b), there are basically two classes of enzymes which are actively involved when polymers are biologically degraded; intracellular depolymerizes and extracellular. In the process of degradation, complex polymers go through a process where they are broken down by exoenzymes produced by microorganisms into smaller molecules with shorter chains such as monomers, oligomers, and dimers. These are the appropriate size to pass through the outer bacterial membranes, which are semipermeable. Once inside the cells, they are then used as energy sources and carbon. This process described above is scientifically known as depolymerisation. Frazer (1994) and Hamilton et al. (1995) note that this process is known as mineralization if it produces H_2O (water), Carbon Dioxide (CO_2) or Methane (CH_4) as an end product. Atlas and Bartha (1997) and Narayan (1993) observe that the degradation and biodeterioration of a polymer does not usually reach 100% because a small part of the polymer gets incorporated into the humus, the microbial biomass, and other natural products.

The environmental conditions usually determine the degenerative pathways and principal groups of microorganisms associated with the degradation of polymers. If there is sufficient oxygen, the destruction of complex polymers is usually a result of the actions of aerobic microorganisms and leads to the production of CO_2 and H_2O as end products. When the conditions are anoxic, consortia of anaerobic microorganisms will be liable for the deterioration of the polymer. Barlaz et al. (1989) identify some of the end products of this process as microbial biomass, H_2O, CO_2, and CH_4 (Figure 1.1).
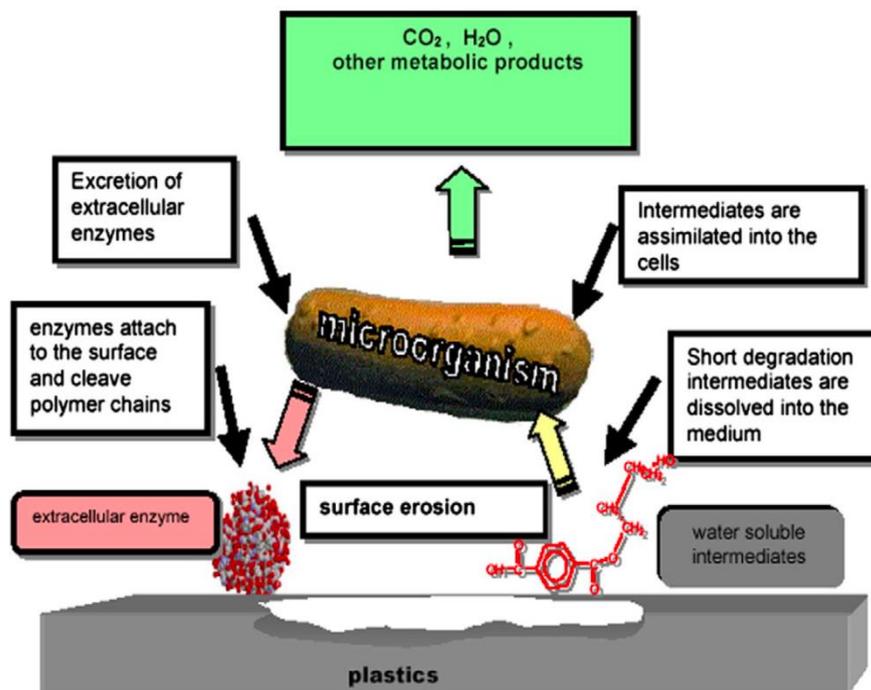
Figure 1.1: Plastic biodegradation mechanism under aerobic conditions (Mueller, 2003).

1.1.5.1. <u>The Aerobic Biodegradation Pathway</u>

A sequence of reactions, most common in microbes, is involved in the bacterial aerobic degradation of phthalate esters. In most cases, the initial step involves the cleavage of the ester linkages between aromatic ring and alkyl chains. This process involves the hydrolysation of phthalate, which turns them into phthalic acid through monoesters. Eventually, one of the key steps in the phthalate ester biodegradation process involves the mineralization of phthalic acid by the dioxygenase-catalyzed pathway. In this mineralization process, there is a difference between the Gram-positive and Gram-negative bacteria (Wen Gao & Dan Wen, 2016).

Phthalic acid is transformed into protocatechuate via 4-dihydroxyphthalate and cis-3,4-dihydro-3,4-dihydroxyphthalate for the Gram-positive bacteria (Figure 1.2). However, in the case of the Gram-negative bacteria, the mineralization process is achieved through 4,5-dihydroxyphthalate and cis-4,5-dihydro-4,5- dihydroxyphthalate. The result of this process is the formation of protocatechuate from phthalic acid. Protocatechuate is a vital intermediary metabolite of aromatic compounds. An ortho- or meta-cleavage pathway, with the assistance of ring cleavage enzymes, converts organic acid into protocatechuate, which results in the tricarboxylic acid cycle (TCA cycle). This product will eventually be converted to CO_2 and H_2O. Liang et al. (2008) have published a detailed explanation on how the enzymes lead to the degradation of

phthalates.

Anumber of aerobic phthalate ester degradation pathways have been proposed. According to Amir et al. (2005) and Wu et al. (2010a) phthalic acid can be formed via the β-oxidation of phthalate esters with long side chains. Wu et al. (2010a) note that the biodegradation of Dioctyl phthalate (DOP) by Gordonia sp. strain JDC-2 indicated that Diethyl phthalate (DEP), Butyl octyl phthalate(BOP), and Di-butyl phthalate (DBP) all appeared as intermediaries while the degradation process was proceeding. This could be an indication that the biodegradation of DOP by strain JDC-2 could happen via β-oxidation by eliminating an ethyl group each time. Additionally, phthalic acid can be formed by phthalate esters with shorter side chains such as DEP and Di-methylphtalate (DMP), in the absence of monoesters (Jackson et al., 1996).

A brief summary of what has been discussed above shows that the biodegradation of aerobic phthalate ester happens in the following manner:

1. As the length of the phthalate ester alkyl chain increases, biodegradability decreases.

2. The hydrolysis of the ester bond is usually where the majority of aerobic biodegradation of phthalate esters begins.

3. The first-order kinetics equation can be used to explain the primary biodegradation of phthalate (Carrara et al., 2011; Cheng et al., 2008; Peng & Li, 2012).

4. Environmental conditions such as the pH balance, microbial populations, temperature, and others affect the biodegradation of phthalate esters.

5. The biodegradation process is inhibited when the concentration of phthalate esters is high (Navacharoen & Vangnai, 2011).

6. The addition of a simple carbon source such as a co-metabolic substrate can boost biodegradability significantly (Yuan et al., 2010; Liao et al., 2010b; Yang et al., 2013).
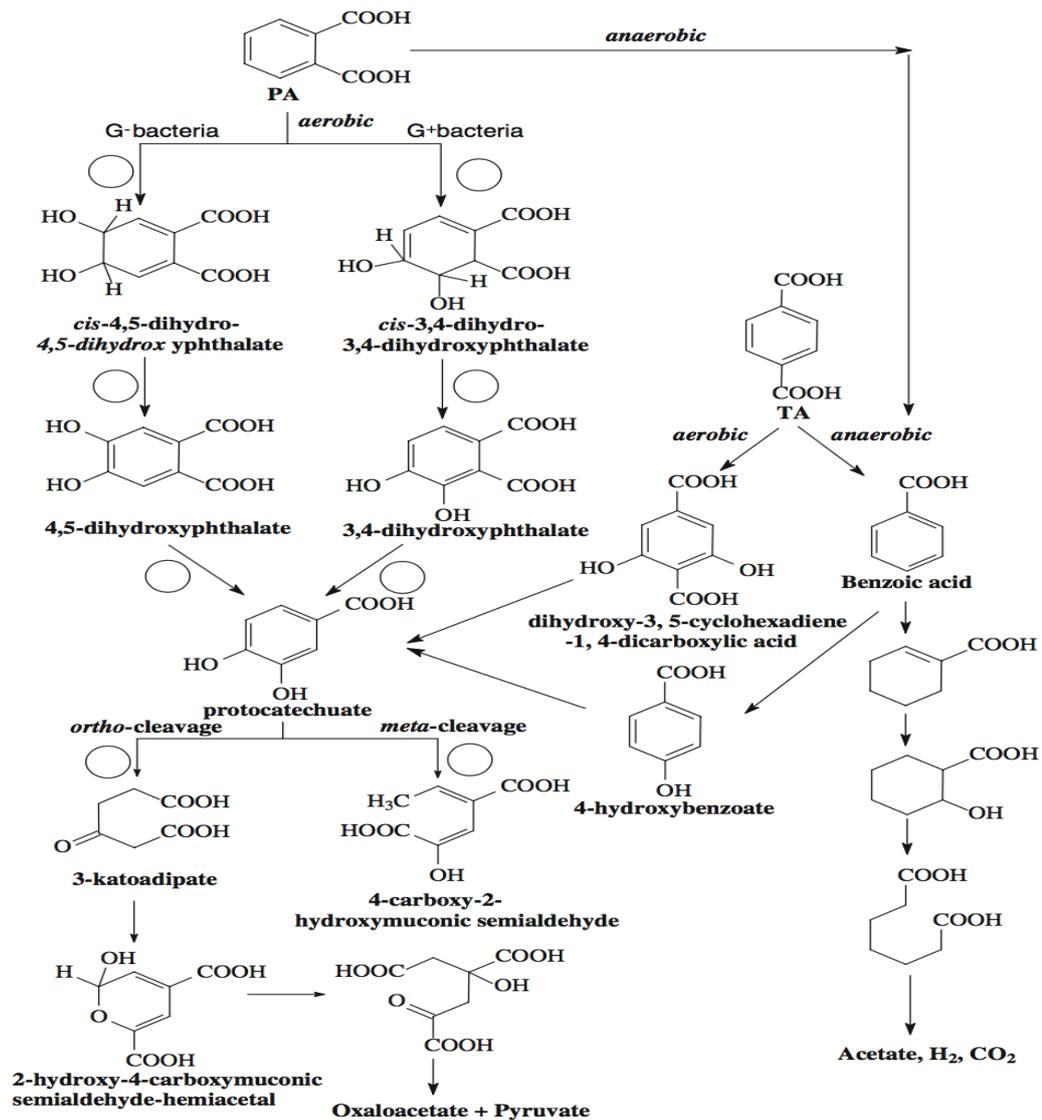
Figure 1.2: Biodegradation pathways of PA and TA via the enzymes E3 PA 4.5-dioxygenase (E.C.1.14.12.7), E4 PA 3,4-dioxygenase, E5 cis-4, 5-dihydroxy-4,5-dihydrophtha- late dehydrogenase, E6 cis-3, 4- dihydroxy-3,4-dihydrophthalate dehydrogenase, E7 4,5- dihydroxyphthalatedecarboxyl- ase (E.C. 4.1.1.55), E8 3,4-dihydroxyphthalatedecarboxyl- ase (E.C. 4.1.1.69), E9 4,5- protocatechuate dioxygenase (EC1.13.11.8), E10 3,4- protocatechuate dioxygenase (EC1.13.11.3) (Wei Liang et al., 2008).

1.1.5.2. <u>The Anaerobic Biodegradation Pathway</u>

Shelton et al. (1984) indicate that in anaerobic conditions, just as is the case in aerobic degradation, the de-esterification of phthalate esters is thought to be the preliminary pathway for degradation. Two common central intermediaries in the anaerobic mineralization of phthalate esters are monoester phthalate and phthalic acid (Liang et al., 2007). According to Kleerebezem et al. (1999c), one of the rate-limiting steps in the anaerobic phthalate ester degradation is the anoxic metabolic process of phthalic acid. Although Phthalic acid must undergo decarboxylation in order to be converted to benzoate (Nozawa & Maruyama, 1988; Liu & Chi, 2003), there is no scientific evidence showing where decarboxylation is specifically located in the

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degradation pathway (Kleerebezem et al., 1999b). The degradation of benzoate takes place through ring cleavage to acetate, hydrogen, and carbon dioxide (Kleerebezem et al., 1999b). Further steps will convert acetate to the production of methane.

Both Kleerebezem et al. (1999a) and Chang et al. (2005b) agreed that the lack of syntrophic conditions makes phthalate esters degrade anaerobically more slower than aerobically. Wang et al. (2000) also note that when compared to long-chained compounds, short-chain compounds have higher anaerobic degradation rates. Molecular phthalates, such as Di-(2-Ethylhexyl) Phthalate (DEHP), which are high molecular weight, show a generally higher toxicity to methanogenesis when they are found in higher concentrations (O'Connor et al., 1989). When the conditions are appropriate, the half-lives of DEHP, DEP, and DBP in anaerobic degradation are 25.70, 15, 40 and 9.40 days respectively (Chang et al., 2005a). According to Xia et al. (2002) the half-lives of anaerobic degradation of 9 phthalate esters including diundecyl phthalate, DMP, dinonyl phthalate, DEP, DOP, DBP, diheptyl phthalate, and diamyl phthalate, range between 81.5 to 445.5 days. However, under aerobic conditions, the half-lives of the 9 named phthalate esters were only 0.30 to 5.65 days (Xia et al., 2002, 2004, 2006; Wu et al., 2005). Chang et al. (2005a) concluded that the anaerobic degradation of DBP, DEP, and DEHP collected from river sediments can be totally biodegraded within periods of 28, 49, and 84 days respectively.

1.1.6. MOST COMMON TYPES OF PLASTIC USED

Plastic is a convenient material because it is cheap, durable and light. The production of plastic at a mass commercial level has been happening since around the 1940s. Since then, the production of plastic has been increasing rapidly (Roy &Visakh, 2015; Hofer, 2008). By 2013, the amount of plastic produced across the world had reached 299 Mt annually (2015). (PE, 2012; see Figure 1.3).

Over 33% of the plastic produced in the United States and Europe is designed to be discarded within a period of three years of production. Most of this plastic is used in disposable products including refuse bags, eating utensils, and packaging (PE, 2015; Barnes et al., 2009). The reason why plastic tends to accumulate in the environment is due to its durability, and therefore the long time it takes to degrade (Barnes et al., 2009). The increasing amounts of plastic in the environment, especially in the marine environment, are growing into an alarming issue (Gregory et al., 2009). The situation is becoming graver as the wind and water currents disperse it in the seawater, and as plastic ages it fragments and is dispersed further (Noone, 2013). This is the reason why

the plastic ends up being found all over the world in varying sizes (Barnes et al., 2009). When the size of the plastic is more than 5mm in diameter it is classified as macroplastic. Microplastic is a class of particles measuring less than 5mm in diameter (Cole, 2011).

Some additives are actually included with the aim of making the plastic mores resistant to degradation (Murphy, 2001). These additives are not properly attached to the polymer and are susceptible to leaching from the plastic into the water during the process of its degradation (Stringer & Johnston, 2001). Deanin (1975) argues that the polymers used in plastics do not generally pose a hazard. However, it needs to be noted that as the plastic ages within the marine environment, it does pose a chemical hazard. This hazard is not only a result of the release of chemicals additives leaching persistent organic pollutants (POPs), it comes about due to chemicals produced while the polymer itself is going through the process of being degraded.
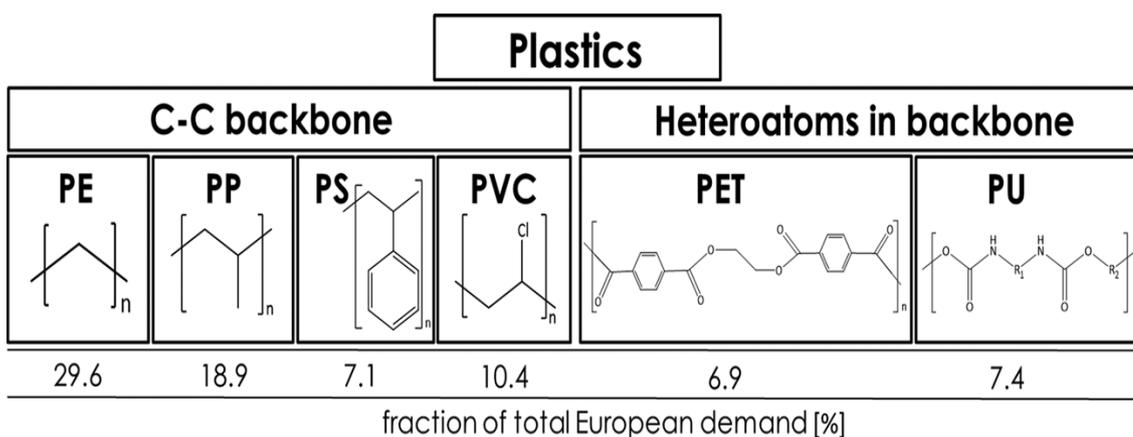
Figure 1.3: Structures and functions of different Polymer within demand in Europe (PE-polyethylene, PP-polypropylene, PS-polystyrene, PVC-poly (vinyl chloride), PET- poly (ethylene terephthalate, and PU- polyurethane) (PE, 2015).

1.1.7. PHTHALATE ESTERS (PAEs)

Phthalate esters are important chemicals that are widely used in the industry (Graham, 1973) as additives to offer flexibility to polyvinyl chloride (PVC) resins. Besides, they are used in various capacities in a host of other resins including polyvinyl acetates, polyurethanes, and cellulosics. Phthalate esters with higher molecular weights are stable, fluid and have low volatility, which are properties that make them good plasticizers. Phthalate esters with medium to high molecular weights are used in PVC manufacturing, whereas di-n-butyl phthalate ester finds its use in cellulose esters and epoxy resins as well as specialized adhesives. Dimethyl and diethyl phthalates are

usually used in the manufacturing of cellulose acetate and cellulose butyrate and other cellulose ester plastics. Plasticizers find their main use in building materials, carpets, transportation, cloth, food packaging and some medical products. Consequences of disposal of phthalate esters into the surrounding environment from industrial areas have been recently reviewed (Wams, 1987, Cadogan et al., 1993).

Since the 1960s, phthalates have been used intensively. Di-(2ethylhexyl) phthalate (DEHP) makes up 50% of the annual production of phthalic acid ester which is estimated at 1,500,000 tons. Figure 1.4 shows the chemical structure of phthalates, which is *o*-benzenedicarboxylic acid (*o*-phthalic acid) dialkylester. The different lengths of their alcohol chain isomeric structure offer them different characteristics. Table 1.2 outlines the properties of the currently existing phthalates, including their abbreviations, molecular formulas, molar masses, and CAS numbers. The following eight are the commercially significant phthalates, namely: di-methylphtalate (DMP), di-ethylphthalate (DEP), di-*i*-butylphthalate (DiBP), di-butylphthalate (DBP), benzylbutylphthalate (BzBP), di-(2-ethylhexyl) phthalate (DEHP), di-*i*nonylphthalate (DiNP) and di-*i*-decylphthalate (DiDP). The last two compounds of this list contain some isomers that have alkyl carbon numbers 9 and 10, respectively (Tienpont, 2004).
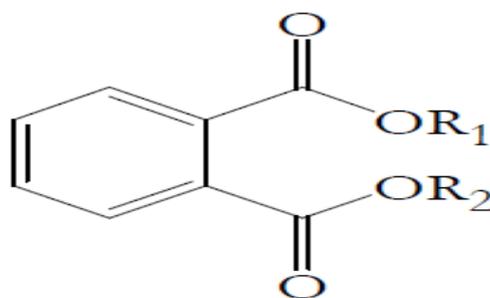
Figure 1.4: Phthalates chemical structure: Phthalic acid di-alkyl esters: R1, R2=alkyl; phthalic acid mono-esters, R1=alkyl, R2=H (Tienpont, 2004).

Low molecular weight phthalates (DEP, DMP, DBP, DiBP, BzBP), on the other hand, are used by utilizing their emulsifying properties; among these DEHP, DiDP and DiNP are added to polymers (e.g. PVC) to serve as plasticizers. As phthalates are easily detached from the products to which they have been added, they have become widespread in the environment (Tienpont, 2004). During the past 20 years, the toxicity of such phthalates has become of growing concern for academic and industrial researchers, leading to increase in the study of their acute as well as chronic toxic effects and environmental distribution (Tienpont, 2004).

Table 1.2: Phthalates List (Tienpont, 2004).

Phthalates	Formula	Abbreviation	Molar Mass	CAS- No
Single isomer phthalates				
Di-methylphthalate	C10H10O4	DMP	194.2	131-11-3
Di-ethyl phthalate	C12H14O4	DEP	222.4	84-66-2
Di-allylphthalate	C14H14O4	DalP	246.3	131-17-9
Di-(1-methylethyl)phthalate (di-*i*-propylphthalate)	C14H18O4	DiPP	250.3	605-45-8
Di-propylphthalate	C14H18O4	DPP	250.3	131-16-8
Butyl-2-methylpropylphthalate	C16H22O4	BMPP	278.4	17851-53-5
Di-butylphthalate	C16H22O4	DBP	278.4	84-74-2
Di-(2-methylpropyl)phthalate (di-*i*-butylphthalate)	C16H22O4	DiBP	278.4	84-69-5
Di (2-ethoxyethyl)phthalate	C16H22O6	DeoEP	310.4	605-54-9
Di-cyclopentylphthalate	C18H22O4	DCPeP	302.4	18699-38-2
Butylcyclohexylphthalate	C18H24O4	BCHP	304.4	84-64-0
Di-(3-methylbutyl)phthalate (di-*i*-pentylphthalate)	C18H26O4	DMBP	306.4	605-50-5
Di-pentylphthalate	C18H26O4	DPeP	306.4	131-18-0
Benzylbutylphthalate	C19H20O4	BzBP	312.4	85-68-7
Di-phenylphthalate	C20H14O4	DPhP	318.3	84-62-8
Di-cyclohexylphthalate	C20H26O4	DCHP	330.4	84-61-7
Butyl 2-ethylhexylphthalate	C20H30O4	BEHP	334.5	85-69-8
Butyloctylphthalate	C20H30O4	BOP	334.5	84-78-6
Di-(2-ethylbutyl)phthalate	C20H30O4	DEBP	334.5	7299-89-0
Di-hexylphtalate	C20H30O4	DHP	334.5	84-75-3
Di-benzylphthalate	C22H18O4	DBzP	346.3	523-31-9
Di-methylcyclohexylphthalate	C22H30O4	DMCHP	358.5	27987-25-3
Butyldecylphthalate	C22H34O4	BDcP	362.6	89-19-0
Di-heptylphthalate	C22H34O4	DHpP	362.5	3648-21-3
Di-(5-methylhexyl)phthalate (*)	C22H34O4	DMHP	362.5	41451-28-9
Benzyl 2-ethylhexylphthalate	C23H28O4	BzEHP	368.6	18750-05-5
Di-(2-ethylhexyl)phthalate	C24H38O4	DEHP	390.6	117-81-7
Di-octylphthalate	C24H38O4	DOP	390.6	117-84-0
Hexyldecylphthalate	C24H38O4	HDcP	390.6	25724-58-7
Octyldecylphthalate	C26H26O4	ODcP	418.6	119-07-3
Di-(7-methyloctyl)phthalate (*)	C26H42O4	DMOP	418.6	28553-12-0
Di-nonylphthalate	C26H42O4	DNP	418.6	84-76-4
Di-(3,3,5-trimethylhexyl)phthalate	C26H42O4	DTMHP	418.6	4628-60-8
Di-decylphthalate	C28H46O4	DDcP	446.7	84-77-5
Di-(8-methylnonyl)phthalate (*)	C28H46O4	DMNP	446.7	89-16-17
Di-undecylphthalate	C30H50O4	DUP	474.7	3648-20-2
Di-dodecylphthalate	C32H54O4	DDDP	502.8	2438-90-8
Isomeric mixture phthalates				
Di-*i*-heptylphthalate	C22H34O4	DiHpP	362.5	-
Di-*i*-nonylphthalate	C26H42O4	DiNP	418.6	-
Di-*i*-decylphthalate	C28H46O4	DiDP	446.7	-
Phthalic acid mono-esters				
Mono-methylphthalate	C9H8O4	MMP	180.2	4376-18-5
Mono-ethylphthalate	C10H10O4	MEP	194.2	2306-33-4
Mono-butylphthalate	C12H14O4	MBP	222.2	131-70-4
Mono-(2-ethylhexyl)phthalate	C16H22O4	MEHP	278.4	4376-20-9
Mono-*i*-nonylphthalate	C16H22O4	MiNP	320.4	-
Mono-*i*-decylphthalate	C16H22O4	MiDP	334.4	-

(*) Compounds found in isomeric mixtures of DiHpP, DiNP and DiDO respectively.

Chou and Wright (2006) pointed to some potential health concerns resulting from phthalate exposure: including toxicity, allergy, asthma, and carcinogenicity. Phthalates in animals were found to affect testicular function by decreasing sperm number and motility, as well as affect hormone production in both female and male animals with DEHP as the most potent phthalates tested. Studies on human urine samples found evidence of phthalates' effects on reproductive. Chou and Wright (2006) stated that some studies associated exposure to Mono-butylphthalate (MBP), Mono-ethylphthalate (MEP) and DEHP with allergy and asthma, of which some appear to result from specific chemical structures and depend on the duration and manner of exposure.

Information about phthalates' effects on reproductive systems is scanty. Studies on male workers exposed to DEHP indicated that the pregnances of their wives were slightly prolonged (Modigh et al., 2002), although pregnancy duration is not regarded as a good measurement of fertility. However, some studies found a significant inverse relationship between the concentration of Monobenzyl phthalate (MBzP) in urine and follicle-stimulating hormone (FSH), one of the reproductive hormones (Duty et al., 2005). High levels of urinary MBP interact with polychlorinated biphenyls (PCBs), the environmental toxin, and their joint exposure decreases sperm motility (Hauser et al., 2005). A study on healthy Swedish men exposed to high levels of MEP found that they suffered from decreased sperm motility and low concentrations of luteinizing hormone (LH), the reproductive hormone, but the study showed no significant effect of MBP (Jonsson et al., 2005). Other studies found some relationship between high levels of phthalate monoesters in the urine and low sperm counts, decreased sperm motility and affected deoxyribonucleic acid (DNA) integrity (Duty et al., 2003a, Duty et al., 2003b). Together these reports raise concerns regarding the effects of phthalates on male reproductive toxicity, although data on female reproductive toxicity of phthalates are relatively scanty (Jung Koo & Mu Lee, 2005). One study found a relationship between endometriosis and high concentrations of serum DEHP (Cobellis et al., 2003), while another study found a relationship between high DEHP and MEHP concentrations in cord blood and shorter gestation periods (Latini et al., 2003). Exposure of animals to moderate levels of DEHP altered the male reproductive hormone levels (Akingbemi et al., 2004; Akingbemi et al., 2001; Kurahashi et al., 2005; Ljungvall et al., 2005; Wang et al., 2005) as well as the female oestrus cycles (Davis et al., 1994;Lovekamp-Swan and Davis, 2003; O'Connor et al., 2002).

1.1.7.1.   **Phthalates Potential Impact on Human Health**

Recent Advances in engineering and materials science led to widespread and varied uses of plastics with the aim of providing cheap, strong, light, safe, more durable versatile consumer goods and products that are apt to improve people's quality of life. Plastics can be so designed as to keep foods fresh for long periods of time, provide medical treatment benefits by means of timely released pharmaceuticals and medical applications, and prevent electrical, electronic and other household articles from igniting or spreading fire (Andrady&Neal 2009; Thompson et al. 2009a,b). However, governments and public and scientific institutions are more concerned about the potential adverse health effects that the general populations are exposed to due to their frequent contact with plastic additives. The chief hypothesis advanced for these increasing concerns is that some chemicals that find use in plastics with the aim of providing good physical qualities, can sometimes work as endocrine-disrupting compounds (EDCs) that can compromise the endocrine function, which can lead to detrimental developmental and reproductive effects (NRC 1999). Environmental or occupational exposure of men to EDCs may compromise the reproductive capacity or may increase the risk of prostate or testicular cancer (Fleming et al.1999 ; Toft et al. 2004). Environmental or occupational exposure of women to these compounds may lead to endocrine changes, and thereby increase the risk of endometriosis and other cancers related to the endocrine glands, poor oocyte or ovarian function, or disrupted menstrual cycle (Nicolopoulou-Stamati&Pitsos, 2001; Pocar et al. 2003).

Early life chemical exposures can decide the individual's health in the future; hence, such exposures can have long-term effects in later life (Gluckman et al. 2008). Contact with plastic additives or other EDCs can bring about changes in the endocrine activity and thereby affect the reproductive development by means of several biological mechanisms, that can impact the hypothalamic, pituitary, and thyroid functions at different levels, ranging from impacts on hormone receptors to impacts on the synthesis, secretion and metabolism of the hormones (Boas et al. 2006; Bretveld et al. 2006).

Despite the growing concern about the impacts of plastic additives on human health, human studies that examined these impacts are still very few. The studies that examined the clinical statistical significance of some of the markers of endocrine or reproductive disruption or developmental changes, did not come up with clear findings regarding effects on the reproductive function, such as decline in the quality of semen or subclinical changes in the levels of the circulating hormones. However, although the

body of evidence for such changes is still limited, it is growing, especially the evidence related to the impacts of the environmental and occupational exposures to the EDCs including plastic additives. The benefits that may be reaped from this evidence is that these markers may be used as intermediate signals indicating that the pathway tying the environmental exposures to the clinical developmental and reproductive impacts comes through changes in the endocrine function. As large numbers of people are frequently exposed to considerable levels of several suspected or confirmed EDCs, even apparent subtle epidemiologic links may lead to great increases in endocrine-related morbidities including reproductive disease among people, which will represent a considerable public health concern (Meeker et al. 2009).

Based on the widespread use of phthalates in various consumer products including personal-care products and cosmetics, human exposure to phthalates is likewise widespread. The general routes of human exposure usually take place through inhalation and external skin contact (Adibi et al. 2003; Rudel et al. 2003). In the case of children and infants, the main routes of high phthalate exposures (e.g. to objects such as plastic toys) include dermal contact with fingers as well as mouthing. Mouthing may also take place through ingestion of phthalates found in infant formula, cow's or breast milk, or some types of food packaging (Sathyanarayana 2008). Exposure to personal-care products that contain phthalates of lower molecular weight is often very high, and has been reported by men who recently used cologne and aftershave lotions (Duty et al. 2005), as well as by the mothers of infants who   reported that they recently used some infant-care products such as shampoos, lotions and powders (Sathyanarayana et al.2008). Infused or injected liquids containing phthalates from medical equipment are also among the important sources of higher exposure to phthalates, especially to DEHP (ATSDR 2002; Green et al. 2005; Weuve et al.2006) in the case of hospital patients.

1.1.7.2.   <u>Di (2-ethylhexyl) phthalate (DEHP)</u>

Di (2-ethylhexyl) phthalate (DEHP) is a chemical usually added to plastics to make them more flexible. The compound is also known by other names such as bis (2-ethylhexyl) phthalate (BEHP). It is a colourless liquid, almost without odour. It evaporates with difficulty, and almost nothing of it is borne in the air, even vinyl materials, where it may make up to 40%, but lower levels are more common. DEHP is used in the manufacturing of many plastic products including wall coverings, floor tiles, tablecloths, shower curtains, furniture upholstery, automobile upholstery, baby pants, toys, dolls, shoes, swimming pool liners, garden hoses, packaging films and sheets,

sheathing for wires and cables, medical tubes, and blood storage bags. Table 1.3 shows the chemical structure of DEHP (ATSDR, 2002). Phthalates degrade rather quickly when added to water. Not all phthalates can bioconcentrate in aquatic systems, but DEHP can do so. However, DEHP's half-life in water is no more than two or three weeks. Currently EPA is evaluating DEHP to test its potential human carcinogenicity (Kang et al., 2014).

The general public daily exposure level to DEHP has been calculated by the U.S. EPA as about 0.02 mg/kg/day, which will make about 1.4 mg/day for a 70-kg person. However, the average human individual's daily exposure to DEHP as estimated by several American studies lies in the range of 0.003-0.03 mg/kg/day as reported by one study (ATSDR, 2002) and 0.006-0.02 mg/kg/day as reported by another (Shea, 2003). The likelihood of children being exposed to phthalates is fairly high, because phthalates are widely used as plasticizers in many common plastic products such as polyvinyl chloride in the U.S.A (USEPA, 2002b; Bouma et al., 2002). Children in the age category of 3-12 months are at a very high risk of exposure to phthalates that may leach from plastic toys and dolls, as result of sucking (ATSDR, 2002).

The present studies deal with the investigation of five phthalate congeners, namely: dimethyl phthalate (DMP), di-n-butyl phthalate (DBP), diethyl phthalate (DEP), dioctyl phthalate (DNOP), and di-(2-ethylhexyl) phthalate (DEHP), all of which are recognized by the US EPA as priority pollutants (USEPA, 2002a). However, only two of these phthalates can be found regularly in environmental samples, namely, DEHP mainly and DBP to a much less extent (Lin et al., 2003).

In this study, di-(2-ethylhexyl) phthalate (DEHP) will be used because it has been recognized by various regulatory agencies as a major pollutant due to its low solubility in water, frequent existence in the environment and high toxicity to the kidney, liver, thyroid, and immune system (Sarkar et al., 2013).

Table 1.3: Chemical Identity of DEHP, CAS = Chemical Abstracts Services; DOT/UN/NA/IMCO = Department of Transportation/United Nations/North America/International Maritime Dangerous Goods Code; EPA = Environmental Protection Agency; HSDB = Hazardous Substances Data Bank; NCI = National Cancer Institute; NIOSH = National Institute for Occupational Safety and Health; NFPA = National Fire Protection Association; OHM/TADS = Oil and Hazardous Materials/Technical Assistance Data System; RTECS = Registry of Toxic Effects of Chemical Substances (ATSDR, 2002).

Characteristic	Information	Reference
Chemical name	Di(2-ethylhexyl) phthalate	RTECS 2000
Synonym(s)	DEHP; dioctylphthalate; bis(2-ethylhexyl) phathalate	RTECS 2000
Registered trade name(s)	Bisoflex 81; Eviplast 80; Octoil; Plantinol DOP; Staflex DOP	RTECS 2000
Chemical formula	$C_{24}H_{38}O_4$	RTECS 2000
Chemical structure		Howard and Meylan 1997
Identification numbers: CAS registry	117-81-7	Cadogan and Howick 1996
NIOSH RTECS	TI0350000	RTECS 2000
EPA hazardous waste	U028	HSDB 1990
OHM/TADS	7216693	HSDB 1990
DOT/UN/NA/IMCO shipping	No data	
HSDB	334	HSDB 1990
NCI	C52733	Montgomery and Welkom 1990
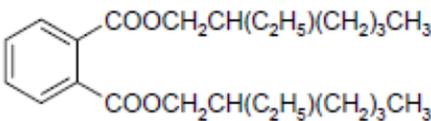
DEHP is the most frequently used plasticizer in PVC formulations for numerous consumer products (Metcalf et al., 1973; Carnevali et al., 2010). Generally, the harmful human health and environmental effects of phthalate esters are well documented (Metcalf et al., 1973; Blount et al., 2000; Ghorpade et al., 2002; Duty et al., 2003b; Lee et al., 2005; Lithner, et al., 2009; Oehlmann et al., 2009), and were found harmful for both marine and land organisms. For instance DEHP had harmful biochemical effects on rats, mice, rabbits and pigs, including the inhibition of cholesterol formation in the liver, adrenal gland, and testes, as well as reduced serum cholesterol levels, and raised fatty acids oxidation in the liver cells mitochondria (Bell, 1982). DEHP in aquatic environments accumulates in plants and animals (Oehlmann et al., 2009). It degrades

slowly in algae, mosquito larvae, clams and snails, resembling Dichloro-Diphenyl-Trichloroethane (DDT) in terms of absorption and storage rates in plant and animal lipids and builds up through the food chains (Metcalf et al., 1973). For example, when channel catfish (*Ictaluruspunctatus*) is exposed for 24 hrs to 1 µg/l of DEHP, its tissue residues build up to 2.6 µg/g (Stalling et al., 1973). Previous studies have shown that plastic leachates can cause acute toxic effects on *Daphnia magna.* Among the 15 different types of plastics tested, PVC was ranked second in toxicity for *D. magna* (Lithner et al., 2009).

Fish are always exposed to phthalates through water, food and sediments in aquatic environments (Oehlmann et al., 2009). When juvenile Atlantic salmon (*Salmosalar*) is exposed to DEHP, the phthalate interferes with gonad differentiation and gives rise to intersex individuals with both ovaries and testis (Norman et al., 2007). DEHP at a concentration level of 104 ppb was detected in samples of freshwater from lakes and rivers (Williams, 1973), which indicates the ubiquitous presence of phthalates in Canadian lakes and rivers due to the common use and release in water of products containing phthalates. The same applies to the Netherlands where DEHP was found in fish to exist at levels of 1.7-141 μg kg^{-1}, which is aggravated by fast DEHP biotransformation in fish (Peijnenburg & Struijs, 2006).

Research in aquatic toxicology has shown evidence of reproductive and development toxicity of DEHP. According to Kim et al. (2002) in Japanese medaka (Oryziaslatipes), it can delay the female oocyte development and in the zebra fish female DEHP weakens the oogenesis and embryo development (Carnevali et al., 2010), and decreases the sexual ability of the zebra fish males (disrupts spermatogenesis) and their capacity to fertilize oocytes (Uren-Webster et al., 2010). During the medaka fry stage, the normal maturation of the fish can be affected by decrease in its gonadal somatic index (GSI) along with decrease in its body weight (Chikae et al., 2004b).

DEHP also negatively affects the Japanese medaka embryos by exacerbating mortality, distorting the species' sex ratio, and reducing the body weight (Chikae et al., 2004a), and decreases its estrogenic potency in both male and female hepatocyte cultures (Maradonna et al., 2013). Furthermore, DEHP alters the transcription data of the genes that play a role in steroid formation, and changes the sex hormones levels of plasma in many fish species that live in freshwater, such as the carp (Cyprinus car-pio) (Thibaut & Porte, 2004), the Chinese minnow (Gobiocyprisrarus) (Wang et al., 2013), and the fathead minnow (Pimephalespromelas) (Crago & Klaper, 2012).

Moreover, DEHP metabolism via hydrolysis to MEHP and subsequently oxidation to mono-(2ethyl-5-hydroxyhexyl)-phthalate (MEHHP) and mono-(2-ethyl-5-oxohexyl)-phthalate (MEOHP) (Monfort et al., 2010), has also shown toxic effects. About 44% of DEHP undergoes metabolism in humans, thus turning into MEHP, MEOHP and MEHHP (Koch et al., 2004). DEHP and its major metabolic products, which include MEHP, MEOHP and MEHHP, have anti-androgenic effects in laboratory studies (Stroheker et al., 2005). Although a considerable amount of literature has tackled the adverse effects of DEHP in marine environments, the harmful effects of DEHP that affect the development and reproduction of marine organisms were limited to some visible features, while the mechanisms that affect their reproduction are not yet adequately understood.

1.1.7.3.   The Sources of DEHP Entering the Marine Environment

- **Emissions to Water Through Indoor Use**
  - *DEHP Leaching from Washing of Clothes That Are PVC Printed*

The annual levels of DEHP release through washing of clothes that are PVC printed are estimated based on an experimental study conducted in Denmark on T-shirts. These levels of phthalates in Denmark were found to average 6.9 tpa or in the range of 1.3-13 tpa. Such large range of estimation points to a considerable variation between different types of clothes. Separate analysis on the part played by DEHP among different phthalates was found to be 1.5 tpa (21%) or in the range of 0.27-2.7 tpa. Applying the Danish use levels of printing clothes with PVC to all the EU countries raises the total EU phthalates emission to 370,000,000 inhabitants (European Union, 2008).

  - *Leaching of DEHP through Washing Polymer Floors*

Levels of DEHP leaching from polymer floors washing and abrasion are based on an experimental study conducted in Sweden. These leaching levels were found to amount to 61.1 + 1,151 = 1,212 tpa. Some argue that DEHP emitted in particles of PVC should be taken into consideration and added to the leached amount. However, as the particle size is thought to be tiny, these particles are supposed to behave in a similar way as that of pure DEHP (European Union, 2008).

- **Emissions to Water Through Out-Door Use**

The most important sources from out-door use include: material used in undercoating cars, roofing, coating coils, coating fabrics, cables and wires, plastic hoses and pipes and shoe soles (European Union, 2008).

1.1.8. PHTHALATE ESTERS DETERMINATION IN NEREIS BY GAS CHROMATOGRAPHY

The most common techniques used to analyse PAEs in water are gas chromatography (GC) with electron capture (EC) (Glaser et al., 1981); mass spectrometry (MS) (Penalver et al., 2000; Serodio and Nogueira, 2006); flame ionization (FI) (Batlle et al., 2004); high performance liquid chromatography (HPLC) with ultraviolet (UV) (Ling et al., 2007) and MS detection (Lopez-Jimenez et al., 2005).

Because of the very low levels of phthalates in water samples in the environment coupled with different matrices complexity, chromatographic methods used directly which can separate the phthalates from the matrices and in some cases, might even need to derivative to make them more volatile. The chromatographic methods check the selectivity and sensitivity of phthalates. In order to increase the dependability of the analytical results, sample preparation before using these analytical methods is essential (Zhao et al., 2005).

The pre-concentration methods traditionally used to observe PEs in water include solid-phase extraction (SPE) (Kato et al., 2003) and liquid–liquid extraction (LLE) (Cai et al., 2007). However, these methods have the shortcomings of being time-consuming, requiring more man -hours and large samples, along with toxic organic solvents.

1.1.8.1. Instrumentation

Two main elements constitute the GC-MS; the mass spectrometer and the gas chromatograph. The gas chromatograph uses capillary columns, that vary by their dimensions (such as length, film thickness, and diameter), together with the stationary phase properties, e.g. 5% phenyl polysiloxane (Figure 1.5). Different injected molecules that form a mixture will be separated by virtue of their different chemical properties. The molecules are separated as they take different time (retention time) to get to the end of the gas chromatography column. This enables the mass spectrometer to convert the molecules individually by ionizing and breaking them down into fragments, and then use their masses to detect them, thus allowing structural identification.

A particular molecule cannot be identified accurately using gas mass spectrometry and gas chromatography alone. Because mass spectrometry usually requires a very pure sample, while gas chromatography, using flame ionization detector, which is a traditional detector used detect multiple molecules, thus taking a lot of time to move down the column and thereby producing two molecules or more in order co-elute. Such two different molecules can have ionized fragments that look alike in the mass spectrum. Combing the two processes, makes error less likely, as it is very unlikely for two molecules that are different in nature to behave similarly in both a mass spectrometer and a gas chromatograph.

Hence, when a recognizing mass spectrum shows up in a combined GC-MS analysis at a specific retention time, usually there will be a high certainty about the product of analysis in question in the sample (Figure1.5) (Sahil et al., 2011).
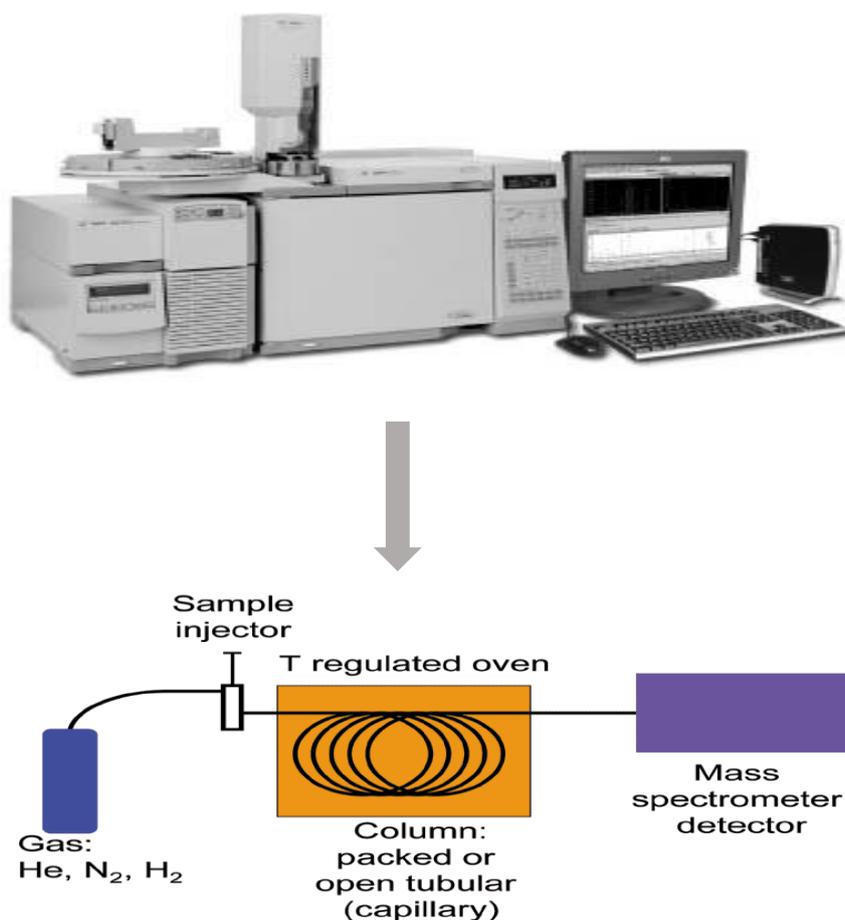
Figure 1.5: GC-MS schematic (Sahil et al., 2011).

The mass spectrometer is a system that converts any compound passing through it into ions. It is the universal standard for detecting gas chromatographs. Another quality that makes it a specific gas chromatographic detector is the highly specific nature of mass spectrum. While the chromatography is good at separating, the mass spectrometry is ideal for identifying. An interfacing arrangement serves to run a mass spectrometer and a gas chromatograph together without affecting the performance of either of them. However, there is a problem in compatibility due to the difference in pressure needed to run a mass spectrometer with a gas chromatograph, because a mass spectrometer runs under high vacuum, while a gas chromatograph operates at high pressures. A problem associated with this is when there is too much carrier gas, and the sample is too little in the effluent for the gas chromatograph. When the gas chromatograph is deploying packed column, it is easy to get a flow of carrier gas above 30ml/min, leading to a collapse of the vacuum within the spectrometer. This would require the carrier gas to be substantially removed, and different designs developed (Sahil et al., 2011).

1.1.8.2. GC-MS Interface

Figure (1.6) shows the main types of interfaces developed for the coupling of GC/MS. The classical molecular jet interface or jet separator was developed for GC/MS using the original Becker separator. The GC flow, in such separators, is allowed to enter into an evacuated chamber via a restricted capillary. A supersonic expanding analyte jet and carrier molecules are formed at the capillary tip and the core flow area is allowed to pass into the mass spectrometer. Compounds of high molecular mass, in an expanding jet, collect in the core flow while the light and diffusive molecules are driven away, partly through collisions. In this way, the core flow sampling produces an enriched analyte. The jet interface is inert, efficient and very versatile, although its efficiency is reduced by the potential plugging problems that take place at the capillary restrictor and due to the more volatile compounds (Abian, 1999).

The interface of the perm-selective membrane, introduced by Llewellyn and Littlejohn, consists of a silicone-rubber membrane which can transmit non-polar organic molecules as well as act as a barrier for carrier gases. Although the procedure is very effective in terms of enrichment, it is marred by discrimination effects creating more polar analytes, and leads to considerable band broadening of the chromatographic peaks of the analytes (Abian, 1999).

The Watson-Biemann or molecular effusion interface is an interface that works according to the principle of gas effluent molecular filtering using porous glass frit. The column of the gas effluent goes through a porous glass frit tube placed in a vacuum chamber. Small molecules move across the tube walls microscopic pores and are evacuated, while molecules with high molecular masses are moved to the source of ions. One of the disadvantages of this interface is the great dead volume and its big surface area that are added. As is the case with the jet separator, here also the interface suffers from discrimination effects towards smaller molecules. These three methods presented herein-above depend on the analyte enrichment in the carrier gas where carrier molecules are eliminated. In this manner, one can introduce sufficient samples into the ion source ensuring that the total gas flows will be compatible with the system's pumping capacity. For these resaons, the jet separator is the most extensively employed and most successful interface (Abian, 1999).

There are alternatives to the reduced total gas flow, the simplest of which is the flow splitting, where no sample enrichment is required and the procedures are very useful when sensitivity is not a key factor. Flow splitting may be carried at the gas chromatograph exit in order to allow the diverted gas to flow to a parallel detector, or to flow at the interface itself as is the case with the open split interface. The latter uses a capillary restrictor which is used in order to limit the flow into the ion source to a constant manageable value. The exit of the GC column lies close to the entrance of the restrictor in an open connector. The restrictor takes samples of the effluent from the exit of the GC column and excess flow from the column is taken away from the connector using helium. The advantage of the open split interface lies in its versatility which offers a variety of column flows without a need to modify the interface (Abian, 1999).

Following the advent and general acceptance of the wall-coated open-tubular (WCOT) fused-silica capillary columns, development and optimization of new GC/MS interfaces was considerably simplified. The optimum gas flow rates of capillary columns are in the range of 1-2 ml min^{-1}, which are much lower compared to those of packed columns of over 10-20 ml min^{-1}. These low rates allow the whole effluent to be carried to the mass spectrometer. In this case direct coupling is used where the exit of the capillary column is entered into the ion source without restriction (Abian, 1999).
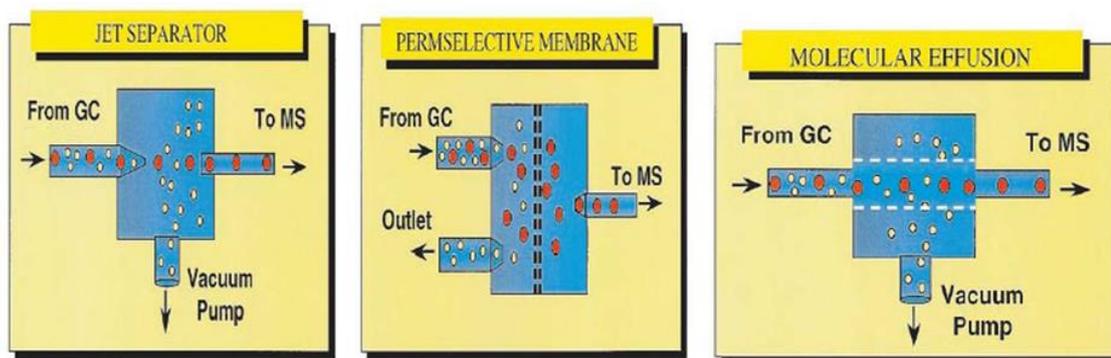
🔴 **Analyte Molecule**

🟡 **Carrier Gas**

Figure 1.6: Coupling interfaces for GC-MS. The molecules of the analyte and the carrier gas are shown by red and yellow circles respectively (Sahil et al., 2011).

1.1.8.3. <u>Mass Spectrometry</u>

Mass spectrometry is capable of accurately measuring the masses of different molecules present in a sample. The procedure involves three main stages:

- **<u>Ionization</u>**

The sample molecules undergo vaporization (conversion to the gas phase through heating). An electron beam is then directed to the vapors in order to convert them to ions. As mass spectroscopy can only measures charged particles masses, ions are detected leaving neutral molecules unseen.

Electrons are given to molecules to produce negatively charged ions or taken away from them to produce positively charged ions (Saxton, 2011).

- **<u>Acceleration and Deflection.</u>**

Thereafter, ions are sorted based on their masses in the stages of acceleration and deflection.

Acceleration is simple attraction where positive ions generated from the ionization stage are attracted towards the negative plates at velocities that depend on their masses. That is to say, the lighter the molecule is, the quicker it is attracted.

Deflection is the change of the course of ions movement by means of a magnetic field, the extent of which also depends on the molecules masses. Here also ions with different masses travel at different speeds. The deflection magnitude depends on:

- The ions masses: The heavier ions don't deflected more than the lighter ions
- The ions charges. The ions charges. The Ions with more positive charges are deflected more than Ions with less positive charge.

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These two factors generate the mass/charge ratio (usually denoted by m/z or sometimes by m/e). For instance an ion with a mass of 28 and positively charged with 1+ charge will have a mass/charge ratio of 28. Conversely, an ion that has a mass of 56 and positively charged with 2+ charge will have a mass/charge ratio of 28 (Saxton, 2011).

- **Detection.**

Ions reach the detector at speeds that depend on their masses, after which the computer eventually generates a spectrum (Saxton, 2011).

1.2. THE BIOMEDICAL AND PHYSIOLOGICAL CHANGES IN STRESSED ANIMALS

1.2.1. OXIDATIVE STRESS AND PEROXISOMES

Marine biota may suffer from oxidative stress as a result of considerable increases in reactive oxygen species (ROS) or reactive nitrogen species (RNS), as well as decrease in the detoxification mechanisms of these species (Schrader & Fahimi, 2006). Oxidative stress also results from environmental oxidants exposure, including heavy metal toxins, heat shock, UV irradiation, and inflammation (Ermak & Davies, 2002). Bio-molecules including DNA, lipids, and proteins suffer toxic effects when exposed to high levels of ROS such as nonenzymatic lipoperoxidation, which results in the build-up of oxidative damage in many cellular locations, as well as deregulate redox-sensitive metabolic pathways, and finally results in pathological conditions. This is because ROS and RNS act as mediators in different important cellular processes and metabolic pathways (Droge, 2003; Saran, 2003). An example here is ROS function in apoptosis. ROS species such as radicals that contain unpaired electrons e.g. the superoxide anion ($O_2^{\cdot-}$) are formed by means of one-electron reduction from Oxygen (O_2) in the reaction $O_2 + e^- \rightarrow O_2^{\cdot-}$. Hydrogen peroxide ($H_2O_2$) acts in a similar way to ROS, despite the fact that it is not a radical, as it does not possess unpaired electrons. It is sometimes formed by a dismutation reaction of $O_2^{\cdot-}$ by catalysis with superoxide dismutases through hydroperoxyl radical ($O_2^{\cdot-} + H^+ \rightarrow HO_2^{\cdot-}$; $2HO_2^{\cdot-} \rightarrow H_2O_2 + O_2$). The hydroxyl radical ($^{\cdot}OH$) is probably the most reactive and hence one of oxygen toxic forms. It is formed when a metal ion (e. g. copper or iron) is catalyzed by H_2O_2 ($H_2O_2 + O_2^{\cdot-} \rightarrow O_2 + OH^- + {^{\cdot}OH}$). RNS possesses radical species e.g. primary nitric oxide ($^{\cdot}NO$). $^{\cdot}NO$ and H_2O_2 can permeate membranes by diffusing through them, but are less reactive and more stable than $^{\cdot}OH$, thus being best suited for intra- and even intercellular signalling. H_2O_2 constitutes no harm until it is converted to the more reactive ROS, and

then attacks cellular thioldisulfide redox systems e.g. glutathione, thioredoxin and peroxiredoxin (Moldovan & Moldovan, 2004; Jezek & Hlavata, 2005).

1.2.2. ROS FUNCTIONS

There is a growing body of knowledge about the mechanisms of the oxidant setting of the cell, showing reactive oxygen species (ROS) as molecules with a signaling capacity as well as being metabolic byproducts. Through the action of hormones, they take many pathways and participate in many networks ranging from transport systems to protein phosphorylation. The action of ROS is not limited to single steps or multistep processes, but they affect all the steps simultaneously, via reaction with numerous compounds and by participating in multiple redox reactions. The signaling pathways and the cell components may be positively or negatively affected depending on the molecular species present, in addition to ROS concentration, and subcellular localization. ROS concentration is thought to be a form of "redox biology" with the task of regulating the physiological functions, including gene expression and proliferation and signal transduction. Physiological and even pathological events are believed to be affected by the "Redox biology" and not by oxidative stress (OS) (Schieber & Chandel, 2014).

The literature has reported data pointing to increases in constant and slow ROS as well as data about stepwise and fast ROS, which are integrated together to account for the typical signaling events that exchange messages among the compartments of the cell. Studies now focus on ROS specificity and dynamics in terms of the effects of ROS concentrations on both intracellular and intercellular networks and signaling pathways. ROS/RNS major target signaling are proteins whose functions sometimes undergo reversible and sometimes irreversible modifications, resulting in cell transformation, growth arrest and death. The reversible oxidation modulation of redox-sensitive proteins greatly contributes to transduction and sensing of the oxygen signal. A receptor-dependent or receptor-nondependent tyrosine kinases such as adaptor protein p66SHC, AMP-activated protein kinases, transcription factors as FOXO (forkhead homeobox type O), p53 (tumor suppressor 53), Nrf2 (nuclear factor E2-related factor 2), AP-1 (activator protein-1), NF-κB (nuclear factor kappa B), PPARγ (peroxisome proliferator-activated receptor gamma), HIF-1a (hypoxia inducible factor-1a), and β-catenin/Wnt signaling (Korbecki et al., 2013; Lavrovsky et al., 2000).

In vitro, ROS mediate responses towards the conditions that exist inside the cell and between cells, including cytokines, growth factors, hypoxia, and nutrients

deprivation, that regulate cell proliferation, apoptosis, and differentiation; in addition to acting as a significant cancer hallmarks (Coso et al., 2012). ROS regulation of self-renewal, aging, quiescence and apoptosis of the cells, is controlled by both extrinsic and intrinsic factors, during tissues repair and homeostasis that take place *in vivo* (Maryanovich & Gross, 2013) which also control stem cells differentiation and proliferation which is induced by ROS. Likewise, ROS play the role of a rheostat, by sensing and translating environmental cues during the stem cells responses, and in this way they balance the output (function) and input (cytokines, nutrients) of the cells. Depending on the levels of ROS, the stem cells sometimes undergo exhaustion (Liang & Ghaffari, 2014). Mitochondrial ROS sometimes create an adaptive response (mitohormesis), in the form of defensive mechanism that promotes health by means of disease prevention or delay and thus extend the cells lifespan (Scheibye-Knudsen et al., 2015). ROS make an integral part of physiopathologic events development such as signaling of mitochondrial death (Dixon & Stockwell, 2014) and autophagy (Filomeni et al., 2015), as well as infection and inflammation (Lei et al., 2015), where ROS pass on immunological changes. Professional cells such as lymphocytes, phagocytes and granulocytes, generate high levels of ROS that serve as defensive agents against microbes (Abais et al., 2015; Cachat et al., 2015). Conversely, any incident that aids chronic OS or NS (nitrosative stress), through more generation of ROS or detoxification, disrupts the signaling networks; changes proteins, lipids and nucleic acids; and generates mechanisms so as to face such changes. Thus, overproduction of ROS hampers the repair of damaged mitochondrial and nuclear DNA, at several steps, thus, leading to genomic instability of the cell (Weyemi & Dupuy, 2012). ROS are known as chief modulators in the processes that lead to chronic build up of oxidized molecules, as in the case of diabetes, hypertension, cardiovascular diseases, ischemia, atherosclerosis, reperfusion injury, rheumatoid arthritis and neuro-degeneration (Phaniendra et al., 2015). ROS also take part in cancer development by means of their intervention with cell proliferation, mutagenesis, and inhibition of apoptosis (Verbon et al., 2012). ROS are allied with NF-κB and p53 to play key roles in tumorigenesis. Energy metabolism synergic effects with OS, stimulate cell growth aided by tumor suppressors or oncogenes (Kang et al., 2015; Reuter et al., 2010). Deregulated production of ROS in cancer cells followed by constitutive OS, sometimes leads to cellular invasive phenotype (Chiarugi, 2008). Despite the fact that ROS functions cannot be easily investigated, several pharmacological studies are underway to generate

ROS homeostasis by decreasing OS and increasing antioxidant defense (Davalli et al., 2012; Assuncao & Andrade, 2015).

1.2.3. ROS EFFECTS

Depending on the types of cells, ROS affect the same targeted cells differently, except for $ONOO^-$ and $^\cdot OH$ in which the effect is always plain toxicity. Some kind of redox homeostasis mechanism is required in order to reach a basal oxidation level which is essential for the cells to be viable and function properly. Fluctuations in radicals are always controlled by their continuous balance via increased energetic demand, that is needed in order to intensify electron flux into the mitochondria, as well as for aging, which compromises mitochondrial efficiency. Several factors aid in redox homeostasis changes including external ROS sources, oxygenases and oxidases, dietary nitrosamines, ultraviolet and infrared radiations, and chemotherapy agents (Caputo et al., 2012). The final ROS effect is not only determined by the concentrations of the different species in the cells, but also by their balance with each other, i.e. the balance of H_2O_2 versus $O_2^{\cdot -}$. In fact, $O_2^{\cdot -}$ of the mitochondria may create signaling pathways during the onset, development, and amplification of cancer in the cells. ROS set off thiol oxidation, nitrosylation, carbonylation, and glutathionylation in specific enzymes and proteins, which then mediate signaling in cell metabolism, although the exact mechanisms remain to be clarified (Chiu et al., 2014; Waypa et al., 2016). Both nuclear and cytosolic proteins are ROS targets that contain cysteine residues that are sensitive to ROS. Such residues play regulatory roles rather than structural ones. These cysteines act as molecular switches in reacting and transduction of redox signals, thus, imparting the proteins with redox activity via their thiol groups. Proteins forms and functions are modified subsequent to undergoing oxidative alterations in which they generate S-hydroxylated derivatives by means of reactions with other cysteines and generation of disulfides either inside the molecules or between them, and thereby create complexes that will perform new functions. Proteins that are redox-activated act as redox sensors inside the cells and help ROS properly adapt to their functions within the redox equilibrium of the cells (Caputo et al., 2012; Winterbourn & Hampton, 2008). Such sensors result can be used to study the pathogenesis and progress of numerous diseases (Siedenburg et al., 2012; Waypa et al., 2016). Among these are physiological traces of H_2O_2 which act as sensors and secondary messengers, as they can cross membranes in order to induce specific cellular signal pathways (Waypa et al., 2016). ROS contribution

to cell homeostasis comes from their activities as "second messengers" via the modulation of the regulatory molecules activities, including G proteins, phosphatases, protein kinases, and transcription factors. Periodic cell redox oscillations regulate the progression of the cell cycle from quiescence (G0) stage to proliferation (G1, S, G2, and M) stage, and then back to quiescence stage. Losing the cell cycle redox control could result in abnormal proliferation, which represents a significant feature in many human pathologies (Sarsour et al., 2009). ROS role features in different physio-pathological conditions such as cell growth, differentiation, proliferation, aging, and combating infectious agents by triggering inflammatory responses (Davalli et al., 2016; Lopez-Otin et al., 2013).

1.2.4. OXIDATIVE STRESS AND DAMAGE OF DNA

Oxidative stress involves the chemicals that generate the reactive oxygen species (ROS), ionizing radiation and ultraviolet (UV) radiation. Oxidative stress can cause damage to different cellular components including DNA, lipids and proteins, resulting in carcinogenesis, aging and other types of disease. DNA damage caused by oxidative stress, in particular, plays a key role in aging and carcinogenesis (Kasai, 1997; Beckman & Ames, 1998). Damaged DNA, in biological systems, is repaired by enzymes, thus helping the cells to recover their normal functions. However, failure to repair the DNA damage may lead to mutations such as deletion and base substitution, thus leading to carcinogenesis (Vineis et al., 1999; Poulsen et al., 1998). DNA damage proceeds in specific sequence which also plays a critical role in the mutation process.

Much of the endogenous damage of DNA comes from oxygen reduction intermediates (Marnett, 2000). ROS are made of oxygen free radicals that include the hydroxyl radical ($\cdot$OH) and the superoxide radical anion ($O_2{}^-$), in addition to non-radical oxidants that include the singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2). Beside ROS, several other free radicals can cause DNA damage, which include lipid hydroperoxides (LOOH), nitric oxide (NO), peroxyl radical ($\cdot$OOH), alkoxyl radical (RO$\cdot$), sulfate radical ($SO_4{}^{\cdot-}$), nitrogen-centered radical and metal–oxygen complexes. These reactive species possess different redox capacities (Buxton et al., 1988; Neta et al., 1988), and their redox capacities play key roles in the specific sequence of DNA damage.

The oxidation capacities of DNA bases can also play a part in the determination of the specificity of DNA damage sequence. Among the four bases of DNA, Guanine is the one that is most easily oxidized because its oxidation capacity versus normal

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hydrogen electrode (NHE) is lower relative to the other three bases of DNA, namely adenine, thymine and cytosine (Burrows &Muller, 1998; Steenken& Jovanovic, 1997). The distribution of the electrons that take part in the chemical reactions with the DNA bases is regarded as an important factor in the determination of the susceptibility to the oxidative attacks. The highest of the occupied molecular orbitals (HOMO), that provide the electrons that have the highest energy, give the DNA bases their specific reactivity. A big part of the HOMO electrons is found on the 5′-G of the sequence of GG in the double-stranded DNA B-form (Sugiyama &Saito, 1996; Saito et al., 1998), which is why guanine is easily oxidized. Therefore, the two factors that determine the specificity of DNA damage sequence appear to be the redox capacities of the reactive species and the DNA bases HOMO distribution. Furthermore, the steric effects on the reactive species interaction with DNA may also contribute to the determination of the specificity of DNA damage sequence (Kawanishi et al., 2001).

1.2.5. DNA METHYLATION BY ROS

The condensed structure of chromatin (heterochromatin) is usually accompanied by genes repression through hypomethylation processes, whereas open chromatin (eu-chromatin) is usually accompanied by genes activation through acetylation processes (Tamaru, 2010). Genes expression is modulated via epigenetic marking by changing the electrostatic nature of chromatin and its protein binding affinity. Methylation of DNA silences the genes by inhibiting the access of the transcriptional activators to the targeted binding sites, or by activating the protein domains that bind the methyl. The function of methyl-binding interferes with histone deacetylases and aids condensation of the chromatin into conformations that are transcriptionally repressive. Hypomethylation and hypermethylation stages take place one after another, indicating the extent to which the methylation of DNA and its binding correlate mechanisms are complex. The alterations that depend on ROS are linked to the methylation and demethylation of DNA, directly or indirectly. The binding of NF-κB to DNA, is methylation-dependent, and leads to alteration in SOD mice that are deficient in Cu/Zn. ROS-dependent alterations are indirectly associated with changes in methylation processes, and this suggests that the alterations that are linked to changed redox mechanisms may constitute a part of the cell signaling pathways (Siomek et al., 2014). It is also thought that deoxy-guanine oxidation of CpG nucleotides to 8-hydroxy-2′-deoxyguanosine (8-OHdG) is oxidative damage proxy marker, in several human diseases (Kroese & Scheffer, 2014). Compounds formed from 8-OHdG interact with

DNA methyl transferases (DNMT) and DNA restriction nucleases, and by doing so change the transcription factors that bind to DNA and lead to general DNA hypomethylation. *In vivo* (Wang et al., 2015) and *in vitro* (Wongpaiboonwattana et al., 2013) studies show that ROS can induce a definite DNA promoters hypomethylation and a general genome hypomethylation, through the upregulation of DNMT and generation of its complexes. Recent studies also demonstrate that a pathway mediated by ROS leads to repression of the gene of protein kinase C epsilon, via its promotor methylation. Such episodes make an important part of *in utero* heart hypoxia, which often results in ischemic injury in people's later life (Patterson et al., 2012).

1.2.6. ANTIOXIDANTS

Antioxidants are the metabolites that carry out the function of cleansing cells of ROS. Among the classic antioxidant examples are vitamin A, vitamin C and vitamin E. A low concentration of antioxidant inhibits or significantly delays the oxidation of oxidizable substrates. Antioxidants are usually broken down into three main groups, namely: enzymatic (Catalase (CAT), Superoxide Dismutase (SOD) and Glutathione peroxidase (GPX)), non-enzymatic (Albumin, homocysteine, L-cysteine and protein sulfhydryl groups) and low molecular weight non-enzymatic antioxidants (ascorbic acid, uric acid α-tocopherol, glutathione, β-carotene and retinol). Mechanisms adopted by body tissue to prevent damage inflicted from free radicals include vitamin E, vitamin C, and β-carotene as major vitamin antioxidants. Besides these, several metalloenzymes such as catalase Iron (Fe), glutathione peroxidase (Se) and superoxide dismutase exert their critical influence in protecting the internal cell components from oxidative damage (Ganaie et al., 2013).

1.2.6.1. <u>Catalase (CAT)</u>

Catalase is an enzyme that contains heme and catalyses the conversion of hydrogen peroxide into oxygen and water. Catalase in the peroxisomes cleanses the mitochondrial and cytosolic peroxides that form during urate oxidation (Oshino et al., 1977). Mitochondrial SOD changes most of the mitochondrial superoxide ions into hydrogen peroxide. Hence, catalase and SOD protect the cellular content from the damage that emanates from the creation of extremely reactive hydroxyl group by changing the superoxide ions into hydrogen peroxide (Miyazaki et al., 1991).

1.2.6.2. <u>Superoxide Dismutase (SOD)</u>

Mann and Keilin (1938) were the first to isolate superoxide dismutase (SOD), which they mistook for a copper storage protein. Hence, SOD was called tetrazolium oxidase and erythrocuprein, indophenol oxidase, until McCord and Fridovich (1969) discovered its catalytic function. The function of SOD is now known as the catalysis of converting the superoxide into oxygen and hydrogen peroxide.

$$°O^-_2 + °O^{-2} + 2H^+ \longrightarrow H_2O_2 + O_2$$
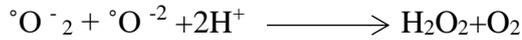
Catalase, SOD and glutathione peroxidase are the major enzymes that detoxify the superoxide anion and the hydrogen peroxide (McCord & Fridovich, 1969; Chance et al., 1979). Pathan et al. (2009) found an increase in the level of SOD as pregnancy advances. They observed that SOD level was considerably higher on the parturition day (3.03 ± 0.13 U/ml) relative to the level 30 days before parturition (2.13 ± 0.19 U/ml).

1.2.7. PEROXISOMES AND ROS

Oxygen is taken up in many different metabolic reactions in various cellular locations, primarily in mitochondria, Endoplasmic Reticulum (ER), and peroxisomes. A respiratory pathway of peroxisomes was described by De Duve and Baudhuin (1966) where O_2 is reduced by electrons derived from different metabolites to H_2O_2, which is then reduced to H_2O. The respiratory pathway of peroxisomes does not include an oxidative phosphorylation, neither does it result in Adenosine Triphosphate (ATP) production. Heat is released as free energy. The high consumption of O_2 by peroxisomes, production of H_2O_2, $O_2^{·-}$ and $·OH$ in peroxisomes, and the recent production of $·NO$ therein (De Duve & Baudhuin, 1966; Stolz et al., 2002), in addition to the discovery of some enzymes that metabolize ROS therein also Tables (1.4) and (1.5) support the notion that such organelles play an important role in the production and foraging of ROS that take place in the cell (Figure 1.7) (Schrader & Fahimi, 2006).

It was initially thought that the primary function of peroxisomes is restricted to H_2O_2 decomposition carried out by various peroxisomal oxidases (primarily flavoproteins) including catalase, the traditional marker enzyme peroxisomes. Now it is evident that peroxisomes participate in many different key cellular functions that take place in the majority of eukaryotic cells (Schrader & Fahimi, 2006).

Hydrogen peroxide is mainly generated by several peroxisomal oxidases such as acyl-CoA oxidase which shares β-oxidation with other fatty acids. Catalase and glutathione peroxidase decompose hydrogen peroxide or change it into hydroxyl

radicals ($^{.}$OH). These hydroxyl radicals can sever the peroxisomal membrane by peroxidating the unsaturated fatty acids. Hydroperoxides formed are broken down by glutathione-peroxidase and catalase. Manganese superoxide-dismutase (MnSOD) and copper zinc superoxide-dismutase (CuZnSOD) clean the superoxide anions ($O_2{}^{.-}$), which are generated by peroxisomal oxidases including xanthine oxidase (XOx). Nitric oxide synthase (NOS) participates in the oxidation of L-arginine (L-Arg) to produce nitric oxide (NO$^{.}$). Nitric oxide can unite with $O_2{}^{.-}$ radicals to produce peroxynitrite ($ONOO^{-}$), the powerful oxidant. Hydrogen peroxide and nitric oxide penetrate the peroxisomal membrane and participate in cellular signaling. PMP20 and Peroxiredoxin 1 participate in hydrogen peroxide degradation, while L-MP and Mpv17 participate in peroxisomal ROS metabolic regulation (Figure 1.7). It was found that some 35% of the H_2O_2 formed in the liver of the rat comes from peroxisomal oxidases (Boveris et al., 1972). ROS are produced by virtue of their metabolic activity, and are degraded in order to make a balance between production and foraging of ROS. For this purpose peroxisomes possess several strong defence mechanisms and have antioxidant enzymes beside catalase (Table 1.5, Figure 1.7) (Schrader & Fahimi, 2006).

Table 1.4: Peroxisomal enzymes that produce ROS (Schrader and Fahimi, 2006).

Enzyme	Substrate	ROS
(1) Acyl-CoA oxidases		
(a) Palimtoyl-CoA oxidase	Long chain fatty acids	H_2O_2
(b) Pristanoyl-CoA oxidase	Methyl branched chain fatty acids	H_2O_2
(c) Trihydroxycoprostanoyl-CoA oxidase	Bile acid intermediates	H_2O_2
(2) Urate oxidase	Uric acid	H_2O_2
(3) Xanthine oxidase	Xanthine	H_2O_2 $O_2{}^{.-}$
(4) D-amino acid oxidase	D-Proline	H_2O_2
(5) Pipecolic acid oxidase	L-pipecolic acid	H_2O_2
(6) D-aspartate oxidase	D-aspartate, N-methyl-D-aspartate	H_2O_2
(7) Sarosine oxidase	Sarcosine, pipecolate	H_2O_2
(8) L-alpha-hydroxy acid oxidase	Glycolate, lactate	H_2O_2
(9) Poly amine oxidase	N-Acetyl spermine/ spermidine	H_2O_2
(10) Nitric oxide synthase	L-Arginine	$^{.}$NO
(11) Plant sulfite oxidase	Sulfite	H_2O_2

Table 1.5: Peroxisomal enzymes that degrade ROS (Schrader and Fahimi, 2006).

Enzyme	Substrate	Enzyme is also present in
(1) Catalase	H_2O_2	Cytoplasm (e. g., erythrocytes) and nucleus, mitochondria (rat heart only)
(2) Glutathione peroxidase	H_2O_2	All cell compartments
(3) Mn SOD	$O_2{}^{\bullet-}$	Mitochondria
(4) Cu, Zn SOD	$O_2{}^{\bullet-}$	Cytoplasm
(5) Epoxide hydrolase	Epoxides	ER and cytoplasm
(6) Peroxireodoxin 1	H_2O_2	Cytoplasm, nucleus, mitochondria
(7) PMP 20	H_2O_2	Peroxisomes
(8) Plant ascorbate– glutathione cycle	H_2O_2	Peroxisomes, chloroplasts, cytoplasm, root nodule mitochondria (plants only)

Among peroxisomes' interesting features is their ability to increase and multiply as well as degrade when induced to do so by environmental and nutritional stimuli (Fahimi et al., 1982). Experiments on rodents indicated that peroxisome's numbers and sizes in addition to generation of peroxisomal β-oxidation enzymes come into play when receptors are activated by the peroxisome proliferator activators (PPARs) such as fibrates or when free fatty acids are used (Fahimi et al., 1982). Such conditions generate oxidative stress induced by peroxisomes, which may exceed the antioxidant capacity and result in cancer. Under certain conditions, iron and copper ions are released such as by xenobiotics, which then catalyze the creation of ·OH via Fenton reaction ($Fe^{2+}+H_2O_2{\rightarrow}Fe^{3+}+OH^-+{\cdot}OH$), thus resulting in lipid peroxidation, which in turn leads to loss of peroxisomal functions through the break of the peroxisomal membrane (Bacon & Britton, 1989; Yokota et al., 2001). By virtue of their oxidative metabolism activities, peroxisomes may be regarded as a cause of oxidative stress. However, they can also fight oxidative stress as well as ROS in other intracellular or extracellular sites, thus protecting the cells against damage due to oxidative stress. Besides, some ROS generated by peroxisome mediate intracellular signalling (Masters, 1996).
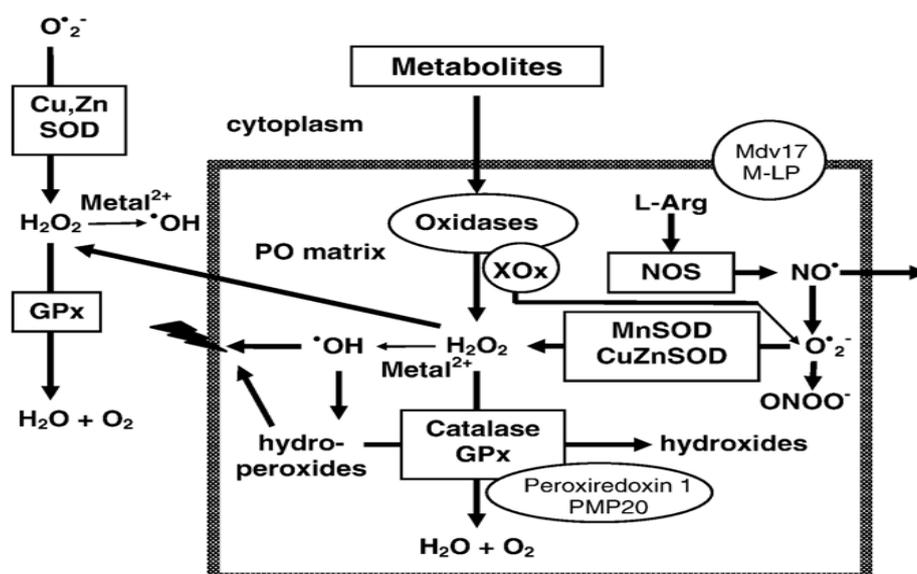
Figure 1.7: Schematic diagram showing peroxisomal enzymes that both generate and degrade ROS (Schrader and Fahimi. 2006.)

1.2.8. ALTERATIONS OF ROS-METABOLIZING ENZYMES

It has been shown that a rise in environmental oxygen concentrations can lead to a considerable increase in the peroxisomes volume density and the amount of their enzymes that forage on ROS (Schrader & Fahimi, 2006). When the cells of the Chinese Hamster Ovary (CHO) are exposed to very high O_2 concentration, they show an increase of twice the volume of peroxisomes and four times the amount of catalase and glutathione peroxidase in addition to increase in manganese, copper- and zinc-SODs (Van Der Valk et al., 1985). Furthermore, peroxisomes are proliferated and fatty acyl-CoA oxidase activity that generates H_2O_2 is observed when peroxisomes are treated with proliferating chemicals (Fahimi et al., 1982; Reddy & Lalwai, 1983). The manner in which oxidative enzymes are induced in peroxisomes by PPARα (the transcription factor), and its co-activators (Reddy, 2004) is somewhat clear, but the manner in which they respond to ROS requires further research. Anti-oxidant enzymes are regulated similar to apoptotic signalling because the oxidative stress has the ability to determine the fate of the cell, i.e. make it either follow the cell cycle or subject itself to apoptosis(McCormick, 1999). In fact, apoptosis can be combated by anti-oxidative enzyme activity, but this may promote carcinogenesis (Corcoran et al., 1994). Anti-oxidant enzymes at low levels (low levels of glutathione peroxidase, catalase and MnSOD) always point to the presence of malignant cells (Oberley & Oberley, 1988; Litwin et al., 1999). Thus, anti-oxidant enzyme regulation appears to be more complex relative to regulation of oxidative enzymes, as they are mainly regulated by PPARα. This transcription factor was also found to regulate the sterol carrier protein 2 (SCP2) in

peroxisomes, which is thought to protect the fatty acids against lipid peroxidation (Dansen et al., 2004). On the other hand, Mpv17 and M-LP, the peroxisomal membrane proteins, have been found to take part in the regulation of the expression of the mammalian antioxidant enzymes (Zwacka et al., 1994; Iida et al., 2003; Iida et al., 2006).

1.2.9. INCREASED PEROXISOME ACTIVITY TO PRODUCE PEROXISOME ENZYMES WITH DEHP

When a rodent is exposed to DEHP, hepatic peroxisomes rise and peroxisome enzymes are induced. High exposure to doses of 1,000 mg/kg/day or more was found to lead to a rise in the peroxisomes in the cells (Ganning et al. 1989; Rhodes et al. 1986; Shin et al. 1999). In such cases, increase of peroxisomes is accompanied by a rise in the presence and activity of several peroxisome enzymes, especially the ones that are associated with fatty acids catabolism. Peroxisomes are cell parts or organelles that take molecular oxygen and make hydrogen peroxide during a catabolism process (Figure 1.8) A variety of enzymes reside in the peroxisomes including oxidases, catalase, and peroxidases (McGilvery & Goldstein, 1983). The fatty acids of the peroxisomes are oxidized following the same pathways used by the mitochondria, but here ATP is not produced and instead of water hydrogen peroxide results. The oxidation of peroxisome fatty acid does not degrade it completely to acetyl-coenzyme A (CoA), but Octanoyl CoA is sent to the mitochondria so as to complete the oxidation by means of electron transport chain and citric acid cycle (Figure 1.8). Hence, rise in peroxisomes numbers and activity and increase in fatty acid catabolism requires a concomitant rise in fatty acid metabolism in the mitochondria (Stott, 1988).
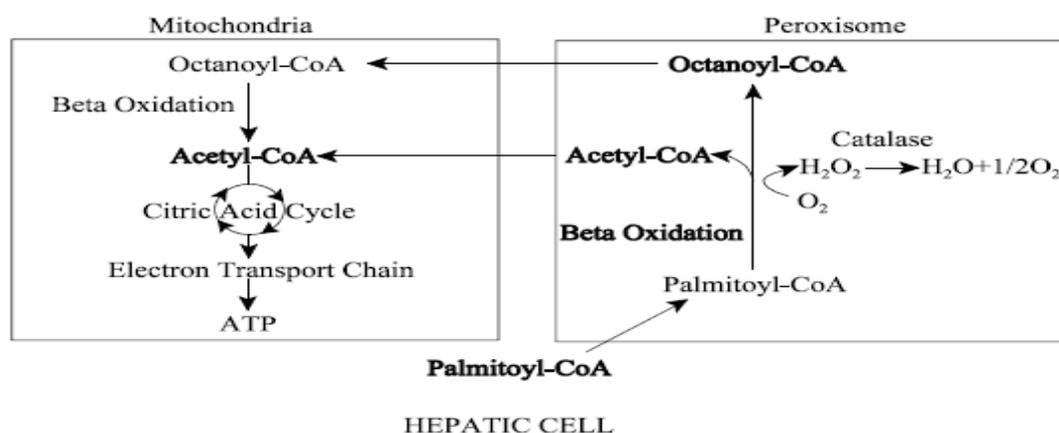
Figure 1.8: Metabolism of Peroxisomal Fatty Acids (Stott, 1988).

Multiple changes take place in the peroxisome enzymes after exposure to DEHP, of which some are caused by peroxisome increase in activity, but some enzymes are also induced. Many studies recorded a rise in the enzyme activities that lead to catabolism of fatty acids (palmitoyl-CoA oxidase, carnitineacyltransferase, enoyl-CoA hydratase, and α-glycerophosphate dehydrogenase) in rodents following exposure to DEHP (Cattley et al., 1988; David et al., 1999; Lake et al., 1986; Poon et al., 1997; Rhodes et al., 1986).

There is little evidence to validate the DEHP impact on catalase in rats. This little evidence points to some dose and duration conditions that lower catalase activity (Ganning et al., 1989; Rao et al., 1987), others that keep it constant (Perera et al., 1986), and a third range of conditions that increase it (Perera et al., 1986; Tamura et al., 1990).

The peroxide that is produced by the peroxisomes is detoxified by catalase and the peroxidases present in the hepatic cells. As the catalase and glutathione peroxidase levels and activities in rats fluctuate following exposure to DEHP, this may indicate that the ability of the peroxisomes to oppose a rise in hydrogen peroxide production is limited. One can also assume the impact of external factors such as type and quantity of food, and other metabolic factors on the liver may influence the ability of the free radical defence of the liver to deal with the rise in hydrogen peroxide production in the peroxisomes. Beside the above-mentioned enzymes, the lipid ubiquinone, which is an antioxidant that protects against lipid, protein and DNA oxidation, significantly increase after treatment (Turunen & Dallner, 1998).

1.3. THE EXPERIMENTAL SPECIES
1.3.1. POLYCHAETA

There are about 10,000 species of Polychaeta, which is the larger of the two major groups of currently known segmented worms (Annelida phylum) – the other being Clitellata phylum (earthworms and leeches). Polychaete worms have elongated, metameric bodies usually with a pair of appendages known as parapodia on each segment (metamere), in addition to tufts of chaetae (spines controlled by muscles which can usually be extended and retracted; thus giving polychaetes the name of bristle worms (deriving from Latin and literally meaning many bristles). Parapodia are diverse in both form and function, with functions that include locomotion, protection, gas exchange, attachment to substrates, and control of water flow; although parapodia are sometimes diminished or lost altogether. The head of a polychaeta is sometimes

provided with several sensory structures including antennae, tentacular palps, and cirri. Carnivorous species may possess large pharyngeal jaws. The segmented body ends with a tail, known as the pygidium, where the anus is located (Brusca & Brusca, 2003).

Some polychaetes live freely, with highly developed muscles that help them move, swim, crawl, or burrow, usually assisted by parapodia that are adapted as legs or paddles. Burrowing polychaetes usually have a muscular proboscis that aids in digging. Conversely, sedentary polychaetes feed using permanent burrows or tubes, either by selective deposit feeding, suspension feeding, or feeding on detritus. The parapodia are usually adapted to circulate water or create turbulence. Sedentary tube-dwellers possess soft muscular bodies and may lose the septa that join the segments. This helps the worm adjust hydrostatic pressure inside its body, which is an important function that helps it anchor its tail to the tube that houses its body. The tube not only provides protection, but also functions as external support for the worm. Tubes range from soft, parchment-like, made of mucus and sand, or hard calcareous tubes, and when several worms are together, a reef structure is formed (Brusca & Brusca, 2003).

In line with the polychaete diversity of lifestyles as well as degree of body segments independence, respiratory and circulatory systems also demonstrate a lot of diversity among the taxa. In many polychaetes, gas exchange is aided by distinct gills, while in some of them gas exchange is carried out by the entire body surface (especially sedentary taxa without parapodia, or worms that have no septa that can separate coelomic spaces). Some taxa enlarge their surface respiratory areas by means of feathery body surface protrusions to allow the coelom to extend. In such taxa, the respiratory and circulatory systems are reduced, and the body distributes most of the oxygen and nutrients in the coelomic fluid. Other taxa, especially sedentary worms, use pumping structures to improve blood flow, as they move parts of their bodies to circulate blood. Most polychaetes possess oxygen-carrying pigments freely moving in the coelomic fluid and circulatory fluid, often taking the form of haeomoglobin. In the same way polychaetes also have diverse sensory structures that include touch receptors; photoreceptors, some of which are developed into anteriorly located eyes or on other parts of the body; statocysts and chemo-receptors (Brusca & Brusca, 2003).

The most common polychaetes, the Nereididae, are commonly known as rag worms. There are some 500 Nereididae species. Fossil discoveries indicate that some Neredidae may have lived 350 million years ago (Rouse & Pleijel, 2001). The habitat of most Nereididae is shallow water, but in the oceans they are found in all types of substrates. Furthermore, some such as the Hediste genus live in brackish water. The

Nereididae size ranges from just a few centimetres (Micronereis) up to more than one metre (Neanthes virens). In the same way the colour varies from transparent to red-brown, but colours out of this range as well as other pigmentation patterns also exist. Some polychaete groups have economic importance, while some are used as bait in fishing and others constitute a delicacy in some south-east Asian cuisines (Rouse & Pleijel, 2001).

1.3.2. POLYCHAETA AS A MODEL ORGANISM

The polychaete species *Capitella capitata* was first used by Reish and Barnard (1960) for toxicological testing, followed by many other authors in this line of research using other polychaete species. By now polychaetes are standard bioassay organisms, being widely used for assessing marine and elutriates sediments toxicity either as regular routine procedures or for impact assessment (Bat, 2005; Dean, 2008).

Polychaetes are regarded as relevant and convenient organisms for sediment toxicological studies, first, because they spend most of their lives living in the sediment and hence are continuously and directly exposed to sediment-bound pollutants (Pocklington & Wells, 1992). Second, many of the polychaete species feed directly ingesting benthic sediments, along with the pollutants that may exist therein, and by doing so provide for study of an additional polluting pathway through the gut wall, while others filter and process large quantities of water for food, or scavenge on other smaller animals. Third, polychaete species are available throughout the year, often occurring in abundance. They tolerate different salinities and sand particle sizes and occur in different geographic areas, and hence are readily available for testing and monitoring, as well as suitable for a broad range of sediments (e.g. (Lana et al., 1997, Oshida et al., 1981).

Polychaetes serve as ideal toxicological testing organisms thanks to their relatively short life cycles, small sizes, and ease of culture maintenance. Polychaetes' life cycles and developmental stages characteristics provide good pollutant monitoring tools and have often been used to this end because they are easier to deal with compared to adult stages. However, factors such as salinity, oxygen content, temperature and presence of organic matter or sediments have confounded the use of this advantage. Due to these confounding factors, the impact of toxic substances on marine animals is difficult to quantify, and the accuracy of the laboratory toxicological results obtained from experiments with these organisms cannot now be taken for granted (Dean, 2008).

1.3.3. THE EXPERIMENTAL SPECIES

1.3.3.1. <u>Taxonomy</u>

Kingdom: Animalia

Phylum: Annelida

Class: Polychaeta

Order: Aciculata

Family: Nereididae

Genus: *Hediste*

Species: *H. diversicolor*

O. F. Müller (1776) defined *Hediste diversicolor* as a polychaeta widely distributed in estuaries and lagoons from North Africa to North Europe (Mettam, 1979, 1981). The species is physiologically highly tolerant to different environmental factors including extreme ones, and is capable of growing and reproducing in different types of sediments and stressful environments (Bartels-Hardege & Zeeck, 1990, Cheggour et al., 1990, Miron & Kristensen, 1993; Scaps, 2002). Not only does it adapt well to different environments, but it also feeds on very different food sources. It is capable of diversifying its food source sizes from micro and macro-zooids and diatoms, to tiny fragments of organic matter contained in silt and stone debris (Reise, 1979; Smith et al., 1996; Lucas & Bertru, 1997) (Figure 1.9).

Hediste diversicolor uses various strategies to obtain food: (1) deposit feeding in which the organism captures food from the sediment surface (Esselink & Zwarts, 1989; Esnault et al., 1990); (2) suspension feeding in which the organism releases a web of mucus and by means of ventral-dorsal movements, it creates a continuous current that sends the phytoplankton to the web, where it is stored to be consumed later (Harley, 1950; Riisgård, 1991; Vedel, 1998). This feeding strategy is adopted by the organism when the water column is full of phytoplankton (Vedel & Riisgård, 1993); (3) herbivorous feeding where the organism consumes fragments of algae and macrophytes (Olivier et al., 1997; Hughes et al., 2000) (4) carnivorous feeding in which the organism acts as a predator preying on different benthic fauna species, thus playing a significant role in the composition of brackish water ecosystems (Rönn et al., 1988). Adoption of one or another of these feeding strategies appears to depend on factors that include availability and quality of food, predators' presence or absence, and tidal heights and seasons (Esselink & Zwarts, 1989).

Figure 1.9: Full body of *Hediste diversicolor* (Garcia-Alonso et al., 2011).

1.3.3.2.   <u>Geographical Distribution</u>

Hediste diversicolor is among the common inhabitants of intertidal mud sediments in estuaries and sea shallow waters and has a very wide geographical distribution (Clay,1967). The species inhabits many parts of the North Eastern Atlantic from Morocco to northern Europe. Several studies conducted on the worm's life history have shown considerable variations in its ecology and biology in different places, depending on different environmental gradients and habitat conditions, but some features of the species ecology and biology are still unknown. Good knowledge about some populations of the organism so far exists but mostly from northern Europe where most of the studies were conducted (Abrantes et al.,1999).

1.3.3.3.   <u>*H. diversicolor* a Form of Bio-Indicator Species in this study</u>

Among the methods of bio-monitoring is to monitor the pollution level of a particular environment using a bio-indicator species. Such a method is applied to quantify contaminant concentrations in the species that can store chemical compounds in their tissues (Durou et al., 2007a). *H. diversicolor* is one of the species that can be used as a bio-indicator because it can take contaminants from the sediment and store them in its tissues. It is also relatively sedentary, and hence can adequately indicate the conditions of the local environment. Thus, by measuring the levels of a particular contaminant in this species, the levels of contamination in a certain marine environment can be monitored (Durou et al., 2007a). As the members of this species are easily available in large numbers and can survive laboratory conditions, they can be used to investigate the contaminant effects. Thus, since the 1950s, the species has been used as an environment monitoring species model (Scaps, 2002).

H. diversicolor is regarded as an ecologically important species (Durou & Mouneyrac, 2007). It has several key roles in many ecosystems. Among these roles is its role as an important source of food for crustaceans, flatfish and wading birds (Durou & Mouneyrac, 2007; Berthet et al., 2003). It is for example the primary source of food for the black head gull in the summer. Female oystercatchers favour *H. diversicolor* throughout the year, and *H. diversicolor,* along with *Scolopus armiger* and *Nephthys hombergi,* make up to 99% of the food sources of the bartailed godwit in the winter (Lawrence & Soame, 2009). The larvae of *H. diversicolor* also make up a source of food for large crabs, shrimps and small fish like the gobies (Scaps, 2002). Since it is closely related to the contaminated sediments and at the same time a source of food for many species of animals, it has also an important role as a means of transport of contaminants up the food chain, and a means of bioaccumulation in the food chain (Kalman et al., 2009).

In addition to being a source of food, *H. diversicolor* also impacts its environment as a food gathering organism. Although it is omnivorous it also engages in active predation, and in filtering and feeding. By means of its effects as an active predator, it regulates the benthic populations (Lawrence & Soame, 2009), and by virtue of its efficiency as a filter feeder, it has also become important in the marine environment. It is estimated, due to the abundance of these worms in the fjords, that they can filter the whole water mass about three times daily, and by doing so they can cut the phytoplankton biomass within 5 hours by 50% (Lawrence & Soame, 2009).

H. diversicolor has a third role in the ecosystems which is the part it plays in sediment bioturbation (Kalman et al., 2009). Bioturbation by *H. diversicolor* is the effective displacement and mixing of the sediment particles, which is very much related to its burrowing behaviours (Bonnard et al., 2009). Bioturbation has a role in creating oxygenated zones in sediments void of oxygen, thus promoting microbial growth (Scaps, 2002). *H. diversicolor* often has a high density in the sediment (37-3700 individuals per square metre), and for this reason it has important impacts on the composition and features of the sediment (Kalman et al., 2009). Among these features is biogeochemical-cycling, both nutrients and contaminants distribution and metal speciation (Durou et al., 2008).

1.4. THE IMPORTANCE OF INFO-CHEMICALS IN THE FUNCTIONAL TRAITS OF AQUATIC ECOSYSTEMS

Organisms are always in quest of fitness, and do so through functional traits that may include morphological, structural, biochemical, physiological, behavioural or phenological characteristics (Nock et al., 2016). Marine organisms in particular adapt their behaviours according to the chemical cues they encounter in their environment. As the aquatic environment where marine organisms live is marred by a complex jumble of chemical background noise, they have to distinguish between different cues through the odours they emit. Most important among the behaviours that are distinguished through chemoreception are feeding and avoidance of predators. Abiotic disruptions that may occur in these vital processes may seriously impact their fitness and even survival (Bronmark and Hansson, 2012).

1.4.1. CHEMORECEPTION

Chemoreception generally refers to the manner in which organisms perceive chemical stimuli, and includes olfaction, which denotes smelling and gustation which denotes tasting. Polychaetes and many other marine organisms use chemical cues including chemoreception to perceive many important ecological behaviors including feeding, reproduction, recruitment and defense (Zimmer and Butman, 2000). Although ecologically important, chemoreception molecular mechanisms of polychaetes are still relatively unknown.

All organisms in the animal kingdom utilise chemical senses; where chemical signals are used in binding to receptor sites, and thereby provide information to the brain (Wyatt, 2010). Chemoreception may either be gustatory (denoting taste), or olfactory (denoting smell) (Drickamer et al., 1996). Chemoreceptors, by means of chemoreception, enable members of the same species to distinguish the gender of each other, and are sensitised to such an extent as to allow them to detect chemicals that are species-specific, even at very low concentrations in the range of few molecules per million (Bronmark and Hansson, 2012). In complex seawater environments, chemoreceptors enable organisms to perceive information and process it, especially when no visual or other cues are available (Bronmark and Hansson, 2012). The importance of chemical signals lies in their ability to elicit essential behaviours including feeding, reproduction and avoidance of predators in many seawater organisms (Dunham, 1978). To be successfully used as a means of communication, the said chemical signals must be produced and released by the sender, and then received and

processed by the receiver (Wyatt, 2003). The signal may get disrupted due to failure of one or more of these processes, which may result in poor information transfer (Wyatt, 2003). Most of these chemical signals function at low concentrations and then degrade rapidly (Bronmark and Hansson, 2012). Besides, the seawater environment contains a cocktail of chemicals between which organisms must distinguish (Derby and Sorenson, 2008). They often exist as complex mixtures, rather than pure compounds (Wyatt, 2010). The evolutionary history of organisms show that they have developed meticulously tuned chemosensory systems in order to be able to detect such compounds (Bronmark and Hansson, 2012). When unable to successfully detect and identify odours due to signal disruption, this may seriously compromise the ability of the organism to survive and live up to the challenges of the environment, especially in the case of organisms that heavily rely upon chemoreception (Wisenden, 2000).

1.4.2. PREDATOR-PREY INTERACTION ECOLOGY IN AQUATIC ECOSYSTEMS

Predator-prey interaction is regarded as a kind of evolutionary arms race. Selection is a key weapon for the predator, if it is to be efficient in detecting and capturing its prey. Likewise, selection plays a key role in aiding the prey to be efficient in detecting and evading its predator. At the selection functional level, claws, teeth and stealth play a key role in the success of the predator, while spines, crypsis, and rapid flight play the role of successful evasion for the prey. At the selection mechanistic level, the predator-prey interaction may represent a race between sensory systems where the predator and prey get rewarded for gaining timely information edge over the other. Early detection plays a key role in gaining an edge as predators easily catch unaware preys. On the other hand a prey that has an early knowledge of the predator's approach easily evades it through crypsis, avoidance of the area, seeking shelter, or early flight in case of an attack (Lima & Dill, 1990; Smith, 1992). As the result of loss of the race differs for the predator and the prey, since it is merely lunch for the former and life for the latter, the selection gradient for the prey is much greater than that of the predator, leading to a high skill in detection and subsequent evasion by the latter (Ferrari et al., 2010).

Predation proceeds through successive intensifying stages from the initial detection of the other (by either prey or predator), through attack, capture, and finally ingestion (Lima & Dill, 1990; Smith, 1992). The various stages of the predation sequence consist of stage-specific patterns of released chemical information, which

factually indicate the existence of the predation risk, as well as provide other information about the characteristics of the risk and its imminence. The initial stage of detection consists of the kairomone, a specific odour of the predator scented by the prey.

A kairomone is a chemical substance released into the environment by a species i.e. the predator and received by another species such as the prey that conveys information to the receiver. The prey is adaptively suited to be the second species rather than the first (Ferrari et al., 2010) but there are cues that also function as allomones and pheromones (Wyatt, 2003). On the other hand, the hormones are produced by the endocrine system to control the physiology and sometimes also behaviour. They are essential for the daily life activities, including the processes of digestion, assimilation, metabolism (anabolism and catabolism), growth and reproduction as they control the physiology of the organisms (Neave, 2008). The kairomone allows the prey to detect the predator and evade it while lying in ambush and before it comes very close.

The literature has characterized many types of kairmones in terms of their morphological and behavioural responses from crustacean plankton through midge larva (*Chaoborus Lichtenstein*) to various fish species (Tollrian & Harvell, 1999; Lass et al., 2005). Another chemical information released by the disturbed or startled prey comes before the attack. Such chemical information constitutes disturbance cues, and the bulk of the literature has characterized this cue as pulses of urinary ammonia (Kiesecker et al., 1999). A third type of cues is the damage alarm cues that also stem from the prey. During the attack and handling of the prey, and before ingestion, the predator first damages the epidermal tissue of the prey. Damaged epidermal tissue releases chemicals that are not released in other contexts, and by doing so factually establishes the existence of active foraging by a predator. From single celled protists to amphibians, all injured conspecifics of aquatic organisms release chemical cues in the form of anti-predator responses (Wisenden, 2003). These chemical compounds, referred to as alarm cues, generate strong behavioural responses and morphological defences, which become so common as to considerably reduce predation. There are also the "dietary cues" which are also alarm cues possibly having similar chemistry to the above alarm cues. They are emitted after ingestion from the digestive system of the predator as it digests the meal and defecates. They also generate behavioural and morphological as well as life history responses. The final class of chemical information used by the prey in predation risk assessment has ecological significance, and the prey acquires it through learning. Minnows serve as a study model for this class of cues (Brown, 2003). Generally, any new chemical stimulus that arouses alarm can be used by the prey as alarm cues and

then used to assess the predation risk (Suboski, 1990; Chivers & Smith, 1994). The remarkable property of this kind of learning is that it requires only a single experience to generate an almost permanent association with predation in many kinds of prey animals (Suboski, 1990). Chivers and Smith (1998) extensively reviewed the chemical ecology related to predator-prey interactions in aquatic organisms.

1.4.3. CHEMICAL SIGNALS AND POLYCHAETE FEEDING

The manner in which marine organisms find food involves different behavioural mechanisms. Some of them detect food items by their shapes, sizes and colours using visual receptors, while others detect food by means of mechanoreceptors. Mechanoreceptors help detect moving preys by means of hydrodynamic disturbances in water columns. Haliplanella luciae and other sea anemones possess plenty of hair bundle mechanoreceptors found on their tentacles, which enable them to respond to stimuli from small swimming organisms carried by water vibrations (Watson and Hessinger, 1989). However, poor visibility that often mar the complex seawater environment, compel many marine organisms to rely heavily on chemoreception in locating food, using a blend of complex chemical signals and several sensory organs to locate food (Bronmark and Hansson, 2000).

Chemoreception plays an important role in the choice of food, for instance in discriminating between nutritious food and harmful or distasteful substances (Wyatt, 2003). Chemicals involved in feeding behaviour are either the ones that affect at a distance, which are classified as attractants, arrestants or repellents, or those that affect by contact, which are classified as incitants or suppressants, and stimulants or deterrents (Bronmark and Hansson, 2012). Nereidid polychaetes and similar organisms can detect food nearby visually within the water column, and hence need to use chemical detection.

Polychaetes ecology and feeding behaviours have been the subject of study for many years. The seminal manuscript of Fauchald and Jumars (1979) entitled "The Diet of Worms" provides good insights in the feeding mechanisms of polychaetes. Feeding behaviours in which chemical processes are involved has been described in many polychaete species. For instance, Copeland and Wieman (1924) described the response of Nereis virens to animal food as it emerges from its burrow in response to crumpled periwinkles. In a recent study, Watson et al. (2005) used such a feeding behavior to investigate the manner in which chemical signals mediate *N. virens* predator-prey interactions, and thereby assessed whether conspecifics extracts, prey species of other

polychaete, or flatfish muscle (as a predator), reduce the feeding of N. virens. Extracts of whole-body conspecifics considerably reduced the activity of polychaetes outside the burrow as well as the number of worms feeding, while flatfish muscle extracts reduced just the number of feeding worms, and *Arenicola marina* extracts did not affect the feeding of *N. virens.* Thus extracts of whole-body conspecifics may have acted as alarm signals that reduced the feeding activity. However, chemical signals released by injured individuals of *N. virens* were not assessed in their resemblance to whole-body extracts homogenates, a matter which deserves study.

Mangum and Cox (1971) reported the feeding behaviour of *Diopatra cuprea* (Bosc), the onuphid polychaete, in relation to 32 different extracts of marine organisms, as well as to several chemicals such as amino acids extracted from bivalve flesh. The responses of worms were positive to almost all organisms, including members of its kind. Some sugars as well as hemoglobin and other polychaete fresh blood, generated feeding currents or induced mouth opening. Worms responded to several amino acids by producing feeding currents, at concentrations that generated feeding currents of 10-5 M to 10-8 M in response to methionine, phenylaline, cysteine, proline, valine, and hydroxyproline. It was noted that the presence of between four and six amino acids existing in two clam species extracts could explain the feeding response of D. cuprea.

As polychaetes that feed on deposit modify the environment of the sediment on a continuous basis while feeding, and by doing so variously mobilize the sediment depending on the food supply, a good amount of literature attempted to answer the question of what determines the amount of food supply in the sediment and make some sediments better than others (e.g. Jumars, 1993). However, the literature is largely deficit in studies that should investigate the signals that trigger ingestion and govern the feeding rates of deposit feeders. Jumars (1993) suggested that several stimuli may be responsible for the regulation of ingestion rates including taste, smell, gut distension and absorbed products detection in body fluids. Chemoreception coordinates feeding by many polychaetes that feed on deposit. For instance, in some spionid polychaetes, the feeding rate is depressed by fresh fecal material (Miller and Jumars, 1986). Phagostimulants are implicated as well. Polychaetes and some other macrofauna that live on the continental slope and in pelagic areas of the sea respond rapidly to fresh phytodetritus (e.g., Levin et al., 1997; Witte et al., 2003).

Duchêne and Rosenberg (2001) reported from laboratory experiments that the addition of a mixture of phytoplankton matter to the sediment surface resulted in an increase in activity of Melinna cristata (Sars), the ampharetid polychaete, on the

sediment surface. Streblospio benedicti, the polychaete that feeds on deposit, prefers organically enriched sediments to unaltered sediments when given the choice (Kihslinger and Woodin, 2000).

1.5. THE EFFECTS OF CHEMICAL STRESS ON BIOCHEMICAL, PHYSIOLOGICAL AND BEHAVIOURAL

When we consider the cascading effects, we find that chemical stress is linked to the local extinction risk in estuarine and coastal areas. This is expressed in the form of early or sensitive indices biological responses impairments from the part of the individuals, through the individual level to the supra-individual level, because of their high ecological relevance (Weis et al., 2001; Amiard-Triquet and Rainbow, 2009) (Figure 1.10). When feeding is reduced and digestive enzymes impaired, this can result in reduced energy intake. Besides, when energy metabolism is impaired and defences activated (metallothionein induction), along with more activation of glutathione-S-transferase (Hoarau et al., 2001; Amiard et al., 2006), this can lead to tolerance, and changes in energy allocation accompanied by changes in growth, reproduction and population levels (Calow, 1991). At the same time, neurotoxic changes may be uncovered by the determination of neurotransmitters levels such as Acetylcholinesterase (AChE), increased activation and a principal biomarker whose link with disturbances in behaviour has been tested before (Amiard-Triquet, 2009). Besides feeding, endobenthic species burrowing is also a behaviour that should be studied because it controls the predator-prey relationships.
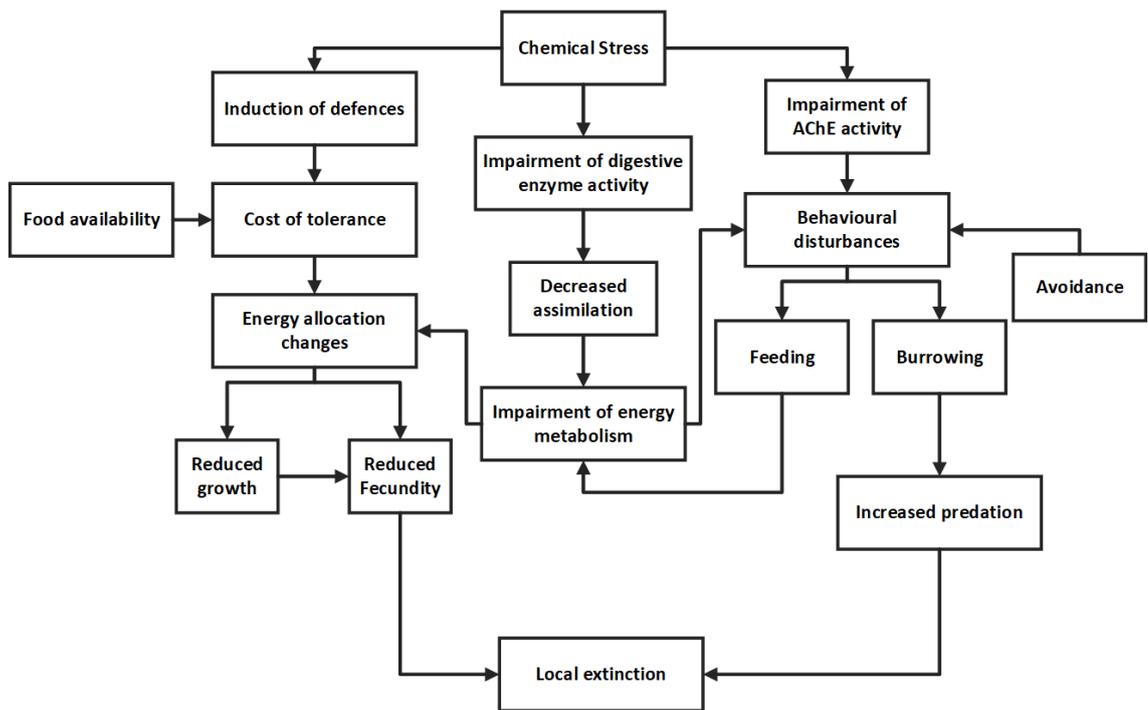
Figure 1.10: Cascading effects of chemical stress at different levels of biological organisation (Boldina-Cosqueric et al., 2010).

1.6. THE RESPONSE OF THE CELL TO SPECIFIC CHEMICALS OF ITS ENVIRONMENT BY MEANS OF SIGNAL-TRANSDUCTION PATHWAYS

Cells have strong responses to some chemicals in their environments. Hormones are among the chemical signals that make the cell respond to changes in environmental conditions. Food molecules or aromas convey taste or smell by means of their interaction with certain sensory cells. One needs to know the manner in how cells respond to change in the environment or to information from there. Cascades of signal-transduction act between sensing and the processing of the stimuli. There are molecular circuits that detect, intensify, and integrate different external signals so as to create responses to the changes that may take place in enzyme activity, ion-channel activity, or gene expression (Figure 1.11). One also needs to know the challenges posed when extracellular information is transferred to the cell's interior (Berg et al., 2002).
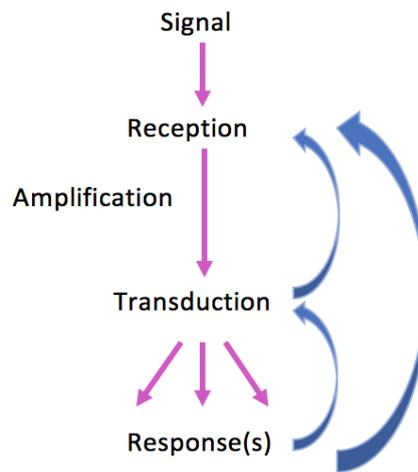
Figure 1.11: Signal Transduction Principles: A signal from the environment, e.g. a hormone, first received by interaction with a component in the cell, such as a receptor on the cell surface. The information about the signal is then changed to a chemical form. The signal often needs to be amplified before it evokes a response (Berg et al., 2002).

1. Membrane receptors take information from the environment and send it to the interior of the cell:

Some nonpolar signal molecules such as steroid hormones, including estrogens, can diffuse across the cell membrane and enter the cell. These molecules enter the cell and bind with proteins which then interact with DNA directly, thus modulating gene transcription. In this way, a chemical signal can enter the cell and directly change its gene-expression patterns. However, most signal molecules cannot pass through the membrane because of their large size and great polarity, and the absence of an appropriate transport system. Thus, information indicating the presence of signal molecules need to be transmitted through the cell membrane without the need for molecules to enter the cell. A receptor protein associated with the membrane often plays the role of an information transferor through the membrane. Such a receptor is a membrane protein that possesses both intracellular and extracellular domains. Signal molecules are recognized by a specific binding site, and these usually exist on a extracellular (or intracellular) domain (often called a ligand). Such a binding site is akin to an enzyme-active site but without catalysis taking place there. The ligand interacts with the receptor and thereby changes the receptor's quaternary or tertiary structure, and also changes the intracellular domain. These changes in structure are not enough to generate a suitable response, since they are limited to a few receptor molecules existing in the cell membrane. The information generated by the ligand's presence, which is referred to as the primary messenger, needs to be transduced into another form that can change the cell's biochemistry (Berg et al., 2002).

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2. Messengers convey information from the receptor-ligand site:

The next stage in the circuit of molecular information is made up by changes in the small molecules concentration, which are called the second messengers. These second messengers are made by the cyclic GMP and cyclic AMP, inositol 1,4,5-trisphosphate (IP_3), calcium ion, and diacylglycerol (DAG). The second messengers are used to perform various functions. First, they can often diffuse freely to other chambers of the cell, including the nucleus, in which they can alter some processes such as gene expression. Second, the signal can be greatly amplified during the generation of these second messengers. During the generation of the second messengers also, enzymes or membrane channels are often activated, while each macromolecule is activated resulting in the creation of numerous second messengers in the cell. In this way, a low concentration signal of even one molecule, can generate a large signal and a big response in the cells. Third, common second messengers used in multiple signalling pathways may create opportunities as well as problems. Input from various signalling pathways, which is often referred to as cross talk, may change the concentrations of the second messengers. Cross talk allows more finely adjusted cell activity regulation relative to independent individual courses of action. However, unsuitable cross talk can lead to misinterpretation of the second messengers (Berg et al., 2002).

3. Phosphorylation of protein is a common way of information transfer:

Many second messengers activate protein kinases in order to generate responses. Phosphoryl groups are transferred by these enzymes from ATP to threonine, serine, and tyrosine residues in the proteins. Protein kinase A is also known as cAMP-dependent protein kinase. This enzyme converts changes in the free second messenger's concentrations into changes in the protein's covalent structures. These changes are more permanent than the changes that take place in secondary-messenger concentrations, but protein phosphorylation can be reversible. Specific phosphoryl groups are removed hydrolytically from modified proteins by protein phosphatases (Berg et al., 2002).

4. Termination of the signal:

One mechanism by which to terminate a signalling process is by protein phosphatases. The initiation of a signalling process results in information being transduced to create change in the cellular processes, and thereafter the signalling processes have to be terminated. This termination allows the cells to respond to new

signals. Furthermore, improper termination of signalling processes may result in uncontrollable cell growth and possibly cancer (Berg et al., 2002).

1.7. THE PROJECT AIMS

The main aim of this study is to investigate and assess the impact of Di (2-ethylhexyl) phthalate (DEHP) on marine polychaetes with long-term at low DEHP concentration and short-term at high DEHP concentration of exposure upon fitness (behaviour) and biochemical biomarker (enzymes activity). The marine polychaetes: *H. diversicolor* was used to achieve these aims (Figure 1.12). In order to achieve the aim the following five objectives will be investigated:

1. *Examine the fate of DEHP in H. diversicolor Culture System Toxicity (Worms, Seawater and Sediment) When They Are Exposed to Low Environmental Concentrations with Long Term of Exposure (Three Months) And High Concentration with Short Term of Exposure (7 Days).*
   Hypothesis: When exposed to DEHP worms will accumulate DEHP over time and in a concentration dependent manner.

2. *Examine the Effects of DEHP In Stability of Functional Traits on H. diversicolor with Short and Long Term of Exposure*

Hypothesis: Functional traits such as feeding behaviour will be negatively impacted upon by DEHP exposure.

3. *Examine the Effects of DEHP in Biochemical Biomarker Development on H. diversicolor with Short and Long Term of Exposure*

Hypothesis: Enzyme activity of oxidative stress regulating enzymes will be upregulted when worms are exposed to DEHP.

4. *Determine the DEHP Level at Different Seasons and Different Populations in UK.*

Hypothesis: DEHP levels in worms collected at different locations in the Humber estuary area will vary and also vary during the seasons.
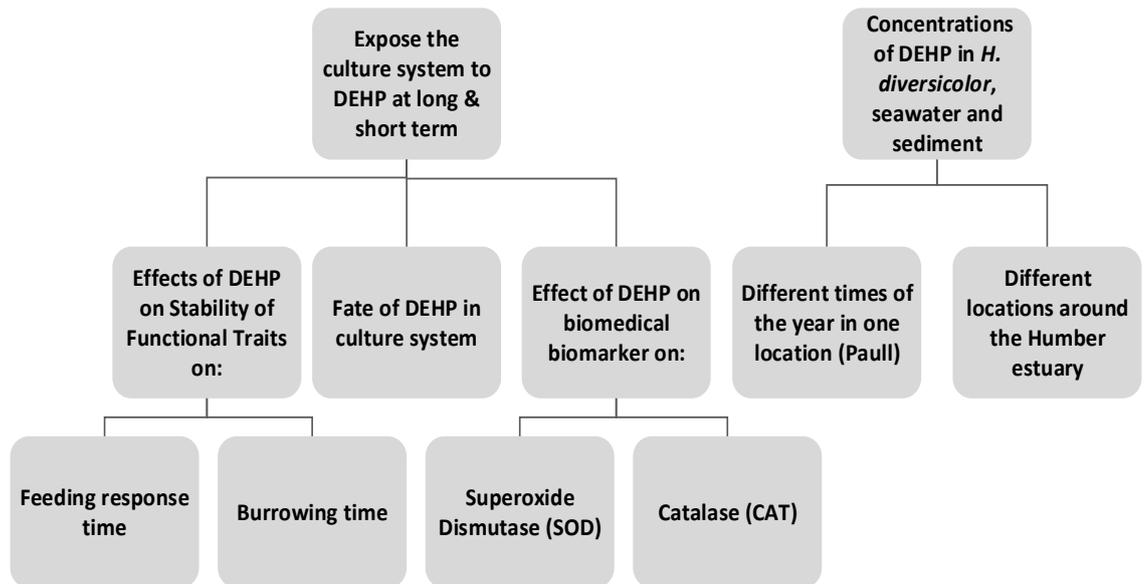
Figure 1.12: The experiments design that show the concentrations of DEHP in culture system and their effects.

CHAPTER 2: GENERAL MATERIALS AND METHODS

2.1. *H. DIVERSICOLOR* COLLECTION DETAILS

H. diversicolor samples were obtained from the foreshore by hand from mudflats at Paull village in East Riding, Yorkshire, England (Figures 2.1 and 2.2). The samples were collected by members of the Functional Ecology Research Group. The specimens were brought to a University of Hull laboratory in containers along with water and sediment from the same place. Then, the samples were washed using filtered seawater at 18-20 ppt salinity (dependent upon the location population), and pH 8.2, under room temperature18°C. Thereafter, they were divided into three categories based on worm size: large size (6-4 cm), medium size (4-2.5 cm) and small size (2.5-2 cm). Worms were then taken to culture tanks and placed there for one week to allow them to properly acclimatize to this new culture environment. These methods were followed as recommended by Hardege et al. (2004).

Figure 2.1: The location of the study areas at Paull, the United Kingdom.

Figure 2.2: Location at Paull, UK from where samples of *H. diversicolor* were taken.

2.2. PREPARATION OF CHEMICALS FOR EXPOSURE

Di (2-ethylhexyl) phthalate (DEHP) was purchased from Sigma-Aldrich United Kingdom, with 99% purity (CAS No. 117-81-7 and Molecular weight 390.56 g/mol). Super stock solutions were prepared in methanol at 1 g/L. The calibration solutions were diluted from the stock solutions with methanol at 0.1g/L. Then, these were serially diluted with purified water to make solutions at concentrations of 500µg/L, 100µg/L, 10µg/L, 2 µg/L and 0.05 µg/L. The internal standard used in GC-MS determination of DEHP and for calibration and quantification, diallyl phthalate (DalP) was purchased from Sigma-Aldrich United Kingdom, with 99% purity. The stock solution were prepared in methanol at 1 g/L and 50ul of this solution is added to each sample. All solutions were kept at 4 °C in the dark as recommended by the material safety data sheet (sigma-Aldrich).

2.3. ECOTOXICOLOGICAL TANKS

2.3.1. ECOTOXICOLOGICAL TANKS FOR DETERMINE THE FATE OF DEHP IN CULTURE SYSTEM OF *H. DIVERSICOLOR* WITH LONG TERM

As Figure 2.3 shows, the ecotoxicological tanks contain four separate re-circulatory systems, each containing two culture tanks. The systems ran for 30 days prior before placing the worms. The reservoir system had a capacity of 50 litres, and each tank was supplied with an aeration pump in order to circulate seawater at a 35 ml/min rate approximately so as to ensure good seawater re-circulation, in order to prevent oxidation. The walls of the ecotoxicological tanks were of polycarbonated glass

of 60X30X20 cm dimension, having layers of 6 Kg coral sand and 9L of seawater. Seawater level above these sediment layers amounted to 4 cm with the layers being controlled by adjusting the height of the draining tube, which in turn allowed excess water to flow into the drain and out of the tank. This excess water was collected in the drain and then led to flow into a biological filter and returned to the reservoir.

Four systems each containing two tanks were used. 100 worms were placed in each culture tank, among which 33 worms were of small size (2-2.5 cm), 33 worms were of medium size (2.5-4 cm) and 34 worms were of large size (4-6 cm). Different sizes of worms were collected in order to have sufficient amounts of worms as some of them inevitably die or reproduce (also results in mortality) during the 3-month experimental period. Also, small worms may have a very different physiology than large, adults that convert energy into gamet development rather than growth, so to include all these one needs a larger pool of animals. Therefore up to 200 worms in total were added per system. In each tank, the worms were given 0.02g of fish food (flakes of tropical fish, Aquarian) twice per week. Grinding 0.02g of fish food flakes with a mortar and pestle, and add distilled water to make it liquid, and distribute slowly using a pipette, and feed the worms with them twice weekly.

The day to night regime was maintained at 16 hours to 8 hours by means of artificial illumination of 200-270 Lux and a low moonlight lamp of 15 watt power (Exo Terra), so as to illuminate the worms for four consecutive nights per month adding up to 28 day intervals, which is similar to the lunar phases. Temperature, salinity, pH and the photoperiod were maintained and monitored daily based on the experimental requirements according to Hardege et al. (2004). For each ecotoxicological tank salinity was monitored using manual refractometers (Fernox 55586 NA Refractometer). PH can be maintained manually either by the addition of CO_2 gas in order to reduce the pH or the addition of liquid NaOH to raise it, as needed (Hanna, HI9126, pH/mv meter with CAL Check[TM]). To reduce the likelihood of errors or anomalous results, the experiments have been repeated twice.
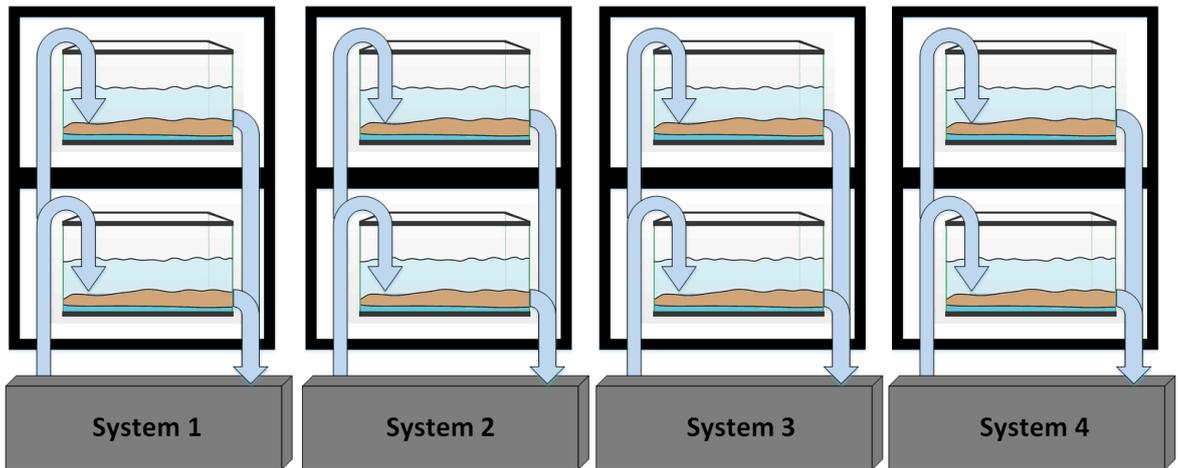
Figure 2.3: Shows four culture systems of eight ecotoxicological tanks that containing 100 worms, seawater and sediment in each tank.

2.3.2. ECOTOXICOLOGICAL TANKS TO DETERMINE THE FATE OF DEHP IN CULTURE SYSTEM OF *H. DIVERSICOLOR* WITH SHORT TERM

As Figure 2.4 shows, the ecotoxicological tanks contained three separate culture tanks. The tanks ran for 7 days prior to placing the worms. The reservoir tanks had a capacity of 50 litres, and each tank was supplied with an aeration pump so as to ensure good seawater re-circulation, in order to prevent oxidation. The walls of the ecotoxicological tanks were of polycarbonated glass of 60X30X20 cm dimension, having layers of 6 Kg coral sand and 9L of seawater. Seawater level above these sediment layers amounted to 4 cm.

Three tanks were used, and 100 worms were placed in each tank, among which 50 worms were of medium size (2.5-4 cm) and 50 worms were of large size (4-6 cm). The day to night regime was maintained at 16 hours light to 8 hours night by means of artificial illumination of 200-270 Lux and a low moonlight lamp of 15 watt power (Exo Terra, programmed with moon cycle data to enable coordination with natural moon cycle). Temperature, salinity, pH and the photoperiod were maintained and monitored daily based on the experimental requirements according to Hardege et al., (2004). Salinity and pH for each ecotoxiclogical tank were monitored using manual refractometers (Fernox 55586 NA Refractometer) for salinity and pH meters (Hanna, HI9126, pH/mv meter with CAL Check[TM]).
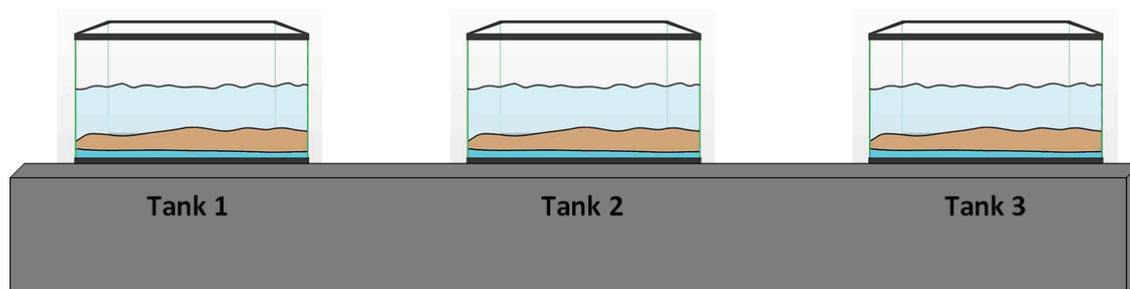
Figure 2.4: Shows three ecotoxicological tanks, each containing 100 worms, seawater and sediment.

2.4. DETERMINATION OF THE CONCENTRATION OF DEHP IN WORM TISSUE, SEAWATER AND SEDIMENT

2.4.1. PREPARARATION OF THE SAMPLES BEFORE ANALYSES BY GC/MS

2.4.1.1. <u>Liquid Extraction of Phthalates from Lipid Rich Substrates (Worm Tissue)</u>

➢ *Preparation of the samples*

Ten *H. diversicolor* worms of 2.5-6 cm sizes were obtained from the DEHP treated and the control (untreated) cultures. Worms were gently and individually taken (using fine animal handling tweezers) to 70mm Pyrex crystallizing dishes containing (free of sediment) filtered seawater which was run through a charcoal filter. They were then left for 6-8 hours in this filtered seawater so as to free their guts from any sediment. Worms were then kept at -20°C and dried by means of a freeze drying device for 48 hours and after that homogenized with a carbide pestle and mortar for chemical analysis.

➢ *Biochemical assays*

This method has been adopted from Guo et al. (2012) as follows: Acetonitrile was saturated with hexane by adding 60 ml acetonitrile to 15 ml hexane (4:1 ratio) in a glass conical flask fitted with a stopper. The mixture was shaken for half a minute and used immediately. The process is best carried out by an orbital shaker, where the flask can be securely fastened down with the stopper fitted so that no solvent spillage will take place.

Freeze dried homogenised worm tissue was placed in a Pyrex Teflon capped test tube (100mm x 6mm). 5 ml hexane saturated acetonitrile was added with 50µl Dalp at a ratio of l mg/ml in acetontrile. The contents were mixed by a whirl-mixer and thereafter

shaken for 15 minutes. These were then centrifuged at a speed of 4,000 rpm for 2 minutes. A glass pasture pipette was used to take the upper hexane layer to a 5 ml glass vial. This hexane layer contained the extracted worms' lipids, which were kept for future analysis. The acetonitrile lower layer contained phthalates. This layer was extracted again twice with 1 ml of hexane as before and all the hexane layers were kept for further lipid analysis.

The other lower acetonitrile layer was placed in a 25 ml rotary evaporator flask while the glass test tube was rinsed with acetonitrile (1 ml) which was also placed in the pear shaped rotary flask. Then 5 ml of milliQ water was added to the pear shaped rotary flask along with 1 ml of hexane. The flask was fitted with a stopper and shaken, and then the contents were placed in a new Pyrex test tube (100mm x 6mm size) and centrifuged in order to collect the upper hexane layer that contained the phthalates and place it in a 150mm x 6mm test tube. The lower water layer was then extracted twice with 1 ml of hexane, and the rotary flask was rinsed each time with l ml of hexane. The total hexane layers were placed in the large test tube after which anhydrous sodium sulphate (l g) was added so as to dry the contents, which were then mixed with a whirl mixer. These were then stored in a fridge for an hour or stored overnight in a freezer.

The hexane was then placed in a rotary evaporator flask of 25 ml capacity, and the hexane was removed to dryness. The phthalates that remained in the rotary flask were now dissolved by means of 1 ml of acetone. The flask was fitted with a stopper and the acetone swirled around many times. The acetone was then taken by a glass Pasteur pipette and placed in an auto-sampler vial to be used in GC-MS analysis (Figure 2.5).
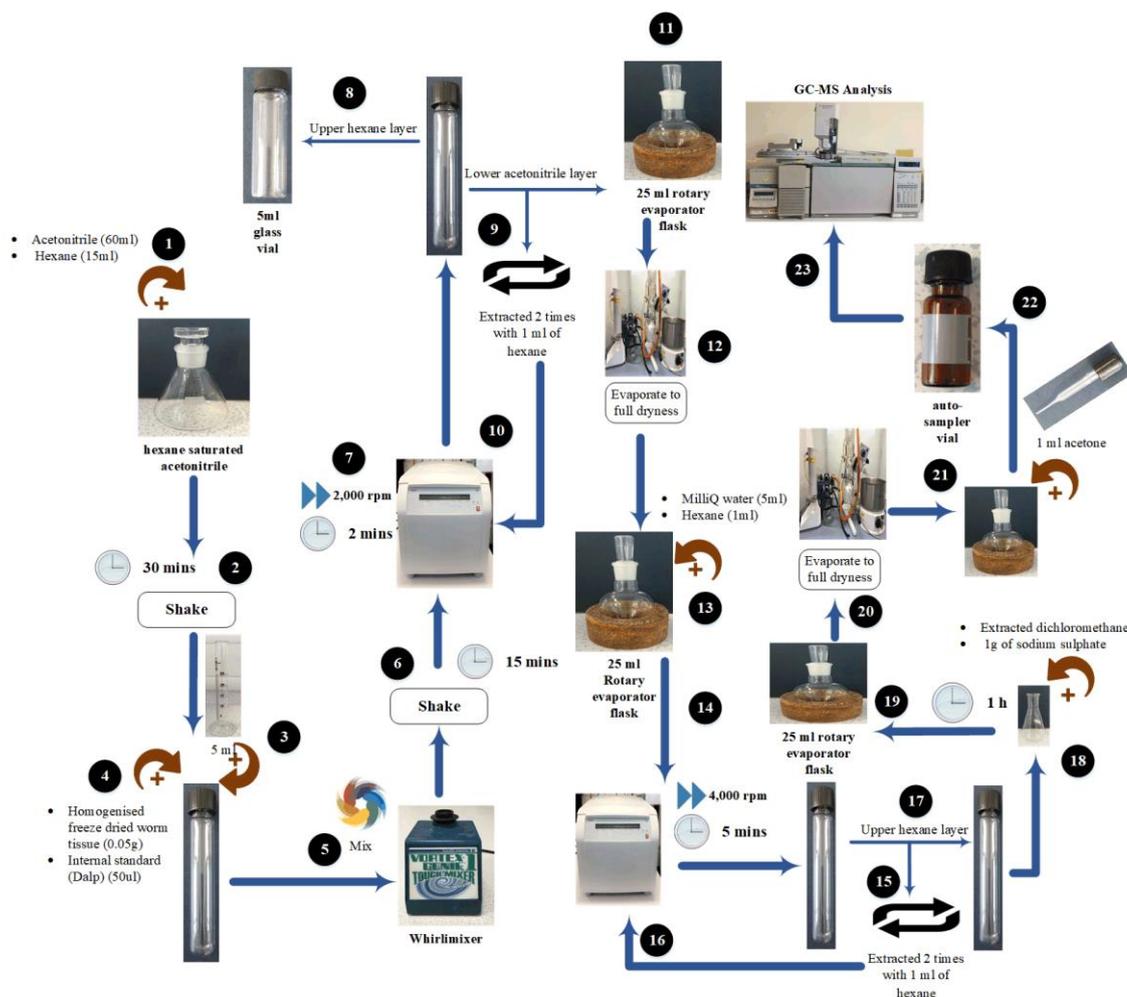
Figure 2.5: Liquid extraction of Phthalates from worm tissue.

2.4.1.2. <u>Liquid Extraction of Phthalates from Water Samples</u>

> ### *Preparation of the samples*

Surface and depth seawater samples (1500 ml) were taken from treated and untreated cultures. Initially and between samples, the container, a glass funnel of 500 ml size, was rinsed with 20 ml of dichloromethane.

> ### *Biochemical assays*

The separatory Funnel liquid-liquid extraction method was adapted from Method 3510C for Phthalate esters (USEPA, 1996). Samples were prepared by adding either 500 ml untreated seawater or 500 ml treated seawater to a separating funnel of 500 ml size. Then 50µl of Dalp was poured in to the seawater. The sample was taken three times by means of adding 25 ml of dichloromethane. Each time this was done by putting 25mls of dichloromethane in the seawater sample and shaken the mixture while

both the stopper and the bottom tap were firmly held for one minute. The separating funnel was then placed in a metal ring set on a retort stand and the contents allowed to settle until two clear layers were formed. Of these two layers, the top was the water layer and the bottom was dichloromethane containing the extracted phthalates. The dichloromethane bottom layer was retained by taking the glass stopper and then opening the tap slowly to allow the dichloromethane to pour into a conical flask placed underneath. The tap was then turned off after passing the dichloromethane layer. The water layer was then retained in the separating funnel, adding the 25 ml of dichloromethane twice after the first addition.

The total extracted dichloromethane (about 75 ml) was then dried by means of anhydrous sodium sulphate (about 3g) added to the conical flask and the contents mixed for half a minute manually and finally the dichloromethane left for one hour to settle.

The semi-dry dichloromethane was now transferred to a l00 ml rotary evaporator flask to evaporate it to full dryness. The conical flask containing the extracts was then rinsed with 5 ml dichloromethane and the extracts allowed to settle. The dichloromethane was then drawn by a Pasteur pipette, and used to rinse the l00 ml rotary evaporator flask after which all the solvent was transferred to a smaller 25 ml rotary evaporator flask. This transfer was repeated by adding another 5 ml of dichloromethane to the quickly fitting conical flask. The contents were then dried thoroughly in the rotary evaporator, and then l ml of acetone was added to the 25 ml rotary evaporator flask, after which the flask was closed with the stopper. The acetone was swirled around the walls of the flask so as to dissolve all the solvent residue and this was placed in a small auto-sampler vial so as to perform the GC analysis (Figure 2.6).
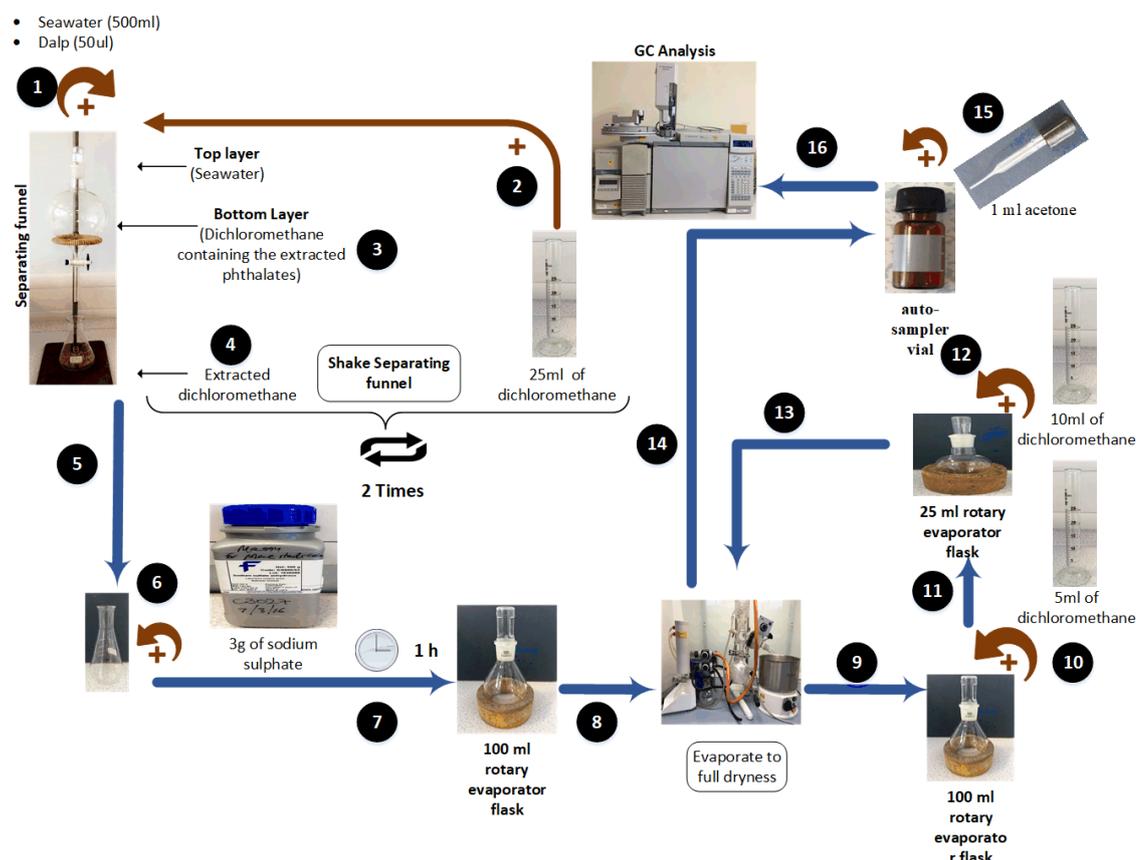
Figure 2.6: Lliquid extraction of phthalates from water samples.

2.4.1.3. <u>Phthalates Extraction from the Sediment Using the Accelerated Solvent Extraction</u>

➢ *<u>Preparation of the samples</u>*

A weight of 240g sediment was taken from the culture systems both treated and untreated. The sediment was placed in aluminium trays and dried in an oven at 110C for 2 hours or more. 40g of this sediment was taken and 50ul of internal standard was added (DalP) to it. The sediment was then taken to the Chemistry Department where it was extracted using 1000 mls of Dichloromethane: Acetone (1:1 parts) by means of an Accelerated Solvent Extractor.

➢ *<u>Biochemical assays</u>*

The florisil method was adapted from Method 3620C Cartridge procedure for phthalate esters (USEPA, 2014). The original method uses 10% acetone in hexane to elute phthalates, but this tends to elute lipids as well. The solvent thus collected was removed and a rotary evaporator was used to evaporate it to dryness. 5 mls of hexane was added to the residue to dissolve it and was then passed through 1g of Florisil SPE cartridge. This process was accomplished as follows:

The column was conditioned using 5mls of hexane. The solvent was soaked in the cartridge for about 5 minutes, after which it was passed under gravity, through the column, until 1-2 mm of the solvent was left over the sorbent bed. The extract was then placed onto the column so as to pass very slowly through it under gravity. Thereafter the rotary evaporator flask was rinsed using an additional amount of 5mls of hexane and the rinse was added to the cartridge and allowed to pass through.

The phthalates were eluted with 10 mls of acetone of 5% strength in hexane by adding it to the cartridge. The eluate of phthalates was then collected in a vial. The solvent was loaded in a 25 ml capacity rotary evaporator flask, and removed to dryness. The remaining phthalates in the rotary flask were dissolved using 1 ml of acetone. The flask was fastened with a stopper to secure the acetone, which was then shaken many times. Thereafter, the acetone was removed by a Pasteur pipette and transferred to an auto-sampler vial for GC-MS analysis (Figure 2.7).
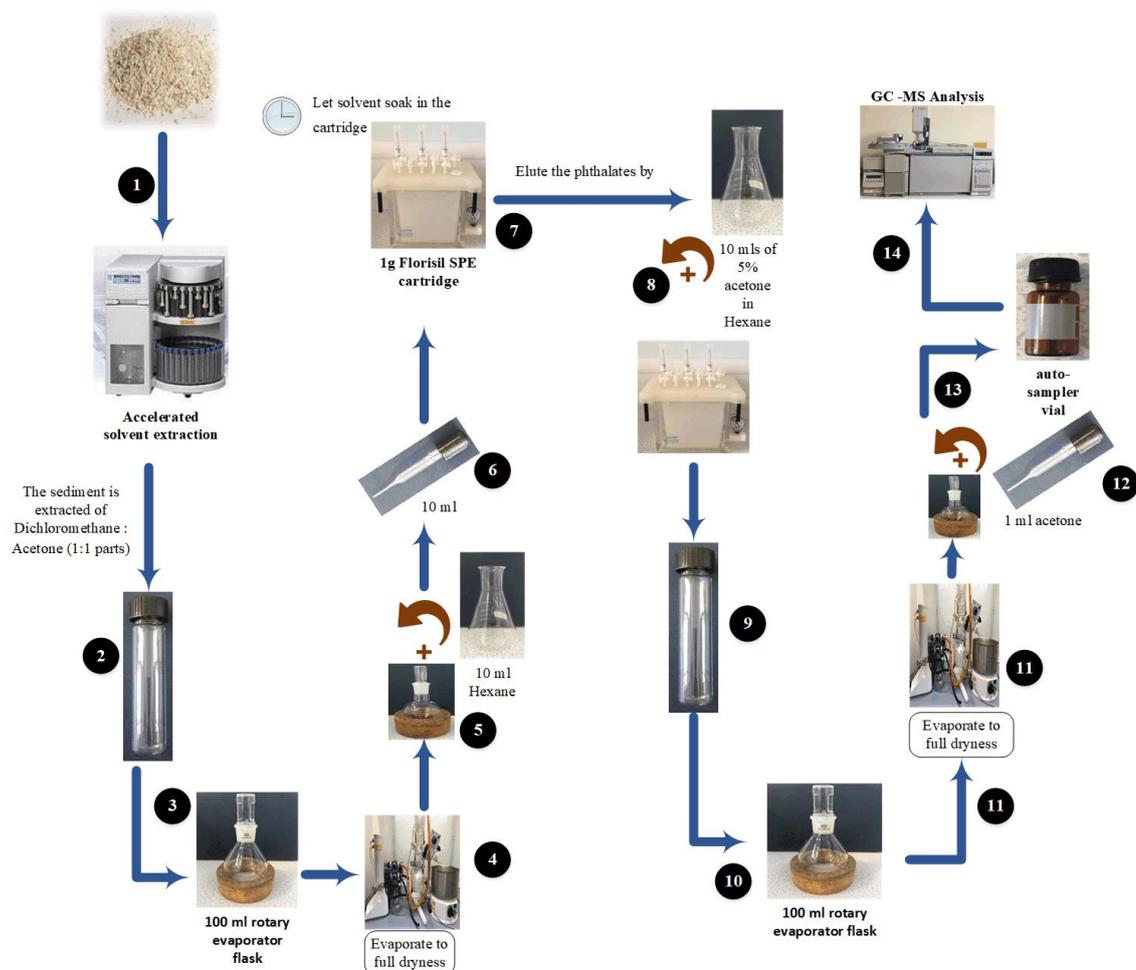
Figure 2.7: Phthalates extraction from the sediment using accelerated solvent extraction.

2.4.2. DETERMINATION OF DEHP CONCENTRATION BY GC-MS IN THE SAMPLES

GC-MS analysis was carried out on a gas chromatograph of Agilent 6890N type which was provided with an automatic liquid sampler of Agilent 7683 type and a mass detector of Agilent 5973N type. GC capillary analysis was carried out on a HP-5MS capillary column with dimensions of 30m x 0.25mm ID x 0.25um, which contained 5% diphenyl, 95% dimethylpolysiloxane and was equipped with a helium carrier gas of 1ml/min speed. GC was performed at 50°C for one minute, and thereafter at 10°C per minute up to 320°C for 2 minutes, at 1.0 mL/min flow rate of the carrier gas. The temperature of injection was 280°C, and the purge delay was 0.75 minutes. The solvent delay was 5 minutes. The mass spectra of the electron impact ionization (EI) was 35-550 m/z in full-scan mode, and the spectra were recorded at an electron energy of 70 eV and at 34.6 mA ionization current and 1376 V multiplier potential. SIM mode was selected to monitor the targeted ions, at 100 ms dwell time for each ion. The targeted ions for each DalP and DEHP are shown in Table 2.1. The limit of Detection (LOD) of DEHP is less than 0.001mg/ml (where a peak is visible at that concentration) and greater than 0.0001mg/ml (where no peaks are visible at that concentration).

Table 2.1: The details of the targeted ions for each DalP and DEHP.

Peak No	Phthalate	Abbreviation	Target ion	Qualifier ion 1
1	Diallylphtlate (internal standards)	DalP	149	189
2	Di (2 ethylhexyl) phthalate	DEHP	149	279

- **GC-MS Analysis Optimum Data Acquisition**

The single ion mode (SIM) was selected for the GC-MS analysis of the present phthalates (DEHP) study (Figure 2.8). By means of SIM the mass spectrometer can detect certain compounds with high accuracy. When the mass spectrometer is in the SIM mode, it only collects data about the masses in question.

As the mass spectrometer gathers only data about the masses in question, it only deals with compounds that have the selected fragments of mass. Generally, the spectrometer focuses only on these selected compounds. In this way a lot of time is devoted to monitoring just a small number of masses and checking them, which leads to greater precision. Moreover, By means of SIM more points are collected across the chromatographic peak, which also improves the sensitivity and accuracy of the quantitative results (Agilent Technologies, 2011).

DEHP were identified based on the mass spectra and retention time using a standard of Diallylphtlate (DalP). For the screening studies, a 149 m/z phthalates base peak was selected and the spectra searched using single ion mode (SIM). Table 2.1 shows the characteristic ions that were selected for the quantitative studies.
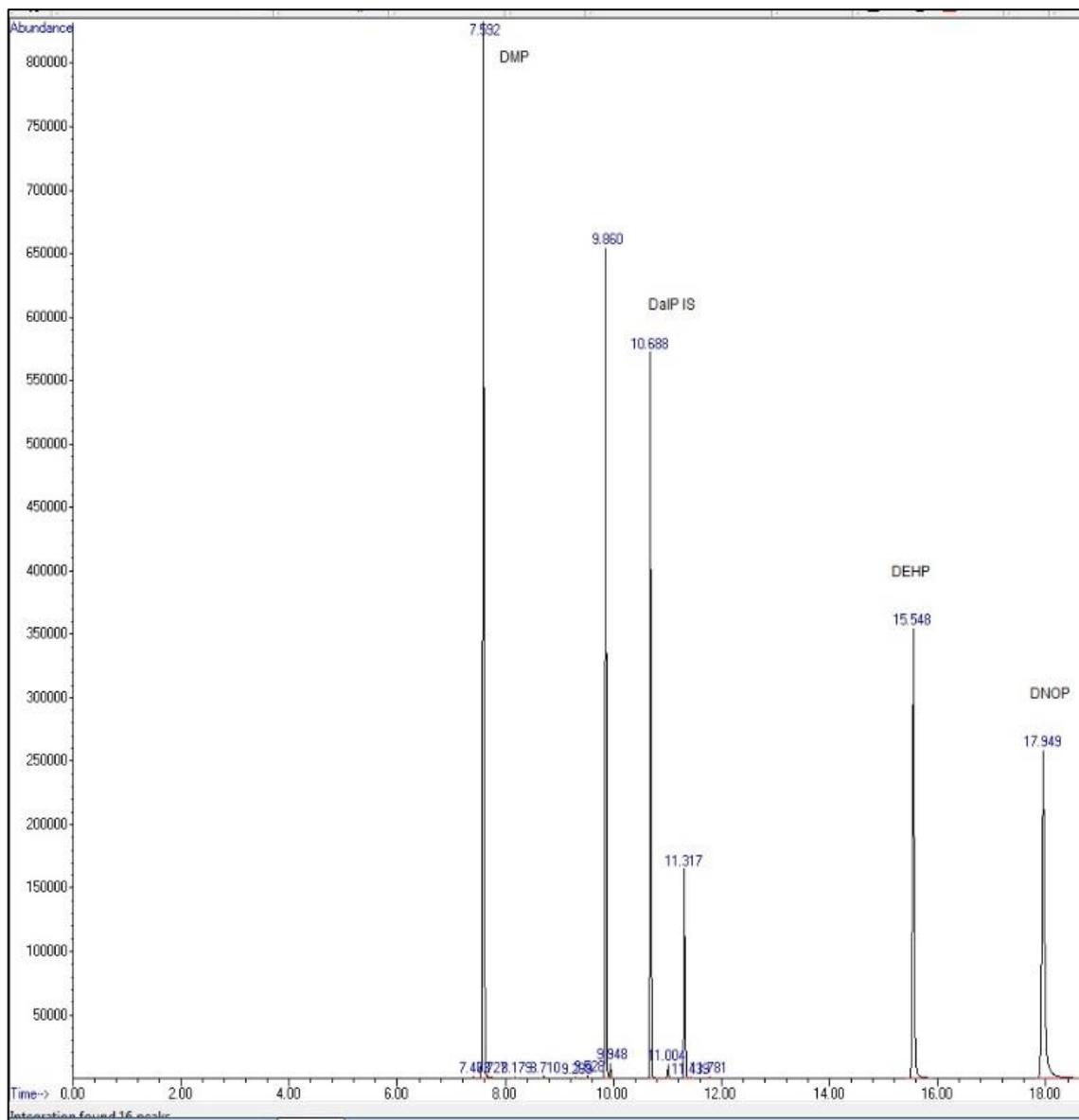
Figure 2.8: Dlap and DEHP standards ion chromatogram obtained using SIM mode.

CHAPTER 3: THE FATE OF DEHP IN *H. DIVERSICOLOR* CULTURE SYSTEMS WITH LONG AND SHORT TERM OF EXPOSURE

3.1. INTRODUCTION

The amount of plastic produced every year is growing by 5% per annum (Andrady and Neal, 2009), often ending up forming part of the anthropogenic debris clogging the marine environment and having an adverse effect on the organisms living it (Laist, 1997; Katsanevakis, 2008; Derraik, 2002; Shomura & Yoshida, 1985). The situation has been deteriorating to the extent that it is now considered a global challenge (UNEP, 2011). Plastic fragments sometimes become less visible as they are scattered in seawater in the form of small particles but they do not actually disappear ('plastic soup') (Andrady, 2015).Large amounts of plastic debris are found on the sea surface in the form of plastic bags, shoes, gloves, children's toys etc. that littered the sea shores after being washed off from ships' containers (Weiss et al., 2006). Other sources of plastics debris come directly from landfill sites that are used to dump plastic items after use, and the seashore was often the place of choice for such landfills (Derraik, 2002; Barnes, 2005). Ivar do Sul and Costa (2007) conducted a study on the seashores of Central and South America where they found huge amounts of marine debris and organisms living in huge build-up of plastic debris, whilst in other places fish and other bigger sea animals created their own mega-environments on plastic debris.

Plastic debris undergoes changes in the natural environment mainly depending on the extent of natural attenuation (Li et al., 2016). Biodegradation rates of plastic debris in the environment depend on the characteristics of the debris (Guo et al., 2012). In general, most plastic debris biodegradation rates in natural water bodies such as oceans and rivers is insignificant. Plastics that have high molecular weights resist biodegradation; and although they fragment gradually, they persist, thus remaining in water as unchanged small fragments of the same plastic polymer. However, after long periods of time, the polymer chains of these plastics reach low molecular weights that allow microorganisms to attack and decompose them easily (Zheng et al., 2005). Plastics created from materials that are readily biodegradable are easily attacked and degraded by fungi and bacteria (Gregory and Andrady, 2003).

Another important factor of natural attenuation besides biodegradation, is photo-degradation. This is a chemical transformation process which may take place directly or indirectly by means of light-induced mechanisms (Yousif & Haddad, 2013). The

process begins when solar ultraviolet rays generate the energetic input required for the inclusion of oxygen atoms in the plastic molecules (Andrady, 2011), thus resulting in the breaking down of the plastic debris into small fragments. Photo-degradation is an effective mechanism for the degradation of PP when exposed to air, e.g. on the beach surface (Andrady, 2011). However, low oxygen concentrations and low temperatures make photo-degradation rates in seawater negligible (Andrady, 2011). Generally, the process of degradation is usually very slow: and may take as much as 50 years or more (Müller et al., 2001). However, one study examined plastics within specific depths in seawater and found that high pressure can facilitate their degradation (Maurizio et al., 2012). Ho et al. (1999) supported these facts by reporting that high humidity and temperature increase the rate of plastic debris degradation because both conditions increase the rate of chemical reaction, which also points to the hindering effects of seawater conditions on plastic degradation. Biodegradation on sediments is more significant since the existence of plastics there allows more exposure to ultraviolet light and mechanical erosion relative to their existence in water (Gregory & Andrady, 2003). Still, the plastic degradation rate in sediments is insignificant, as labile minerals such as feldspars, which have their chemical and mechanical effects on plastics, are easily carried to the seawater (Corcoran et al., 2009). In general, natural attenuation has a minor impact on plastic degradation. Most plastics resist degradation and are only fragmented into small pieces (micro-plastics), that continue to exist on the sea shores and in seawater. Thus, plastic debris poses continuous ingestion and entanglement hazards to the organisms of the natural environment.

DEHP is released to the environment during production, processing, formulation, transport and storage of polymers (PVC) and non-polymers. Furthermore, plasticisers do not bind chemically to the polymer matrix in the case of flexible PVC and similar plastics. Hence, plasticisers are often removed from the finished products while in use and after disposal. DEHP comes to the environment primarily through direct release to the waste water and air, from sewage and solid waste. It may exist in the air in the form of vapour or solid particles. Such DEHP particles may either be in the form of DEHP-containing polymer particles or aggregated pure DEHP. When polymer products weather and form particles, most probably they will become a key route for DEHP distribution. As about 800 industrial sites in the European Union use DEHP or products containing DEHP, wastes released from these sites may cause a high local exposure of DEHP (European Union, 2008).

Phthalate esters (PAEs) have been investigated in many marine ecosystems and their concentrations were reported to range from negligible to 300 µg/L in superficial marine water (Gledhill et al., 1980; Walker et al., 1984; Preston & Alomran, 1986; Staples et al., 1997b; Xie et al., 2005; Peijnenburg & Struijs, 2006), from negligible to 3 µg/g in superficial marine sediments (Lin et al., 2003; Mackintosh et al., 2006), and from negligible to 0.00407 µg/g in seawater organisms (Lin et al., 2003; Mackintosh et al., 2004).

The phthalate most persistent in waste water is DEHP (Gavala et al., 2003; Chaler et al., 2004). It makes about half of the concentrations of all phthalates, being in the range of 0.00033-0.0978 µg/ml in superficial water, 0.00174-0.182 µg/ml in flowing sewage, 27.9-154 µg/g (dw) in sludge sewage and 0.21-8.44 µg/g in the sediment (Fromme et al., 2002). Concentrations of DEHP in seawater lie within the boundaries of the magnitude of their concentrations in fresh water environments (Peijnenburg & Struijs, 2006).

Kwak and Lee (2005) noted that when the midge (*Chironomus riparius*) is exposed to concentrations of 0.3-30 µg/L of DEHP, reproductive effects are observed (Kwak and Lee, 2005), while changes in the midge (*Chironomus tentans*) occur in gene expression (Lee et al., 2006) at concentrations of 500-5000 µg/L as well as increased mortality in human cells (Park & Choi, 2007) at higher concentrations of 1124 µg/L. Kim et al. (2002) observed insignificant effects of DEHP in Medaka (*Oryzias latipes*) adults at concentrations of 10, 50, or 100 µg/L in 5 days.

DEHP is less prone to biodegradation (Wang et al., 2000; Chang et al., 2004) at low concentrations in aquatic systems (Ye et al., 2014). Under optimal and anaerobic conditions, the half-life of DEHP degradation is 25.70 days, (Chang et al., 2005a). However, it half-life in aerobic conditions does not exceed 2-3 weeks (Staples et al., 1997; Ye et al., 2014; Xia et al., 2002, 2004, 2006). Studies conducted on DEHP anaerobic degradation in Taiwan river sediments suggests after quantifying the remaining amounts that it may be entirely biodegraded within a period of 84 days (Chang et al., 2005a).

3.1.1. BIODEGRADATION PATHWAYS

Examination of the degradation pathways of phthalates may help in understanding their mineralization processes and their metabolites' toxicological behaviours (Nozawa and Mar- uyama,1988; Horn et al., 2004). Generally, the biodegradation pathways of phthalates go through two processes, of which primary

biodegradation results in phthalate diesters (PDEs) changing into phthalate monoesters (PMEs), and subsequently into phthalic acid (PA); while the other process, the final biodegradation, involves a change of PA into CH_4 or CO_2 or both (Staples et al., 1997) (Figure 3.1).

3.1.1.1. <u>The Primary Degradation Pathway</u>

As shown in Figure 3.1, the primary degradation involves different pathway types, namely: de-esterification (dealkylation), followed by β-oxidation, and then trans-esterification. (1) De-esterification, which is most common, and is the serial change of PDEs into PMEs and PA. Such a degradation pathway does not vary in aerobic or anaerobic conditions (Shelton et al., 1984) and is aided by over 20 genera of bacteria (Eaton and Ribbons, 1982). (2) β-oxidation: involves phthalates that possess side chains that are longer than diethyl phthalate (DEP) and are occasionally changed by β-oxidation into ones with shorter chains, each time removing one ethyl group (Amir et al., 2005). Furthermore, DEP is changed into PA via two pathways, namely: de-esterification and a pathway of trans-esterification. (3) Trans-esterification (demethylation): Here DEP is degraded by substituting in each step an ethyl group by a methyl group, thus producing dimethyl phthalate (DMP) and ethyl-methyl phthalate, in a process known as trans-esterification (Cartwright et al., 2000).

Not all phthalates biodegradation pathways follow the general course described here, as the dotted arrows in Figure 3.1 show exceptions. For example, Arthrobacter sp. degrade DMP via the pathway of DMP hydrolysis directly into PA. Besides, DEP may also undergo direct degradation into PA by Aureo-bacterium saperdae without passing through the intermediates of MEP (Jackson et al. 1996).
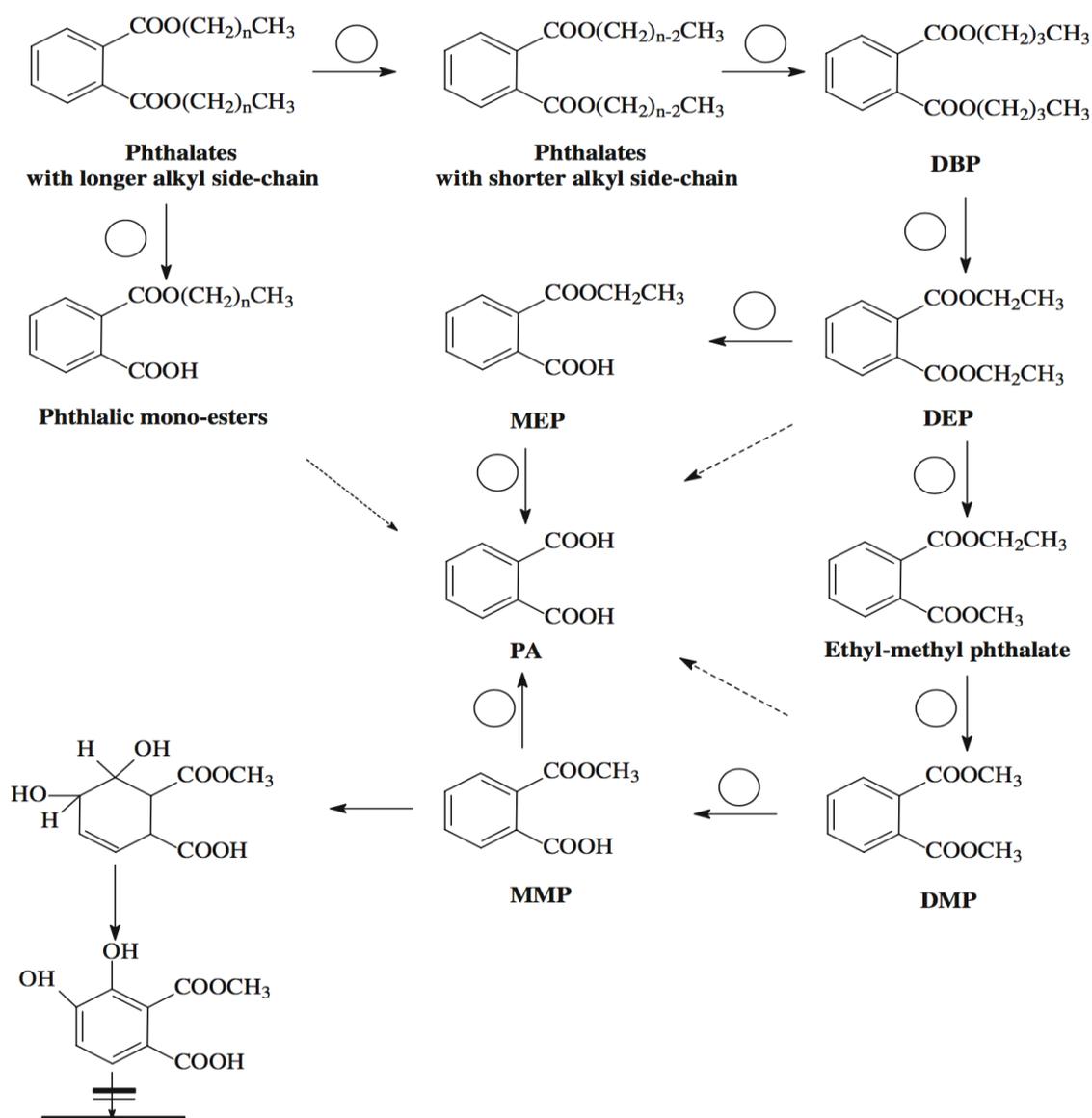
Figure 3.1: Pathways of phthalates degradation by means of E2 MAP esterase enzyme and E1 DAP esterase enzyme (Wei Liang et al., 2008).

3.1.1.2. Ultimate Degradation Pathway

PA plays the role of a major intermediate in phthalates biodegradation as well as polycyclic aromatic hydrocarbons including phenanthrene (Kiyohara and Nagao, 1978), fluoranthene (Eaton, 2001) and fluorene (Grifoll et al., 1994). The pathways of PA ring cleavage under aerobic conditions differ from those under anaerobic conditions (Cartwright et al., 2000).

As shown in Figure 1.2, PA is degraded under aerobic conditions, via two pathways catalyzed by dioxygenase, resulting in protocatechuate 3,4-dihydroxy

benzoate which is a common intermediate (Eaton and Ribbons, 1982; Nomura et al., 1992).

Gram-negative bacteria use dioxygenase as a catalyst to form cis-4,5-dihydro-4,5-dihydroxyphthalate. This product is oxidized by dehydrogenase which depends on NAD, and this results in the formation of 4,5-dihydroxyphthalate. The formation of protocatechuate takes place when 4,5-dihydroxyphthalate is decarboxylated under decarboxylases catalyzation. Gram-positive bacterium Arthrobacterkeyseri 12B, converts PA to protocatechuate via 3,4-dihydroxyphthalate and cis-3,4-dihydro-3,4-dihydroxyphthalate (Eaton and Ribbons, 1982).

Protocatechuate is then metabolized by ring cleavage enzymes via either an ortho- or a meta-cleavage pathway (Eaton and Ribbons, 1982). Protocatechuate, in the meta-cleavage pathway, is changed to 4-carboxy-2-hydroxymu-conic semialdehyde, followed by 2-hydroxy-4-carboxymuconic semi-aldehyde-hemiacetal, then to 4-oxalocitramalate, and finally to pyruvate and oxaloacetate. Protocatechuate, in a pathway that is ortho-cleavage, and also called the ketoadipate pathway, is degraded to 3-ketoadipate. The ortho-cleavage pathway is used by P. fluorescens and P. putida (Dennis et al., 1973), while the meta-cleavage pathway is used by P. acidovorans and P. testosterone (Nakazawa and Hayashi, 1977). The meta-cleavage pathway is also used by A. keyseri 12B (Keyser et al. 1976), but 12C, the 12B's mutant strain uses the ortho-cleavage pathway (Eaton and Ribbons, 1982).

Aerobic bacteria use terephthalic acid (TA) as a carbon source (Keyser et al., 1976). The pathway that is used by aerobic bacteria to act on TA differs from the pathway of PA, because TA changes to protocatechuate through benzoic acid, 4-hydroxybenzoate (Naumova et al., 1986), or changes to it through dihydroxy-3,5-cyclohexa-diene-1,4-dicarboxylic acid (Schlafli et al., 1994).

PA has been reported to be degraded in anaerobic conditions via decarboxylation to benzoate (Kleerebezem et al., 1999b; Liu and Chi, 2003). Then, the benzoate gets cleaved via β-oxidation, and undergoes degradation to carbon dioxide, hydrogen, and acetate (Elder and Kelly, 1994). Anaerobic degradation of phthalates from PA to benzoate, is generally regarded as the rate-limiting stage in the whole degradation process (Kleerebezem et al., 1999c).

3.1.2. RATIONALE OF THIS STUDY

Exposure of *H. diversicolor* to DEHP for short and long periods of time is used to determine the ragworm's acclimatisation ability. Acclimation is defined as the

tolerance changes under laboratory or under other experimental conditions for short periods of time (Brown, 1997). Species that live for relatively short periods of time such as *H. diversicolor*, are used for such a purpose due to the ability to use multiple generations over an experimental period as they have a short generation time, and the fact that they can be cultured in the laboratory, thus making the study of their adaptation possible in experimental studies. This study uses this species to investigate its adaptation potential to DEHP.

Based on the above studies and the concentration and degradation of DEHP, chapters 3, 4, and 5 will examine the exposure of *H. diversicolor* in culture systems to low concentration of DEHP at long term (0.05, 2 and 10 μg/L) and high concentration of DEHP at short term (100 and 500 μg/L). The following chapters will then also study the fate and the impacts of DEHP exposure upon the behaviour of *H. diversicolor*, as a biochemical biomarker associated with detoxification stress.

3.2. AIMS & OBJECTIVES
3.2.1. THE AIMS OF THIS CHAPTER

➢ Determine the half-life of DEHP at concentrations of 10,100 and 500 μg/L, before the exposure.

➢ Determine the recovery and fate of DEHP in the culture system of *H. diversicolor* when the worms are kept in clean seawater and coral sand with low environmental concentrations of 0.05, 2 and 10 μg/L in the case of long term exposure (3 months).

➢ Determine the fate of DEHP in the culture system of *H. diversicolor* when the worms are kept in clean seawater and coral sand at high DEHP concentrations (100 and 500 μg/L) in the case of short term exposure (7 days).

3.2.2. THE OBJECTIVES OF THIS CHAPTER ARE TO ANSWER THE FOLLOWING QUESTIONS:

➢ Is DEHP half-life in marine waters in the range of <1 day - 2 weeks (Amir et al., 2005)?

➢ Have the levels of DEHP in the body of *H. diversicolor* decreased in low contamination scenarios when the exposure was stopped and replaced by a clean culture?

> ➢ In case that DEHP levels have decreased to background noise levels in the worms, where has the DEHP gone?

> ➢ In case that DEHP levels have increased in the worms, in what concentration?

Based on the above objective, it is hypothesised that:

The half-life of DEHP in seawater ranges between <1 day and 2 weeks depending on DEHP concentrations.

- Worms can be recovered from DEHP exposure with low environmental concentrations at long term.

- DEHP will accumulate more in *H. diversicolor* followed by sediments whereas it will degrade quickly in seawater in both long and short term.

3.3.   MATERIALS AND METHODS

3.3.1. EXPOSURE PROTOCOL

3.3.1.1. <u>Exposure of seawater to DEHP in order to determine the half-life of DEHP at concentrations of (10,100 and 500 µg/L)</u>

Figure 3.2 shows the four ecotoxicological exposure tanks. The capacity of the reservoir tanks was 50 litres, and each tank was supplied with an aeration pump so as to ensure good seawater re-circulation. The walls of the ecotoxicological tanks were of polycarbonated glass of 60x30x20 cm dimensions. Each tank contained 9 litre of seawater

The first tank contained untreated seawater and the second one contained seawater with 10 µg/L of DEHP, while the third one contained seawater with 100 µg/L of DEHP and the fourth one contained seawater with 500 µg/L of DEHP. Seawater underwent analyses after 1 hour, 2 hours, 4 hours, 6 hours, 8 hours, 1 day, 2 days, 4 days, 6 days and 8 days, in order to determine how long the DEHP took to degrade in different concentrations. The total experiment was conducted with 8 days.
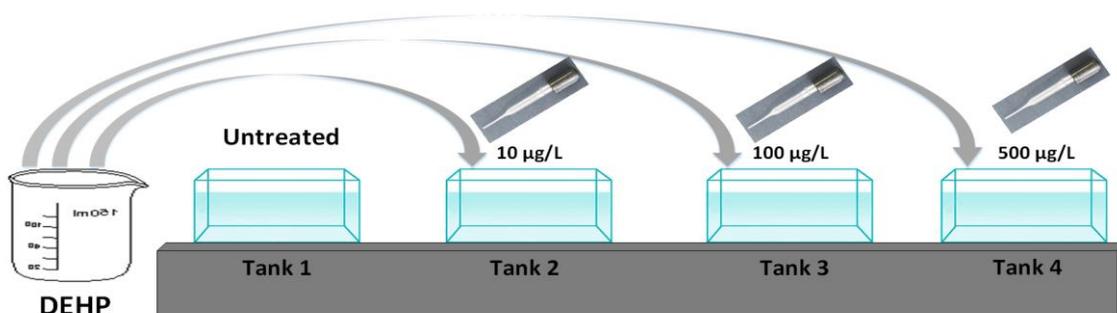
Figure 3.2: Shows four ecotoxicological tanks containing seawater. Three tanks were exposed to different concentrations of 10 µg/L, 100 µg/L and 500 µg/L for 8 days.

3.3.1.2. <u>Long term exposure of the culture systems to DEHP (chronic stress)</u>

Chronic stress in this study is defined as response to repeated exposure to DEHP over a long period of time (Nagaraja & Jeganathan, 1999). The environmental concentrations of DEHP used in the long-term experiments of this study (0.05 μg/L, 2 μg/L, and 10 μg/L) were designed as concentrations lower than the levels of DEHP in contaminated estuarine environments like the Humber estuary (sampling point at Paull, Hull) where worm samples were collected. However, it should be noted that studies by Zhou et al. (2011) and Liu et al. (2009) have recommended this range of DEHP concentrations, and using these three concentrations also enabled looking at the potential of animals 'recovering' from DEHP exposure. Liu et al. (2009) used different concentrations of DEHP in the embryonic development of the abalone *Haliotis diversicolor supertexta* that led to changes in the endocrine activity and thereby affect the early life stage. Therefore, this study used their concentrations in *Hediste diversicolor* to examine the functional traits and biochemical biomarker.

The worms in System 1 (Figure 3.3) were fed two bottles of a mixture of food and a solution of DEHP of 10 µg/L concentration. The same procedures were repeated with System 2 but with a concentration of 2 µg/L of DEHP. In System 3 the concentration of DEHP was 0.05 µg/L, while in System 4 the worms were fed two bottles of a mixture of food and water. The phthalates were introduced at the beginning of each month without further influencing the concentration. This was designed to give the bacteria in the system time to modify the concentrations over the three months period of exposure time. After three months of exposure, the worms, seawater and sediments were taken for phthalate analysis.
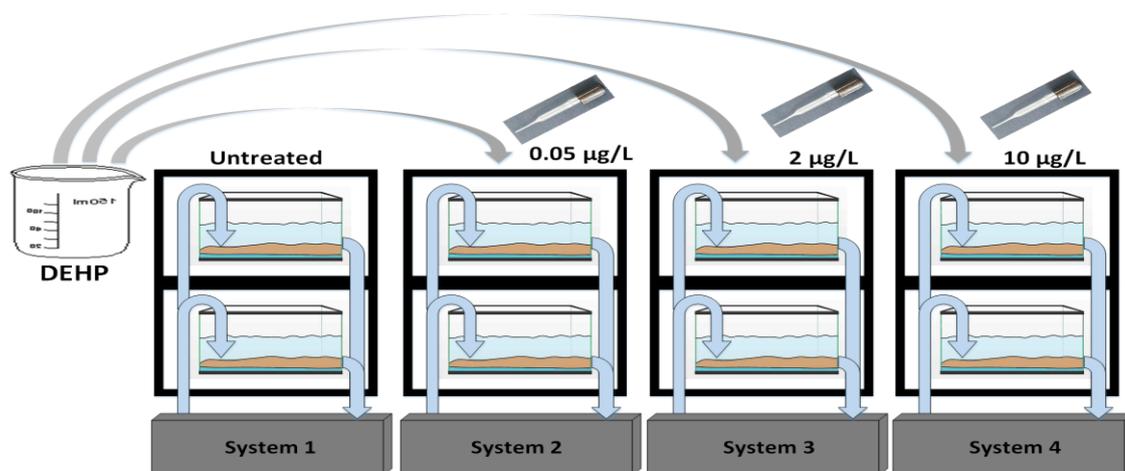
Figure 3.3: shows four culture systems of eight ecotoxicological tanks each containing 100 worms, seawater and sediment. System 1 is an untreated system and the other three culture systems are exposed to different low concentrations of DEHP of 0.05 μg/L, 2 μg/L and 10 μg/L for 3 months.

3.3.1.3. <u>Short term exposure of the culture systems to DEHP (acute stress)</u>

Acute stress in this study is defined as a single exposure of DEHP (Nagaraja & Jeganathan, 1999). In this study, *H. diversicolor* was examined to see whether it can reach homeostasis when exposed to high DEHP concentrations in short-term exposures (7 days). Two concentrations, 100 µg/L and 500 µg/L were used as high, but environmentally relevant DEHP concentrations in the short-term experiments (Figure 3.4). The worms in tank 1 were fed with a bottle of a mixture of food and a solution of DEHP of 500 µg/L concentration. The same procedures were repeated with tank 2 but with a concentration of 100 µg/L of DEHP. In tank 3 the worms were fed a bottle of a mixture of food and water. The exposure procedures were repeated depending on the half-life of DEHP at concentrations 100 and 500 µg/L. The tanks were re-exposed to a concentration of 500 µg/L after 4 days, but were re-exposed to a concentration of 100 µg/L after 1 day. After 7 days of exposure, worms, seawater and sediments were taken for phthalate analysis.
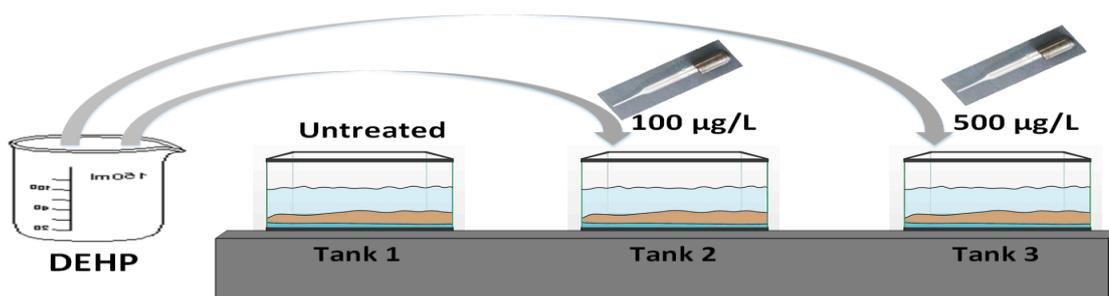
Figure 3.4: Shows three ecotoxicological tanks each containing 100 worms, seawater and sediment. Tank 1 is an untreated tank and the other two tanks are exposed to high different concentrations of DEHP of 100 µg/L and 500 µg/L for 7 days.

3.3.2. Determination of DEHP concentration in worm tissue, seawater and sediments by GC/MS

(See Chapter 2, Section 2.4 pages 72-77)

- **DATA ANALYSIS**

SPSS (Statistical Package for the Social Sciences) was used for the statistical analyses of the data collected. First, descriptive statistics were performed. Then, Shapiro-Wilk's test was used to test the normality of data. Homogeneity of variance was checked by Levene's test. For data that fulfilled both conditions, multiple comparisons were assessed by one-way analysis of variance (ANOVA) with Tukey's test as a post-hoc test. A Dunnett's test performed to compare untreated group with other groups. However, Welch ANOVA was carried out with a Games-Howell post-hoc

when the homogeneity of variances was violated. If data were not normally distributed, a log transformation was conducted to satisfy parametric conditions. However, when the log transformation failed normality test, a non-parametric Kruskal–Wallis test was performed with multiple Mann Whitney comparison tests as post hoc tests. A Bonferoni correction used with Mann Whitney comparison tests to minimise the error rate.

3.4. RESULTS

3.4.1. DETERMINATION OF THE HALF-LIFE OF DEHP IN SEAWATER AT CONCENTRATIONS OF 10,100 AND 500 µg/L

Figure 3.5 shows the level of DEHP in seawater exposed to three different concentrations over time highlighting a fast, concentration dependent decrease of phthalate levels within 2 hours, 1 day, 6 days at 10, 100 and 500 µg/L. The DEHP levels reached 0.00000053 ±0.000000119, 0.00000075 ±0.0000003016, 0.00000209 ±0.000001824 and 0.00000708 ±0.000006498 µg/ml for the untreated, 10, 100 and 500 µg/L, respectively.

When a Shapiro-Wilk's test (p=0.00) was used followed by data's histograms visual inspection, normal Q-Q plots and then box plots, it transpired that the data were not normal distribution. Therefore, after a log transformation was conducted, a Shapiro-Wilk's test (p=0.000) used, followed by data's histograms visual inspection, normal Q-Q plots and then box plots, it was then found that the data were still not normally distributed. Therefore, a Kruskal Wallis test was conducted.

Concertation groups analysis

It was evident that there is a significant difference between groups, $X^2(3) = 12.280$, p=0.006. It was found that the highest mean rank was for 500µg/L group followed by 100µg/L, 10µg/L and untreated groups respectively.

Multiple Mann-Whitney U test with a Bonferoni correction were conducted to measure differences between any two groups. A significant difference was found between untreated and 500µg/L p=0.001, whereas, no significant difference was found between other groups.

Duration groups analysis

It was evident that there is no significant difference between groups, $X^2(9) = 14.557$, p=0.104. It was found that the highest mean rank was for 1h group followed by 2h, 6h, 4h, 8h, 1d, 4d, 2d, 8d and 6d groups respectively.
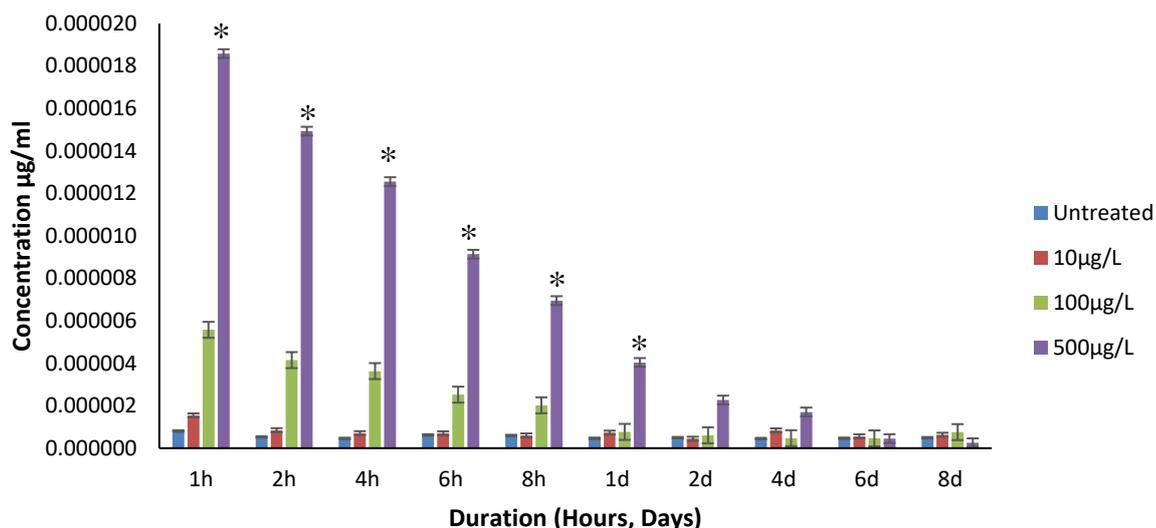
Figure 3.5: Mean Levels of DEHP in exposed seawater (± SE) at concentration 10µg/L, 100µg/L and 500µg/L over 8 days of exposure. n= 3 sample of seawater (500ml), Asterisk indicates significant difference from untreated group (p<0.05).

3.4.2. DETERMINATION OF THE FATE OF DEHP IN THE LONG-TERM EXPOSURE CULTURE SYSTEMS OF *H. DIVERSICOLOR*

➢ *H. diversicolor* **tissue**

Figure 3.6 shows the increasing level of DEHP in tissue of *H. diversicolor* after a long term of exposure (3 months). The DEHP levels reached 0.08510 ±0.03762, 0.01671 ±0.00788, 0.02121 ±0.01085, 0.05766 ±0.04854 and 0.06663 ±0.04203 µg/g for Day 1 (worms that were analysed on the day of collection), untreated, 0.05, 2 and 10 µg/L, respectively. The highest accumulation of DEHP was with worms exposed to 10 µg/L.

When a Shapiro-Wilk's test (p=0.00) was used followed by data's histograms visual inspection, normal Q-Q plots and then box plots, it transpired that the data were not normal distribution. Therefore, after a log transformation was conducted, a Shapiro-Wilk's test (p=0.574) used, followed by data's histograms visual inspection, normal Q-Q plots and then box plots, it was then found that the data were approximately normally distributed. The Levene's test has verified the variances equality (variance homogeneity) in the samples (p>0.05).

Therefore, One-way ANOVA test was conducted, and it was evident that there was a statistically significant difference between groups ($F_{(4,45)} = 16.102$, $p = 0.000$).

A Dunnett's test performed and a significant difference was found between untreated and Day 1, 2µg/L and 10µg/L p=0.00.

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In addition, a Tukey post hoc test found a significant difference between Day 1 and 0.05 µg/L p=0.00, and between 0.05µg/L and both 2µg/L p=0.019 and 10µg/L p=0.01. However, no significant difference was found between other groups (p>0.05).
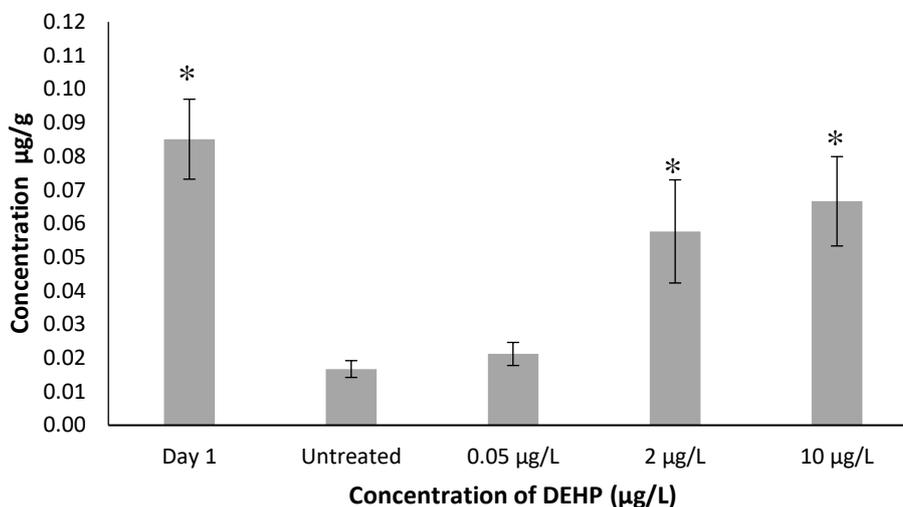
Figure 3.6: Mean level of DEHP for _H. diversicolor_ tissue (± SE) in five culture systems that each contains 10 worms. Day 1 is worms that were analysed on the day of collection, whereas treated group expose to DEHP for 3 months (chronic stress) at different concentrations (0.05, 2 and 10 µg/L). Asterisk indicates significant difference from untreated group (p<0.05).

➢ **Sediment**

Figure 3.7 shows the level of DEHP in sediment after a long term of exposure. The DEHP levels reached 0.000077 ±0.000028, 0.000072 ±0.000032, 0.000136 ±0.000120, 0.000386 ±0.000218 and 0.000614 ±0.000202 µg/g for Day 1 (worms that were analysed on the day of collection), untreated, 0.05, 2 and 10 µg/L, respectively. The highest accumulation of DEHP was with sediment exposed to 10 µg/L.

When a Shapiro-Wilk's test (p=0.005) was used followed by data's histograms visual inspection, normal Q-Q plots and then box plots, it transpired that the data were not normal distribution. Therefore, after a log transformation was conducted, a Shapiro-Wilk's test (p=0.144) used, followed by data's histograms visual inspection, normal Q-Q plots and then box plots, it was then found that the data were normally distributed. The Levene's test has verified the variances equality (variance homogeneity) in the samples (p>0.05).

Therefore, one-way ANOVA test was conducted, and it was evident that there was a statistically significant difference between groups (F (4,10) = 2.709, p = 0.011).

A Dunnett's test performed and a significant difference was found between untreated and 10µg/L p=0.10.

In addition, a Tukey post hoc test found a significant difference between Day 1 and 10 µg/L p=0.024. However, no significant difference was found between other groups (p>0.05).
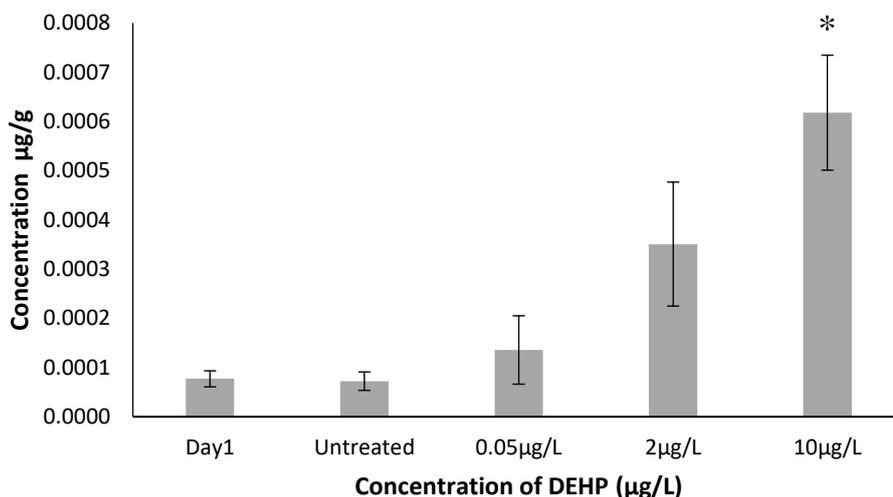
Figure 3.7: Mean level of DEHP for sediment (± SE) in the five culture systems. Three samples from each system have been analysed. Day 1 is sediment that was analysed on the day of collection, whereas treated group expose to DEHP for 3 months (chronic stress) at different concentrations (0.05, 2 and 10 µg/L). Asterisk indicates significant difference from untreated group (p<0.05).

➢ **Seawater**

Figure 3.8 shows the level of DEHP in seawater after a long term of exposure. The DEHP levels reached 0.0000028 ±0.00000006, 0.0000027 ±0.00000036, 0.0000041 ±0.00000085, 0.0000044 ±0.00000095 and 0.0000045 ±0.00000132 µg/ml for Day 1 (worms that were analysed on the day of collection), untreated, 0.05, 2 and 10 µg/L, respectively. The accumulation of DEHP was similar in all exposed seawater.

A Shapiro-Wilk's test (p=0.297) was used followed by data's histograms visual inspection, normal Q-Q plots and then box plots, it transpired that the data were normal distributed. . The Levene's test did not verify the equality of variances (homogeneity of variance) in the samples (p<0.05).

Therefore, a Welch ANOVA test was conducted, and it was evident that there was no statistically significant difference between groups (F (4,2.044) = 1.690, $p =$ 0.401).
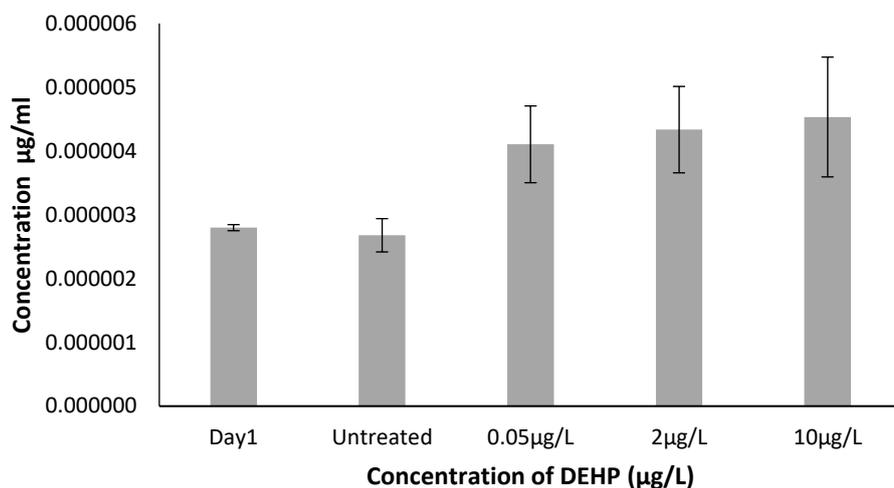
Figure 3.8: Mean level of DEHP for seawater (± SE) in five culture systems. Three samples from each system have been analysed. Day 1 is seawater that was analysed on the day of collection, whereas treated group expose to DEHP for 3 months (chronic stress) at different concentrations (0.05, 2 and 10 µg/L).

3.4.3. DETERMINATION OF THE FATE OF DEHP IN THE SHORT-TERM CULTURE SYSTEMS OF *H. DIVERSICOLOR*

➢ *H. diversicolor* Tissue

Figure 3.9 shows the increasing level of DEHP in tissue of *H. diversicolor* after short term of exposure. The DEHP levels reached 0.08510 ±0.03762, 0.02994 ±0.01863, 0.05593 ±0.03454 and 0.09733 ±0.04178 µg/g for Day 1 (worms that were analysed on the day of collection), untreated, 100 and 500µg/L, respectively. The highest accumulation of DEHP was with worms exposed to 500µg/L.

When a Shapiro-Wilk's test (p=0.002) was used followed by data's histograms visual inspection, normal Q-Q plots and then box plots, it transpired that the data were not normal distribution. Therefore, after a log transformation was conducted, a Shapiro-Wilk's test (p=0.005) used, followed by data's histograms visual inspection, normal Q-Q plots and then box plots, it was then found that the data were still not normally distributed.

Therefore, a Kruskal Wallis test was conducted, and it was evident that there is a significant difference between groups, $X^2(3) = 15.449$, p=0.001. It was found that the highest mean rank was for the 500µg/L group followed by the Day 1, 100µg/L and untreated groups respectively.

Multiple Mann-Whitney U test with a Bonferoni correction were conducted to measure differences between any two groups. A significant difference was found between the Day 1 and untreated group p=0.001, and between the untreated and

96

500µg/L group, p=0.001. However, no significant difference was found between other groups.
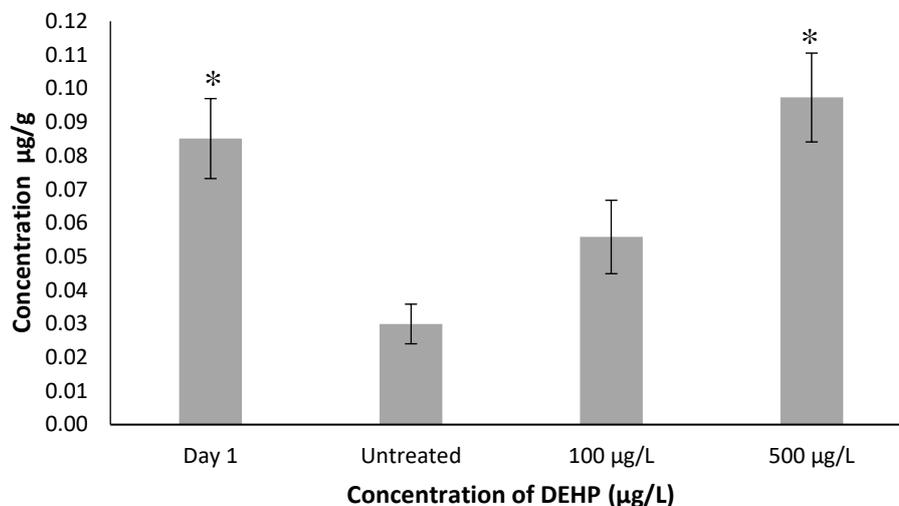
Figure 3.9: Mean level of DEHP for *H. diversicolor* tissue (± SE) in four culture systems that each contains 10 worms. Day 1 is worms that were analysed on the day of collection, whereas treated group expose to DEHP for 7 days (acute stress) at different concentrations (100 and 500 µg/L). Asterisk indicates significant difference from untreated group (p<0.05).

➢ **Sediment**

Figure 3.10 shows the increasing level of DEHP in sediment after a short term of exposure. The DEHP levels reached 0.000077 ±0.000028, 0.000058 ±0.000019, 0.001344 ±0.000341 and 0.003868 ±0.000282 µg/g for Day 1 (worms that were analysed on the day of collection), untreated, 100 and 500µg/L, respectively. The highest accumulation of DEHP was with sediment exposed to 500µg/L.

When a Shapiro-Wilk's test (p=0.004) was used followed by data's histograms visual inspection, normal Q-Q plots and then box plots, it transpired that the data were not normal distribution. Therefore, after a log transformation was conducted, a Shapiro-Wilk's test (p=0.018) used, followed by data's histograms visual inspection, normal Q-Q plots and then box plots, it was then found that the data were still not normally distributed.

Therefore, a Kruskal Wallis test was conducted, and it was evident that there was a significant difference between groups, $X^2(3) = 9.462$, p=0.024. It was found that the highest mean rank was for the 500µg/L group, followed by the 100µg/L, Day 1 and untreated groups respectively.

Multiple Mann-Whitney U test with a Bonferoni correction were conducted to measure differences between any two groups. A significant difference was found

between Day 1 and 500µg/L p=0.017, and between untreated and 500µg/L p=0.007. However, no significant difference was found between other groups.
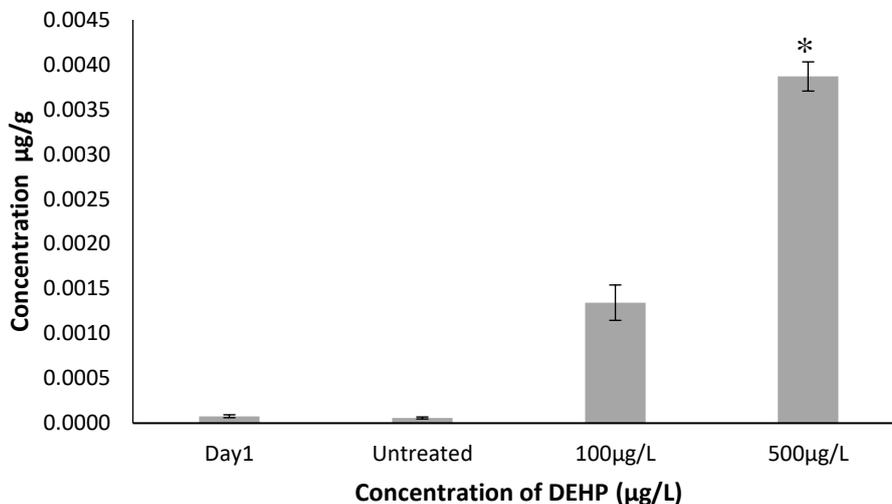
Figure 3.10: Mean level of DEHP for sediment (± SE) in four culture systems. Three samples from each system have been analysed. Day 1 is sediment that was analysed on the day of collection, whereas treated group expose to DEHP for 7 days (acute stress) at different concentrations (100 and 500 µg/L). Asterisk indicates significant difference from untreated group (p<0.05).

➢ **Seawater**

Figure 3.11 shows the increasing level of DEHP in seawater after a short term of exposure. The DEHP levels reached 0.0000028 ±0.00000006, 0.0000016 ±0.0000015, 0.0000219 ±0.0000171 and 0.0001934 ±0.000050 µg/ml for Day 1 (worms that were analysed on the day of collection), untreated, 100 and 500µg/L, respectively. The highest accumulation of DEHP was with seawater exposed to 500µg/L.

When a Shapiro-Wilk's test (p=0.055) was used followed by data's histograms visual inspection, normal Q-Q plots and then box plots, it transpired that the data were normal distribution. The Levene's test has verified the variances equality (variance homogeneity) in the samples (p>0.05).

Therefore, one-way ANOVA test was conducted, and it was evident that there was a statistically significant difference between groups (F (3,4) = 30.707, p = 0.003).

A Dunnett's test performed and a significant difference was found between untreated and both 100 µg/L p=0.006 and 500 µg/L p=0.003.

In addition, a Tukey post hoc test found a significant difference between Day 1 and both 100µg/L p=0.021 and 500µg/L p=0.009. However, no significant difference was found between other groups.
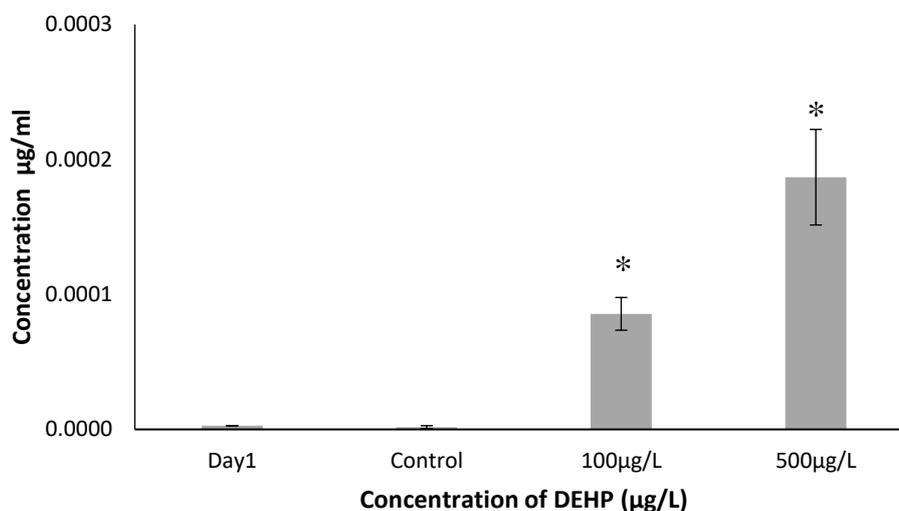
Figure 3.11: : Mean level of DEHP for seawater (± SE) in four culture systems. Three samples from each system have been analysed. Day 1 is seawater that was analysed on the day of collection, whereas treated group expose to DEHP for 7 days (acute stress) at different concentrations (100 and 500 µg/L). Asterisk indicates significant difference from untreated group (p<0.05).

3.5. DISCUSSION

3.5.1. DEHP HALF-LIFE IN SEAWATER

This chapter focuses on determining the concentrations of DEHP in seawater, as well as in the sediment and in *H. diversicolor*. Selection fell on DEHP as the center of attention because it is a major pollutant of the aquatic environment owing to its low solubility in seawater, as well as its frequent presence in the environment (Sarkar et al., 2013). Four diverse factors interfere with the existence of chemical pollution in aquatic organisms, namely: the environmental and bio-physiological characteristics of the various organisms; the amount of the dissolved chemicals; the solid forms of the ingested chemicals; and the physical and chemical properties of seawater (Jenne and Luoma, 1977).

Tanks were exposed to DEHP in the ecotoxicological tanks with seawater at different levels of DEHP concentrations (10, 100 and 500 µg/L) (see figure 3.2). Seawater samples were analysed after 1, 2, 4, 6 and 8 hours; and 1, 2, 4, 6 and 8 days. Results indicated that the level of DEHP concentration in the untreated seawater was about 0.00000053 µg/ml. Comparing treated and untreated seawaters, the degradation

times of the 10, 100 and 500 µg/L levels of concentration, were before 2, 1 and 6 days, respectively.

The treated seawater had the ability of degrading DEHP, which shows that aerobic conditions were satisfied along with a sufficient supply of oxygen. Furthermore, the half-life of DEHP was found to depend on the concentration that was used, which was in the range of <1 day - 1 week in seawater. This is consistent with previous studies that show the half-life of DEHP in marine waters range of <1 day to 2 weeks (Amir et al., 2005, Staples et al., 1997). The rate of plastic degradation in seawater is slightly slower at low temperatures and oxygen concentrations (Andrady, 2011). The total degradation process progresses very slowly and full degradation may require more than 50 years (Müller et al., 2001). However, a study by Maurizio et al. (2012) demonstrated that as plastic nears certain depths in the sea, the increasing pressure makes their degradation much easier. Another study by Ho et al. (1999) showed that the rate of plastic debris degradation increases with high temperature and humidity, as these two accelerate the rate of chemical reaction. In this way, seawater conditions may accelerate or slow plastic degradation. Biodegradation in sediment is of particular significance because of the higher exposure to ultraviolet light (UV) and higher mechanical erosion relative to these conditions in water (Gregory & Andrady, 2003). Despite all this, the rate of plastic degradation in sediment is still very low because of the very slow chemical and mechanical changes that take place in minerals, and feldspars and clay minerals can easily wash out to the open sea (Corcoran et al., 2009).

The Sun produces ultraviolet radiation in a continuous spectrum, while it is produced in a gaseous discharge tube by atomic excitation in the form of a wavelength discrete spectrum. Oxygen absorbs most of the sunlight ultraviolet radiation (Andrady, 2011).

Degradation of the polymer is first initiated by ultraviolet radiation and then proceeds for some time thermo-oxidatively without having to be exposed to ultraviolet radiation. The sequence of the autocatalytic degradation reaction can advance depending on the availability of oxygen to the system. As a result of degradation the polymer's molecular weight decreases and generates oxygen-rich functional groups in the polymer. Biodegradation by light-induced oxidation exceeds other types of biodegradation in speed by orders of magnitude. Plastics and all other biomaterials are susceptible to biodegradation in the seawater environment. Hydrolysis in seawater is not considered as a significant mechanism. Generally, biodegradation of plastics by

ultraviolet light, even in benthic sediment, is several orders of magnitude speedier than other mechanisms of biodegradation (Andrady, 2011).

Plastics lying on the beach surface and exposed to the air are most efficiently degraded when degradation is initiated by solar ultraviolet radiation. However, degradation of plastic material floating on the surface of seawater and exposed to solar ultraviolet radiation at the same site is much slower. Andrady et al. (1993) and Andrady and Pegram (1990, 1989a, b) compared the degradation of several common gear-related and packaging plastics floating on the surface of seawater and exposed to sunlight with those freely exposed to the air at the same location (in Biscayne Bay, FL and Pugeot Sound, WA.) and found the latter much speedier.

Slow degradation of plastics exposed to sunlight while floating on the surface of seawater is mainly due to lower oxygen concentration and lower temperatures in the seawater environment. Unlike exposure of plastics to air, the water temperatures are lower, thus retarding the reactions of degradation. The difference in degradation rates between air exposures and floating exposures is more aggravated by fouling effects. Extensive surface fouling of floating plastics is rapid in the seawater environments, first generating a biofilm on the plastics surface which in turn attracts algae and colonies of invertebrates (Muthukumar et al., 2011).

DEHP adsorption in marine sediments could be greater than its adsorption in freshwater sediments, due to DEHP low solubility in saltwater (ATSDR, 2002). The study conducted by Xu and Li (2009) indicated that the phthalate sorption on marine sediments may be attributed to the seawater's organic content and increase in sorption generated by increase in salinity. However, phthalate sorption by suspended solids or freshwater sediments depends on the relative hydrophobicity of such chemicals, especially in the case of longer alkyl chain phthalates such as DEHP. The dominant degradation mechanism of phthalates released to the water in the form of liquid wastes from sewage treatment plants, is thought to be biodegradation, as many bacterial species and actinomycetes are capable of degrading phthalates under aerobic as well as anaerobic conditions (Petrovic et al., 2001).

Several degradation pathways are followed by phthalates, and hence they may not be regarded as chemicals that persist in seawater. Visible light easily degrades phthalates under normal atmospheric conditions with expected half-life of about 1 day, and they are also biodegradable by actinomycetes and bacteria, which may be the dominant mechanism of loss in sediments and surface water (Staples et al., 1997). Generally, degradation half-lives in marine surface waters is in the range of <1 day - 2

weeks, while in the soil it is in the range of less than one week up to several months. Data of aerobic biodegradation tests that utilize sewage sludge show that around 50% of the phthalate esters undergo final degradation within about 28 days (Staples et al., 1997).

DEHP biodegradation has been thoroughly studied under a variety environmental conditions including freshwater, waste water resulting from different treatment processes, sediments, soils, sludge, and landfill sites (Wei Liang, 2008). Phthalate biodegradation in different environments is characterized by the following common features: (1) the longer the side chain of the phthalate, the lower is its biodegradability; (2) phthalates degradation in anaerobic conditions is slower than their degradation in aerobic conditions (Staples et al., 1997; Yuan et al., 2002); (3) phthalates' primary biodegradation never exceeds first-order kinetics (Wang et al., 1997b; Gavala et al., 2003); (4) when phthalates or their metabolites are highly concentrated they tend to resist biodegradation. Different studies reported different phthalate degradation half-lives (Table 3.1).

Table 3.1: Half-life time of DEHP.

Environment	*Half- lifes time*	*Reference*
DEHP in water	2000 years	(Staples et al.,1997)
DEHP in lagoon sludge and activated sludge under aerobic conditions	45.4 and 28.9 days respectively	(Amir et al., 2005)
DEHP in the river sediment	2.5-14.8 (aerobically) and 14.4 - 34.7 days (anaerobically)	(Turner and Rawling, 2000)
DEHP in bacteria enrichment into a soil-slurry sequential batch reactor (SBR)	2-5 days at ambient temperature (20–22°C)	(Juneson et al., 2001).
DEHP in Taiwan river Sediments	14.8 days under aerobic conditions 34.7 days under anaerobic conditions	(Yuan et al., 2002)

3.5.2. LONG AND SHORT TERM EXPOSURE OF THE CULTURE SYSTEMS OF *H. DIVERSICOLOR* BASED ON THE RESULT OF THE HALF-LIFE OF DEHP IN SEAWATER

3.5.2.1. <u>Long term exposure of the culture systems to DEHP</u>

The DEHP concentrations were analysed in the culture systems by GC/MS. The culture systems were exposed once in a month in order to allow the worms to be detoxified of the DEHP. The worms that were taken from a contaminated environment (Paull, Humber Estuary) were investigated in terms of their ability to recover from

DEHP, and to determine the fate of DEHP when exposure was stopped and the culture was replaced by a culture at low contamination. Worms were kept under different environmental concentrations of low DEHP levels (0.05, 2 and 10 μg/L) as well as under 'clean' seawater and coral sand. Based on the DEHP degradation time for 10 μg/L (figure 3.3), the culture systems should have been almost free of DEHP for 1 month before the next exposure.

After long term of exposure to DEHP, it accumulated significantly in the worms and sediment, whereas it degraded quickly in seawater (Figures 3.6, 3.7 and 3.8). Hence, the results suggest that the presence of sufficient oxygen supply and an aerobic condition in the culture systems and active/passive uptake of DEHP by the animals. DEHP accumulated more in the worms than in the sediment. By comparing, the worms on Day 1, worms that were analysed on the day of collection, with the untreated worms that recovered from DEHP by the detoxification and/or metabolisation or active/passive release of DEHP. Furthermore, the worms treated with low 0.05 μg/L concentration recovered from DEHP, because they had a potential ability of detoxification by means of active metabolism. On the other hands, the worms treated with concentrations of 2 μg/L and 10 μg/L had slightly lower and similar concentrations of DEHP to the worms of Day 1 (Paull). The reason behind this might be due to the diffusion-limited exchange across the body surfaces. However, these results show that the worms can recover from DEHP if they are kept under the conditions of a clean environment.

Moreover, the results suggested that the accumulation of DEHP increased proportionately in treated sediment, coral sand, with the different concentrations (0.05, 2 and 10 μg/L) (Figure 3.7). It seems that the sediment can act in aquatic environments as a sink for DEHP. DEHP transport from water to the sediment is just the result of the suspended material taking the adsorbed chemical down with it when it settles. The concentration of DEHP in the sediment depends on the supply rates of DEHP during sedimentation and degradation (Staples et al., 1997). Furthermore, DEHP is inclined to bio-accumulate primarily in fats, and a significant transfer of DEHP may occur from the sediment to aquatic organisms (Chaler et al., 2004, Huang et al., 2008).

3.5.2.2. Short term exposure of the culture systems to DEHP

DEHP concentrations were analysed in the culture systems by GC/MS. The culture systems were exposed based on the result of DEHP half-life, which is shown in Figure 3.5. Hence, the tanks with the concentration of 100 μg/L were exposed daily, whereas the tanks with the concentration of 500 μg/L were exposed after 4 days. Worms

taken from a contaminated environment (sampling point at the Humber estuary, Paull, Hull) were investigated to determine the fate of DEHP in the short-term culture systems with these high DEHP concentrations. Figures 3.9, 3.10 and 3.11 show the accumulation of DEHP in the worms, sediment and seawater at both concentrations. The concentration of DEHP in the worms treated with 500μg/L was approximately three times higher than in the untreated worms. The concentration of DEHP in the treated worms was 0.05593 μg/g in the 100μg/L exposure and 0.09733 μg/g in the 500μg/L exposure. By comparing the worms in Day 1 with the untreated worms, Figure 3.9, we find that the untreated worms recovered from DEHP within 1 week.

After short term of exposure to DEHP, it was found that the accumulation of DEHP increased in treated samples at different concentrations (100 and 500 μg/L) more than the untreated control samples in worms, seawater and sediment. However, the concentration of DEHP in the 500 μg/L exposure sample was higher than that of the 100μg/L exposure sample. Due to the degradation time in seawater which was after 1 day for 100μg/L and 4 days for 500μg/L, the concentration of DEHP accumulated more in worms and sediment. The concentration of DEHP in the Day 1 exposure sample (Humber estuary) was 0.08510μg/g, and this indicates that the worms in the Humber estuary were exposed daily to a high concentration of DEHP (=~500 μg/L).

However, the concentration of DEHP was high in worm tissue after both long and short term exposure. This might be due to the worms' tolerance (homeostasis) in dealing with significant amounts of DEHP. This homeostasis is apt to exist more in several *Nereidid* polychaetes, including *H. diversicolor* where higher tolerance to zinc has been reported (Mouneyrac et al., 2003; Bryan et al., 1983; Grant et al., 1989; Hateley et al., 1989). Furthermore, regarding the effect of DEHP on the worms' active metabolism, it has been found that a decrease in aquatic animals' active metabolism leads to a decrease in their ability of detoxification from DEHP (Wofford et al.,1981).

In *H. diversicolor* tissue, the concentration level of DEHP on Day 1 was high because the DEHP that comes from factories and enters the Humber Estuary as well as from natural vegetation, sewage and other sources. According to the environment Agency, Paull is a polluted place because it has industrial pollution from chemicals, power stations, metal, minerals and fuel. Furthermore, Paull is exposed to the heavy industry of BP's chemical works.

The level of DEHP in the untreated worms after 7 days (short term) was similar to the concentration of DEHP in the untreated worms after 3 months of exposure (long term). By comparing the level of DEHP in exposed *H. diversicolor* tissue at long-term

and Day 1 (Paull, polluted sites), the level of DEHP was decreased at concentration 0.05 µg/L which explains that the worms recovered from DEHP when kept in a low environmental concentration of DEHP. Regarding the effect of DEHP on worms' active metabolism, it has been found that a decrease in the worms' active metabolism leads to a decrease in their ability of detoxification from DEHP, as Wofford et al. (1981) showed in aquatic animals. In contrast, the level of DEHP on *H. diversicolor* tissue with concentration 500µg/L was higher than Day 1. However, the worms treated with concentrations 2 µg/L ,10 µg/L and 100 µg/L were similar in the concentration of DEHP to the worms of Day 1. However, the increase of the concentration of DEHP in the worms and sediment was potentially due to leaching DEHP into the culture system water from the polycarbonate tanks of the culture system. Polycarbonate is inclined to hydrolyze or hydrolyze by base-catalysis, mainly at high temperatures, rather than at ambient temperatures at which it remains resistant to hydrolysis (Thompson and Klemchuk 1996). Some studies (e.g. Casajuana and Lacorte, 2003; Guart et al. 2014; Keresztes et al. 2013; Leivadara et al. 2008) reported increasing concentrations of DEHP when its levels in the water bottles is examined over time, while others (e.g. Al-Saleh et al. 2011; Diana and Dimitra, 2011; Guart et al. 2014; Keresztes et al. 2013) reported constant or decreasing DEHP concentrations. Not only that, but DEHP concentrations have been reported at the level of individual studies as both increasing and decreasing over time (Guart et al. 2014; Keresztes et al. 2013). A decrease in DEHP concentration may point to a breakdown in the compound of some kind, which looks unlikely.

Published reports about DEHP concentrations in marine animal tissues are very few. DEHP concentrations in this study were higher than the concentrations in other marine animals in some studies. Brown and Thompson (1982a) found the range of the concentration of DEHP in the tissues of *Daphnia magna* between 0.00000053 µg/g and 0.0000267 µg/g, while these authors (1982b) found the concentration of DEHP in *Mytilus edulis* in the range of 0.0000097- 0.0001106 µg/g. Furthermore, Mackintosh et al. (2004) and Lin et al. (2003) found DEHP concentrations in marine organisms to amount to 0.00407 µg/g.

However, the DEHP concentrations in this study were lower than the concentrations in other studies. For example, Vethaak et al. (2000) in the Netherlands found DEHP concentrations up to a maximum of 1.5 µg/g in fish and up to a maximum of 0.4 µg/g in mussels. Moreover, Huang et al. (2008) found DEHP concentrations in

fish samples in Taiwanese rivers to amount to 253.9 µg/g $^{-1}$dw in *Liza subviridis* and 129.5 µg/g $^{-1}$dw in *Oreochromis niloticus*.

DEHP also accumulated in the sediment on both monthly and daily exposures. Adding higher concentrations of DEHP led to increased accumulation in the sediment. It seems that sediment makes a good sink for DEHP in aquatic environments. DEHP is transported from seawater to sediment, mainly as a result of the settlement of suspended material that carries the adsorbed chemicals with it (Staples et al., 1997). The concentration of DEHP in the sediment depends on its rates of supply by means of sedimentation. the DEHP concentrations of this study were lower than the concentrations found in some studies, for example, DEHP concentrations in the sediments of the Mersey Estuary (UK), Nueces Estuary (Texas), Lake Yssel (Rhine Estuary) and Mississippi Estuary were in the ranges of 1.20, 0.040-16.0, 12.0-25.0 and 0.069 µg/g^{-1}, respectively (Preston & Al-Omran,1989; Ritsema et al., 1989; Ray et al., 1983; Giam et al., 1978). Moreover, Huang et al. (2008) investigated the average and the range of DEHP concentrations in the sediments of 17 Taiwanese rivers and found that they were 4.1 (<0.05-46.5) µg/g^{-1}dw in the low-flow season, and 1.2 (<0.05-13.1) µg/g^{-1}dw in the high-flow season (Magdouli et al., 2013).

Most Nereids are capable of forming burrow structures in the sediment column that are to some extent complex and somewhat permanent (Neuhoff, 1979). The worms actively ventilate the burrow structures in the sediment constantly or intermittently and evacuate the metabolites from them in order to supply burrows with fresh oxygen (Scaps, 2002). The few destructive measurements that are used to calculate the bio-turbation in the sediments and in the ventilated burrows, (Kristensen, 2001) indicate that *H. diversicolor* bio-turbs the water column that exists inside the burrow and expels a water volume of excellent quantity enough to pump into the sediment other big volumes of water. *H. diversicolor* individuals (1000-3000 m-2) are capable of passing a water column of 3-9m into their burrows daily (Kristensen, 2001). In this way, *H. diversicolor* worms can constantly flush their burrows with excess of water, and by doing so create extremely toxic as well as oxidized conditions in their burrows and the sediment that surrounds it (Kristensen, 2001).

According to Mouneyrac et al., (2003), the main route by which metals are absorbed by *H. diversicolor* is through sediment ingestion. Some studies indicate that 95% or more of the accumulated metals (Co, Cd, Se and Zn), come from sediment ingestion thanks to the higher ingestion rate of the worms, although the bioavailability

of the dissolved metals to these worms is low (Casado-Martinez et al., 2009). A big quantity of the ingested sediment exists on the surface of the sediment, after being excreted (Esselink and Zwarts, 1989). Bat (1998) reported that the concentration of Zn in the tissues of *H. diversicolor*, increases as metal sediment concentrations increase.

Zn accumulates in polychaetes in their mid-gut cells, while Cu is deposited in the pre-intestinal and hypodermal tissues (Gibbs et al., 2000). Ag accumulates in the connective tissues of the worms (Koechlin and Grasset, 1988), as well as in the cuticle and nephridia of some worms (Vovelle and Grasset, 1991). Furthermore, Pirie et al., (1985) found that Zn is deposited in the epithelial cells of the intestines of *H. diversicolor* as spherical granules. Toxicants are absorbed by the worms through two main routes of deposit-feeding, the first of which is by absorption from the surrounding seawater, which is either interstitial or across the respiratory tract of the worms and other surfaces of the body. The second route is through the absorption of contaminated material by ingestion of food and digestion of the sediment where uptake takes place through the gut walls (Weston et al., 1999). Bioturbation in aquatic sediments results from the worms activities (Rhoads, 1967; Francois et al., 1997; Pearson, 2001), as is the case with *H. diversicolor*, through which it distributes oxygen in the sediment and transports toxicants.

However, the level of DEHP in exposed seawater at long term was at a similar level to that before exposure. The DEHP levels reached 0.0000028, 0.0000027, 0.0000041, 0.0000044 and 0.0000045 µg/ml for Day 1, untreated, 0.05, 2 and 10 µg/L, respectively. However, it was lower than the level of DEHP in treated seawater at high concentrations in short-term (0.0000219 and 0.0001934 µg/ml for concentrations 100 and 500µg/L respectively). DEHP concentrations of this study with low concentrations at long-term were consistent with the DEHP concentrations found in the North Atlantic (Open Ocean) in the range of 0.0000049 µg/ml^{-1} (Giam et al. 1978). Furthermore, the DEHP concentrations in some studies were consistent with concentrations found with short-term exposuer. For example, in the Mersey Estuary (UK), Nueces Estuary (Texas), Lake Yssel (Rhine Estuary) and Mississippi Estuary, concentrations were in the ranges of 0.00013-0.00069, 0.00021-0.00077, <0.00010 - 0.0003and 0.000070 µg/ml $^{-1}$ respectively (Preston & Al-Omran,1989; Ritsema et al., 1989; Ray et al., 1983; Giam et al., 1978). In addition, in Germany, Greece and Croatia, the concentrations were 0.00005 - 0.00006 µg/ml^{-1}, 0.00093µg/ml^{-1} and 0.000247µg/ml^{-1}, respectively (Magdouli et al., 2013).

However, the DEHP concentrations of this study in both long and short term were lower than the concentrations found in some studies. For example, in Sweden and Denmark the DEHP concentrations in the surface water were in the range of LOD-0.0004µg/ml (Furtmann, 1999; Vikelsoe, 1999; Remberger, 1999), while the ranges in the Netherlands were between 0.002 µg/ml and 0.005 µg /ml (Vethaak et al.2000). In 2000, the European Community listed DEHP among the 33 hazardous substances that should be controlled and combated in surface water. In 2007 a European Directive recommended that DEHP concentrations in surface water should be controlled at 0.0013µg/ml^{-1}. Khan and Jung (2008) found that DEHP concentrations in surface water were between 0.00033µg/ml^{-1} and 0.09787µg/ml^{-1}. Yuwatini et al. (2006) reported the range of DEHP concentration in river water between 0.008µg/ml^{-1}- 0.025 µg/ml^{-1}.

In this chapter, we can conclude that long and short term results indicate that DEHP accumulation in worm tissue and sediment is greater than in seawater. Adding higher concentrations of DEHP led to increased accumulation in the worms and sediment. Furthermore, *H. diversicolor* can recover from DEHP if they are kept under the conditions of a clean environment.

CHAPTER 4: THE EFFECTS OF DEHP ON BIOCHEMICAL BIOMARKERS IN *H. DIVERSICOLOR* AFTER SHORT AND LONG-TERM EXPOSURE TO DEHP

4.1. INTRODUCTION

As the early toxic effects of chemicals on organisms take place mainly at the biochemical and cellular levels, studies have focused on the measurements of these effects as early indicators examining whether exposure to contaminants had harmful effects (Zhou et al., 2004; Li et al., 2005; Wang & Zhou, 2005, 2006a). Biomarkers are regarded as useful tools to monitor the impact of contaminants and anthropogenic activities on marine organisms and the health of ecological systems (Wang & Zhou, 2006b; Zhou, 2006). When marine organisms are exposed to very low doses of contaminants, this can result in various biological effects including oxidative stress (Lauwerys et al., 1995; Wang & Zhou, 2006a), as a result of the production of reactive oxygen species (ROS) or oxygen free radicals, such as hydrogen peroxide (H_2O_2), superoxide radicals (O_2.$^-$), and hydroxyl radical (OH.).

ROS production aided by anthropogenic activities may include organic contaminants, phthalates, heavy metals, and air and soil contaminants. Such contaminants are capable of increasing ROS production by means of various mechanisms, of which some are direct and others indirect. These may include redox reactions with O_2, enzyme induction of electron transport disruption and antioxidant defences depletion such as reduced glutathione (GSH) that participates in phase II of organic contaminants biotransformation (Livingstone, 2001; Zhou et al., 2004; Liu et al., 2005).

Antioxidant systems resulting from enzyme activities such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and glutathione peroxidase (GPx), can act against oxidative stresses as well as serve as oxidative stress biomarkers. Sensitive biomarkers that can monitor the level of water contamination and the degree of environmental safety are frequently used to compensate for ecotoxicity bioassays and chemical analyses that are performed on environmental samples in cases of acute toxic effects (Sun & Zhou, 2007). Unlike the methods used to measure contaminants accumulating in tissues, biomarkers can give more relevant ecological information about the possible effects of toxicity on living organisms health, and most important is their ability to allow early detection of contaminants in ecosystems (Ferrat et al., 2003; Almedia et al., 2002). As oxidative stress biomarkers of aquatic environments,

antioxidant enzymes have also been described in polychaete species e.g. *Heteromastus filiformis* (Abele et al., 1998), *Arenicola marina* (Buchner et al., 1996), *Eurythoe complanata* (Nusetti et al., 2001), *Sabella spallanzanii* (Bocchetti et al., 2004), *Laeonereisacuta* (Geracitano et al., 2002), *H. diversicolor* (Pe´rez et al., 2004; Ait Alla et al., 2006; Moreira et al., 2006) and some other aquatic invertebrates (Sole´ et al., 1996; Francesco et al., 1998; Cossu et al., 1997, 2000; Narbonne et al., 1999).

4.1.1. MECHANISMS AND PATHWAY OF PRODUCTION OF FREE RADICALS AND THE ENZYMES INVOLVED IN THE PROCESS

The majority of ROS are generated from electrons that escape from a mitochondrial electron-transport chain (ETC) primarily to molecular oxygen from coenzyme Q. The electrons that escape from ETC react with molecular oxygen giving $O_2^{\cdot-}$. Thereafter, it is converted to H_2O_2 and $HO\cdot$ either enzymatically or spontaneously. The amount of electrons that escape from ETC varies to a great extent depending on the physiological state of the organisms. The total ROS amount produced by the mitochondria is not much controlled at this stage by the cells, which use finely regulated antioxidants in order to keep up low steady-state level of ROS. Moreover, ETC located in or at the endoplasmic reticulum (ER), plasmatic, or nuclear membranes, produce little amounts of ROS and some oxidases. ROS production in ER is primarily connected with the hydroxylation system operation represented by the cytochrome P450 family of enzymes. Substrates oxidation involves the escape of some electrons from ETC to react with O_2 and give $O_2^{\cdot-}$ as well as other products from this transformation. The products that are formed during the process of hydroxylation may also generate ROS by entering the autoxidation cycles (Lushchak, 2014).

Different kinds of oxidases also produce ROS. They do so by oxidizing carbohydrates, amino acids, aldehydes, and heterocyclic compounds, among others. Among these oxidses, xanthine oxidase, which received a considerable attention from the part of researchers, plays a physiological role in the total balance of ROS, especially in animals. Under hypoxic conditions, this enzyme is believed to be the main producer of ROS (Griguer ,2006; Nanduri et al., 2013). However, the best studied oxidase that generates ROS in a finely controlled manner is NADPH oxidase complex (Nox). This complex was discovered in the late 1970s (Briggs et al., 1977; Yamaguchi et al., 1980) and was found to inactivate bacteria and other pathogens. It exists in nonimmune cells

and was found to produce ROS in a delicately controlled manner in order to carry out specific spatiotemporal functions (Brandes et al., 2014; Nayernia et al., 2014).

Another source of ROS is the one related to the autoxidation of various small endogenous and exogenous molecules. An example of endogenous molecules that undergo autoxidation accompanied by ROS production are epinephrine (adrenalin) and norepinephrine (Miller et al., 1996; Saller et al., 2012), while many xenobiotics, particularly various homocyclic and heterocyclic compounds, were found to be related to this type of ROS production (Renaud et al., 2012; Michail et al., 2013).

Steady-state level of ROS is not only controlled during their production, but also during their elimination. Living organisms enjoy a complicated multilevel antioxidant system that operates either to minimize the negative effects of ROS or eliminate them completely. These systems are classified via several methods, and in the following the most appreciated one will be used based on their molecular masses.

Based on this system, antioxidants are classified into two groups: antioxidants with low molecular masses, usually below one kilodalton, and antioxidants with high molecular masses, higher than one and up to ten kilodaltons. The group of antioxidants with low molecular masses includes compounds with different chemical characteristics, but familiar to the readers, including vitamins such as vitamin C (ascorbic acid) and vitamin E (tocopherol), uric acid, carotenoids, anthocyanins and polyphenols. Most of these compounds are consumed by humans in the form of food or nutritional supplements (Lushchak, 2014). However, glutathione (tripeptide c-glutam- yl-cysteinyl-glycine, GSH) which is a very important antioxidant, is synthesized by the majority of living organisms which use it to control the levels of ROS, either through direct interaction with ROS, or synergistically with ROS-detoxifying enzymes (Lushchak, 2012). Glutathione level is delicately controlled by organisms based on specific conditions through several regulatory pathways. It is worth-mentioning here that GSH, as well as thiols, sometimes react with nitric oxide to neutralize glutathione and simultaneously provide additional regulatory mechanisms for processes related to ROS, such as s-nitrosylation (Lushchak, 2012; Uys et al., 2014). This pathway does not only lower the level of nitric oxide, but also generates a buffer for this type of gaseous signal transmitter and transports it for relatively long distances, as well as prevents irreversible oxidation of the thiol groups during oxidative boots (Lushchak, 2012).

In the past few decades, most ROS researches focused on investigating the operation of antioxidants with high molecular masses, primary antioxidant and enzymes associated with them. The first significant discovery in this area was the description of

superoxide dismutase (McCord & Fridovich, 1969). The reaction: (1) $O_2^{\bullet-} + O_2^{\bullet-}$ +2H$^+$ → H$_2$O$_2$+ O$_2$ is catalysed by the enzyme; and the formed H$_2$O is either dismutated by catalase: (2) 2 H$_2$O→ 2H$_2$O$_2$+ O$_2$ or reduced by various peroxidases such as glutathione-dependent peroxidases which along with H$_2$O$_2$ (3) H$_2$O$_2$+ 2GSH → H$_2$O + GSSG also reduce lipid peroxides (4) LOOH +2GSH → LOH+ GSSG.

4.1.2. BIOMARKERS THAT MONITOR IMPACTS OF CONTAMINANTS ON *H. DIVERSICOLOR*

Most chemicals have effects on the biochemistry of organisms via mechanisms that can be metabolically toxic or genotoxic (Depledge, 1989). *H. diversicolor* and other marine invertebrates take up chemicals through the apical membranes found on their surface epithelial cells by means of diffusion facilitated by transporter proteins. Anthropogenic contamination affects the body concentrations of proteins, lipids and glycogen (Durou et al., 2007b). *H. diversicolor* from the highly contaminated French Seine estuary's were assessed and compared with *H. diversicolor* from the relatively clean French Authie estuary (Durou et al., 2007b). The worms of the Authie estuary were found to have considerably higher levels of proteins, lipids and glycogen (Durou et al., 2007b). However, the focus of this study was not on particular contaminants but on the effects of the Seine estuary general contaminant chemistry.

H. diversicolor worms are frequently taken from contaminated sites and assessed for enzymes, including catalase (CAT) and superoxide dismutase (SOD) (Moreira et al., 2006; Poirier et al., 2006; Kalman et al., 2009). CAT and SOD are involved in the protection of cells against oxidative stress (Durou et al., 2007a). When *H. diversicolor* worms are suffering from oxidative stress, CAT increases in order to act as an antioxidant by breaking down H$_2$O$_2$ into hydrogen and oxygen. By doing so CAT prevents the membrane lipids' peroxidation, thus protecting the worms from physiological damage (Durou et al., 2007a). Oxidation can also exert harmful effects on the respiratory haemoglobin of the worms. Oxidation of haemoglobin leads to the formation of meta-haemoglobin, which has no physiological function. Here also, CAT and SOD can serve an antioxidant function by protecting the worms against the build up of meta-haemoglobin (Geracitano et al., 2002). CAT and SOD levels have been measured in *Laeonereis culveri* (*Laeonereis* acuta), the annelid worm, after exposure to copper (Geracitano et al., 2002), with the result of both CAT and SOD increasing to act against copper, even at low concentrations (Geracitano et al., 2002). When *H.*

diversicolor worms are exposed to cadmium, the activity of CAT increases (Banni et al., 2009), which is also accompanied by an increase in SOD activity (Sun & Zhou, 2007).

4.2. AIMS & OBJECTIVES

4.2.1. THE AIM OF THIS CHAPTER

Investigate and assess the effect of DEHP exposure on the enzyme activities as biochemical biomarkers in exposed *H. diversicolor* in the long-term (low DEHP concentration) and short-term (high DEHP concentration).

4.2.2. THE OBJECTIVE OF THIS CHAPTER IS TO ANSWER THE FOLLOWING QUESTIONS:

➢ Did the exposure of *H. diversicolor to* DEHP affect the enzyme activities in the case of long and short term exposures?

➢ At which concentrations were the enzymes more effective in both long and short term exposures?

Based on the above objective, it was hypothesised that:

➢ DEHP affects the enzyme activities of *H. diversicolor* in both the long and short- term exposed worms.

➢ High concentrations of DEHP increase the rate of the enzyme activities in the treated worms.

➢ Untreated worms have lower rates of enzyme activity compared with DEHP treated worms.

4.3. MATERIALS AND METHODS

4.3.1. PREPARATION OF TISSUE EXTRACTS (SAMPLES) AND THE ENZYME ACTIVITY ASSAY

4.3.1.1. Preparation of worm tissues (samples) for the enzyme activity assay

The whole body of the worms were homogenised by means of ice-cold buffer (50mM Tris pH 7.5, 1mM EDTA, 1mM DL- dithiothreitol, 150 mM NaCl) using a Teflon pestle for grinding. The homogenate was then centrifuged at 12,000g for 20 minutes under refrigeration, and the supernatants were taken and used for the enzyme activity assay and then stored at the degree of –80C° for analysis based on the method of Kang et al. (2010). A catalase assay kit was used to measure Catalase (CAT) activity,

while a superoxide dismutase (SOD) kit was used to measure SOD activity. These kits were purchased from Sigma-Aldrich United Kingdom.

4.3.1.2. <u>Catalase (CAT) Assay Kit</u>

This kit offers an easy and simple colorimetric assay for studying the catalase activity in the worm tissues. The colorimetric assay procedure is used to measure the hydrogen peroxide substrate that remains after the catalase action. First, catalase converts hydrogen peroxide into water and oxygen (termed the catalatic pathway). Sodium azide is used to stop this enzymatic reaction. The reaction mix aliquot is then assayed to measure the hydrogen peroxide amount that remains after using the colorimetric method. This method employs a substituted phenol (3,5-dichloro-2-hydroxybenzene-sulfonic acid), which uses oxidation in the presence of H_2O_2 and horseradish peroxidase (HRP) to couple and form 4-aminoantipyrine, thus giving a red quinoneimine dye (N-(4-antipyryl)-3-chloro-5-sulfonate-p-benzoquinone-monoimine) that absorbs at the wavelength of 520 nm. Catalase activity is measured at a concentration of non-saturating substrate (H2O2), because it is not easy to saturate the enzyme along with the substrate at a concentration greater than 1 M.

- *Kit Reagents*

Assay buffer 10x (potassium phosphate buffer of 500 mM concentration and 7.0 pH), stop solution (sodium azide of 15 mM concentration in water), chromogen reagent, catalase positive control (0.1% thymol crystalline suspension in water), 3% (w/w) H_2O_2 solution, peroxidase with enzyme dilution buffer (potassium phosphate buffer of 50 mM concentration and 7.0 pH, containing 0.1% TRITON$^®$ X-100).

- *Preparation Instructions*
  - ➤ <u>1x Assay buffer:</u> 2 ml of the assay buffer 10 x is diluted with ultrapure water to 20 ml. The buffer is stored at room temperature.
  - ➤ <u>Peroxidase Solution:</u> 1 mg of solid peroxidise is weighed and dissolved in 1.45 ml of the 1x assay buffer. The peroxidase solution is stored for 2 weeks at 4°C.
  - ➤ <u>Colour reagent:</u> Potassium phosphate buffer (150 mM) and 7.0 pH consist of 0.25 mM 4-aminoantipyrine and 2 mM 3,5-dichloro-2-hydroxybenzenesulfonic acid. In order to prepare 200 ml of chromogen solution, 60 ml of assay buffer 10x☐ is mixed in a 250ml beaker with 140 ml of ultrapure water. 10 ml of

diluted buffer is added to the chromogen reagent in a vial and mixed till it completely dissolves. The chromogen solution is transferred to the beaker that contains the buffer and mixed well. Ten suitable aliquots are divided and stored at –20 °C. This solution remains stable for about 12 months. To prepare the colour reagent, 20 µl of the peroxidase solution is added to each 20 ml of the chromogen solution. The colour reagent is kept for three days at 4 °C if necessary.

➢ **Catalase Control:** Catalase enzyme is a water-based crystalline suspension in which the crystals fall down to the bottom of the tube. The catalase tube is then shaken vigorously till a homogenous suspension is obtained, and immediately 10 µl of the suspension is taken. This 10 µl of enzyme suspension is diluted serially 10,000-fold [for example this 10 µl of enzyme suspension is diluted with the enzyme dilution buffer down to 400 µl (1:20), then 10 µl of the first diluted solution is diluted to 400 µl (1:400), and finally, 10 µl of the second solution is diluted to 500 µl (1:10,000)]. 2 µl of the final dilution is used in each reaction. Catalase control should be prepared fresh each day.

➢ **Colorimetric Assay Substrate Solution (200 mM H_2O_2):** 3% H_2O_2 concentration ranges between 3 and 4%. The exact concentration must be determined spectrophotometrically and corrected to 200 mM concentration before the substrate solution of the colorimetric assay is used in the assay. 200 µl of the 3% H_2O_2 is diluted with 1x assay buffer to 1 ml. The exact concentration of the substrate solution is determined by diluting 50 µl of the above solution to 1 ml (20 times) with 1x assay buffer. The range of the expected concentration is 10-15 mM. The concentration can be determined using the absorbance of UV.

The actual concentration of H_2O_2 is calculated using Beer's Law ($\sum^{mM}$ = 0.0436):

$$[H_2O_2] \ (mM) = \frac{A_{240}}{0.0436}$$

The substrate solution of the colorimetric assay is adjusted with 1x assay buffer to a final concentration of exactly 200 mM. The standardized substrate solution of the colorimetric assay is stored at 4 °C for 6 days.

➢ **10 m MH_2O_2 Solution**: This solution is used to obtain a standard absorbance curve of the red quinoneimine dye against H_2O_2 concentration. 200 µl of the standardized substrate solution of the colorimetric assay (H_2O_2 of 200 mM

concentration) is diluted with 1x assay buffer to 4 ml. This solution is stored at 4 °C for 6 days.

➢ **Preparation of the Standard Curve**: The red quinoneimine dye absorbance is plotted against the amount of H_2O_2 (0.0125-0.075 μmole).

1. Series of H_2O_2 standard solutions are prepared by placing 0, 125, 250, 500, and 750 µl of H_2O_2 solution of 10 mM concentration into microcenterfuge tubes and then 1x assay buffer is added to give a final volume of 1.0 ml (Table 4.1). The solutions are mixed by inversion.

Table 4.1: Dilutions used for the preparation of the hydrogen peroxide standard curve.

Volume of 10 mM H_2O_2	1x Assay Buffer	H_2O_2 in standard solution mM	H_2O_2 in Reaction Mixture* mM
0 µl	1000 µl	0	0
125 µl	875 µl	1.25	0.0125
250 µl	750 µl	2.5	0.0250
500 µl	500 µl	5.0	0.0500
750 µl	250 µl	7.5	0.0750

2. The process involves transferring from each solution an amount of 10 µl aliquot to another tube and adding 1 ml from the colour reagent. The absorbance is read 15 minutes later at 520 nm. H_2O_2 series of standard solutions should be freshly prepared each day.

3. A standard curve of absorbance at 520 nm is plotted against the H_2O_2 final amount in the reaction mixture.

➢ **Colorimetric Assay Reaction:** The assay reaction is carried out at room temperature (about 25 °C). Table 4.2 shows the catalase enzymatic reaction.

Table 4.2: The Scheme of the catalase colorimetric enzymatic reaction.

	Sample Volume	1x Assay Buffer	200 mM H_2O_2 Solution
Zero	0	100 µl	0
Blank	0	75 µl	25 µl
Sample	x µl	75-x µl	25 µl

73 µl of 1x assay buffer is added to the micro-centrifuge tube. The reaction is started by adding 25 µl of the substrate solution of the colorimetric assay. The solution is then mixed by inversion and incubated for 3 minutes. Finally, 900 µl of the stop solution is added and the tube is inverted. A 10 µl aliquot from each solution is transferred to a second tube and 1 ml of the colour reagent is added. After waiting at least 15 minutes, then the absorbance is read at 520 nm.

- *Calculations*

1. The amount of H_2O_2 (μmoles) that remained in the mixture of the colorimetric reaction is determined using the standard curve of H_2O_2.

2. Δμmoles (H_2O_2) = μmoles of blank H_2O_2 – μmoles of sample H_2O_2. Δμmoles (H_2O_2) denotes the difference between the amount of H_2O_2 added to the colorimetric reaction in the blank and that in the sample.

3. The value from the above calculation is used to determine the activity of the catalase:

Activity (μmoles/min/ml) $= \frac{\Delta \mu moles(H2O2) x d x 100}{v x t}$

- ✓ Δμmoles (H_2O_2) = The difference between the H_2O_2 amount added to the colorimetric reaction in the blank and in the sample.
- ✓ d = The original sample dilution for the catalase reaction.
- ✓ t = The duration of the catalase reaction in minutes.
- ✓ V = The volume of the sample in the catalase reaction (x μl = 0.00x ml).
- ✓ 100 = The dilution of the aliquot remaining from the catalase reaction in the colorimetric reaction (10 μl from 1 ml).

4.3.1.3. Assay Kit for determination of Superoxide dismutase (SOD)

This kit offers an easy and simple colorimetric assay for studying superoxide dismutase (SOD) activities in worm tissues. The superoxide anion ($O_2.-$) dismutation is catalyzed by SOD into molecular oxygen and H_2O_2. SOD is a very significant antioxidative enzyme. The Kit-WST of the SOD assay is an expedient assaying method that uses Dojindo's tetrazolium salt WST-1 (2-(4-Iodophenyl)- 3-(4-nitrophenyl)-5-(2,4-disulfophenyl)- 2H- tetrazolium, monosodium salt) which is extremely soluble in water, and yields formazan dye which is soluble in water when a superoxide anion reduces it. As figure 4.1. shows, the reduction rate with O_2 is linearly related to the activity of xanthine oxidase (XO), and SOD inhibits it. Therefore, for the IC50 to be determined, a colorimetric method should be used (50% SOD inhibition activity or inhibition activity of SOD-like materials). As the absorbance at a wavelength of 440 nm is proportional to the superoxide anion amount, the SOD inhibition activity can be measured by measuring the colour development reduction at 440 nm.
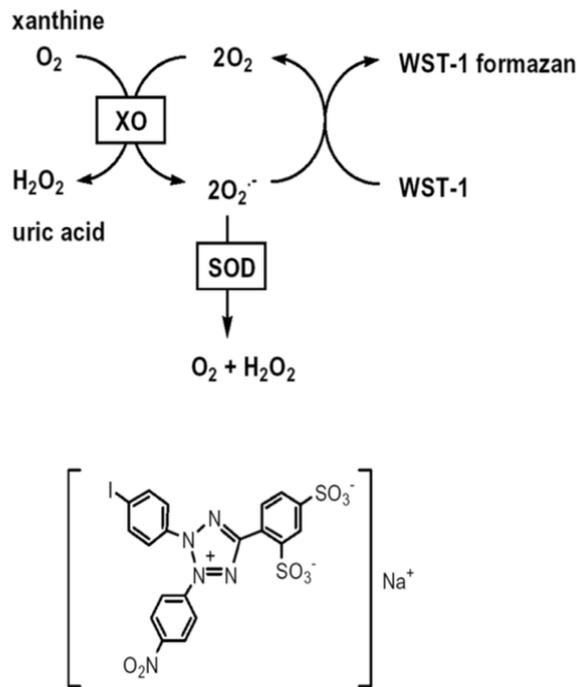
Figure 4.1: The SOD assay kit principle (Sigma-Aldrich, 2014).

- *Kit Reagents*

  5 ml of WST Solution, 100 μl of enzyme solution, 100 ml of buffer solution and 50 ml of dilution buffer.

- *Preparation Instructions*

  - **WST working solution:** Dilute 1 ml of WST solution by adding 19 ml from the buffer solution.

  - **Enzyme working solution:** Centrifuge the enzyme solution tube for 5 seconds, mix the solution, and dilute 15 μl of the enzyme solution by adding 2.5 ml of the dilution buffer.

  - **SOD Solution:** SOD is diluted using the dilution buffer, and SOD standard solution is prepared using the following concentrations: 200 U/ml, 100 U/ml, 50 U/ml, 20 U/ml, 10 U/ml, 5 U/ml, 1 U/ml, 0.1 U/ml, 0.05 U/ml, 0.01 U/ml, 0.001 U/ml.

- *Methods*

  1. Table 4.3. shows the amounts of solutions of each well. A SOD standard is used to set up wells in the same way as the sample.

2. Add 20 μl from the sample solution to each sample and to the blank well 2; and add 20 μl of distilled water to blank well 1 and to blank well 3.

3. Add 200 μl of WST working solution to each well, and mix.

4. Add 20 μl of dilution buffer to blank well 2 and blank well 3.

5. Add 20 μl of the enzyme working solution to blank well 1 and to each sample, and mix thoroughly.

6. Incubate the plate at 37 °C for 20 min.

7. Read the absorbance at 450 nm using a microplate reader.

Table 4.3: Amount of each solution in the sample, and blank wells 1, 2 and 3.

	Sample	Blank 1	Blank 2	Blank 3
Sample solution	20 μl		20 μl	
ddH$_2$O		20 μl		20 μl
WST working solution	200 μl	200 μl	200 μl	200 μl
Enzyme working solution	20 μl	20 μl		
Dilution buffer			20 μl	20 μl

- *Calculations*

SOD activity, measured as inhibition rate%, can be calculated via the following equation:

SOD activity (inhibition rate %) = {[(Ablank 1 - Ablank 3) – (Asample - Ablank 2)]/ (Ablank 1 - Ablank 3)} x 100.

- **DATA ANALYSIS**

SPSS was used in the statistical analyses. First, descriptive statistics were performed. Then, Shapiro-Wilk's test was used to test the normality of data. Homogeneity of variance was checked by Levene's test. For data that fulfilled both conditions, multiple comparisons were assessed by one-way analysis of variance (ANOVA) with Tukey's test as a post-hoc test. A Dunnett's test performed to compare untreated group with other groups. However, Welch ANOVA was carried out with a Games-Howell post-hoc when the homogeneity of variances was violated. If data were not normally distributed, a log transformation was conducted to satisfy parametric conditions. However, when the log transformation failed normality test, a non-parametric Kruskal–Wallis test was performed with multiple Mann Whitney comparison tests as post hoc tests. A Bonferoni correction used with Mann Whitney comparison tests to minimise the error rate.

4.4. RESULTS

4.4.1. THE EFFECT OF DEHP ON CATALASE ACTIVITY IN *H. DIVERSICOLOR* AFTER LONG-TERM EXPOSURE

Figure 4.2 below shows the mean catalase activity level on exposed *H. diversicolor* to DEHP at long-term (3 months) with different low concentrations 0.05 µg/L, 2 µg/Land 10 µg/L. The mean catalase activity level was increased (940 ±577.4, 1405 ±233.6 and 1545.87 ±469.7 mM) for 0.05 µg/L, 2 µg/L and 10 µg/L respectively, comparing to untreated worms (836.17 ±550.4 mM).

When a Shapiro-Wilk's test (p=0.166) was used followed by data's histograms visual inspection, normal Q-Q plots and then box plots, it transpired that the data were approximately normally distributed. The Levene's test has verified the variances equality (variance homogeneity) in the samples (p>0.05).

Therefore, a one-way ANOVA test was conducted, and it was evident that there was a statistically significant difference between groups (F (3,32) = 4.746, p = 0.008).

A Dunnett's test performed and a significant difference was found between untreated and both 2 µg/L p=0.043 and 10 µg/L p=0.010.

However, no significant difference was found between other groups (p>0.05).
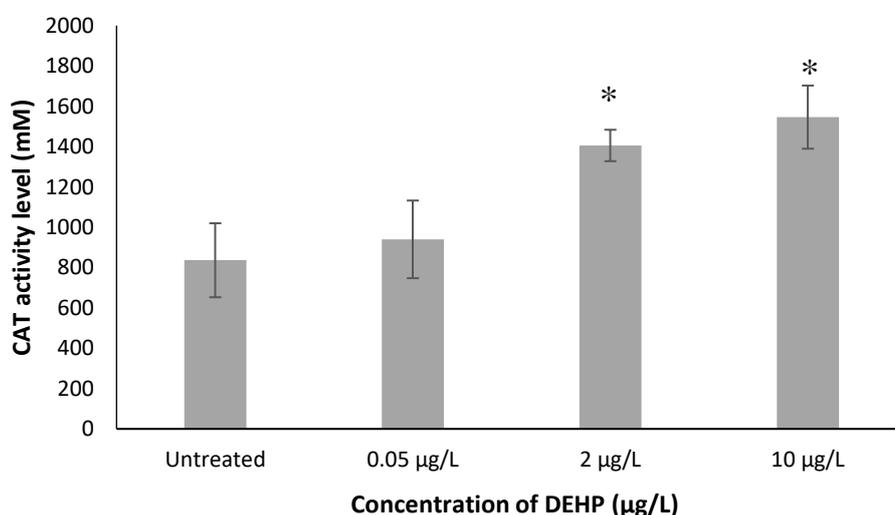
Figure 4.2: Mean catalase activity level (± SE) of long term (3 months) DEHP exposed *H. diversicolor*, n=10, Asterisk indicates significant difference from untreated group (p<0.05).

4.4.2. THE EFFECT OF DEHP ON CATALASE ACTIVITY IN *H. DIVERSICOLOR* AFTER THE SHORT-TERM EXPOSURE TO DEHP

Figure 4.3 below shows the mean catalase activity level on exposed *H.*

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diversicolor to DEHP after short-term exposure (7 days) with two different high concentrations, 100 µg/L and 500 µg/L. The mean catalase activity level was increased (1336.78 ±575 and 1691.04 ±486 mM) for 100 µg/L and 500 µg/L respectively, compared to untreated worms (1048.04 ±489.8 mM).

When a Shapiro-Wilk's test (p=0.149) was used followed by data's histograms visual inspection, normal Q-Q plots and then box plots, it transpired that the data were approximately normally distributed. The Levene's test has verified the variances equality (variance homogeneity) in the samples (p>0.05).

Therefore, one-way ANOVA test was conducted, and it was evident that there was a statistically significant difference between groups ($F_{(2,27)} = 3.857$, $p = 0.034$).

A Dunnett's test performed and a significant difference was found between untreated and 500 µg/L p=0.019.

However, no significant difference was found between other groups (p>0.05).
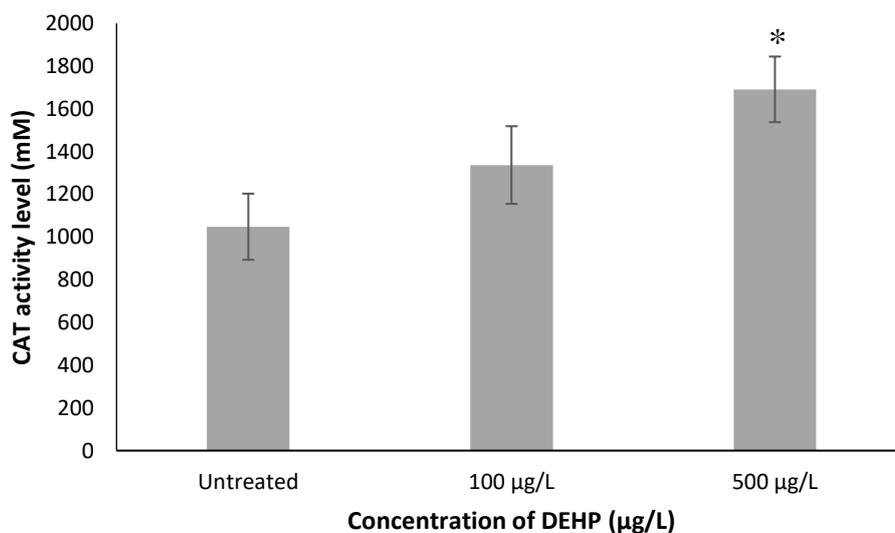
Figure 4.3: Mean catalase activity level (± SE) of short term (7 days) DEHP exposed *H. diversicolor*, n=10, Asterisk indicates significant difference from untreated group (p<0.05).

4.4.3. THE EFFECTS OF DEHP EXPOSURE ON SUPEROXIDE DISMUTASE (SOD) ACTIVITY IN *H. DIVERSICOLOR* AFTER THE LONG-TERM OF EXPOSURE

Figure 4.4 below shows the mean superoxide dismutase activity level on exposed *H. diversicolor* to DEHP at long-term (3 months) with different low concentrations 0.05 µg/L, 2 µg/L and 10 µg/L. The mean superoxide dismutase activity

level was almost stable (65.25 ±4.1, 65.38 ±5.7 and 65.61 ±9.3 mM) for 0.05 µg/L, 2 µg/L and 10 µg/L respectively compared to untreated worms (64.02 ±3.8 mM).

When a Shapiro-Wilk's test (p=0.075) was used followed by data's histograms visual inspection, normal Q-Q plots and then box plots, it transpired that the data approximately normally distributed. The Levene's test did not verify the equality of variances (homogeneity of variance) in the samples (p<0.05).

Therefore, a Welch ANOVA test was conducted, and it was evident that there was no a statistically significant difference between groups (F (3,19.267) = 1.338, p = 0.291).
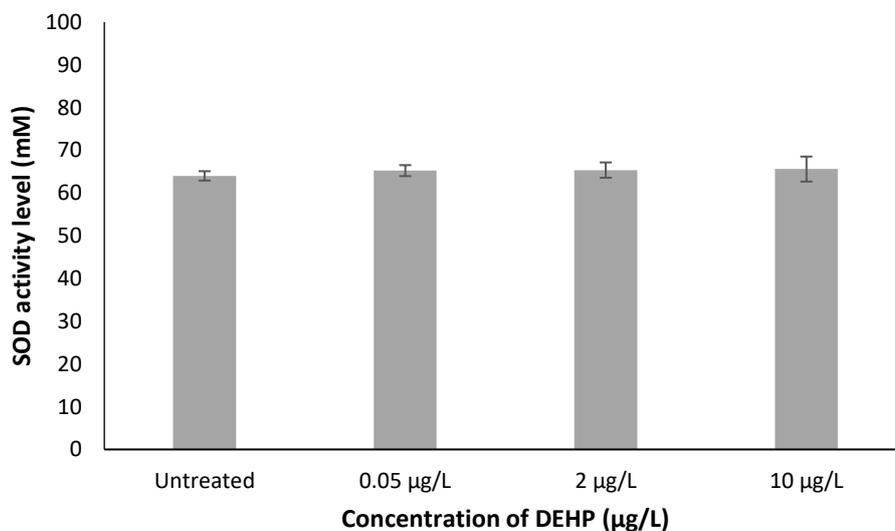
Figure 4.4: Mean SOD activity level (± SE) of long term (3 months) DEHP exposed *H. diversicolor*, n=10.

4.4.4. THE EFFECT OF SHORT-TERM OF EXPOSURE WITH DEHP ON SUPEROXIDE DISMUTASE ACTIVITY IN *H. DIVERSICOLOR*

Figure 4.5 below shows the mean superoxide dismutase activity level on exposed *H. diversicolor* to DEHP at short-term (7 days) with different high concentrations 100 µg/L and 500 µg/L. The mean superoxide dismutase activity level was almost stable (64.92 ±1.8 and 66.07 ±10.2. mM) for 100 µg/L and 500 µg/L respectively, compared to untreated worms (63.73 ±5.3 mM).

When a Shapiro-Wilk's test (p=0.00) was used followed by data's histograms visual inspection, normal Q-Q plots and then box plots, it transpired that the data were not normal distribution. Therefore, after a log transformation was conducted, a Shapiro-Wilk's test (p=0.000) used, followed by data's histograms visual inspection, normal Q-Q plots and then box plots, it was then found that the data were still not normally distributed.

Therefore, a Kruskal Wallis test was conducted, and it was evident that there was no significant difference between the groups, at $X^2(2) = 4.721$, p=0.094. It was found that the highest mean rank was for the 500 µg/L, followed by the untreated and 100 µg/L groups, respectively.
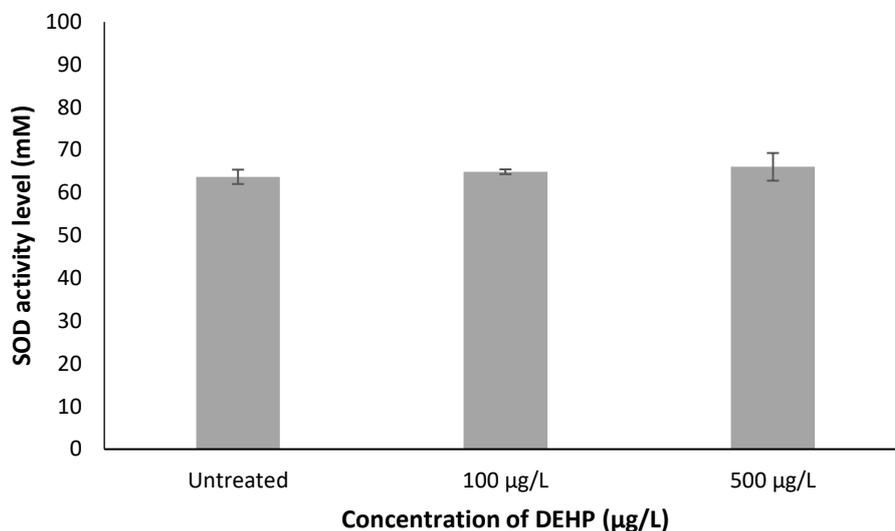
Figure 4.5: Mean SOD activity level (± SE) of short term (7 days) DEHP exposed *H. diversicolor,* n=10.

4.5. DISCUSSION

This chapter focuses on the relationship between accumulation of different concentrations of DEHP and the molecular level of *H. diversicolor*, including potential changes in enzyme activities. Antioxidant defences are among the biochemical systems that are receiving increasing interest, because they play a key role in adapting to extreme environments as well as in revealing organisms' responses to stressful conditions (Bocchetti et al., 2004). Extremely reactive oxygen species (ROS) are produced on a continuous basis in a number of aerobic metabolic pathways, but the presence of complex groups of antioxidant enzymes and scavengers of low molecular weights, normally counteracts the potential toxicity of ROS to biological components (Bocchetti et al., 2004). Antioxidant systems' efficiency under normal physiological conditions represents the basic pro-oxidant pressure to which the organism is subjected, and in different species it is influenced by a number of environmental and biological factors including the oxygen present, degree of exposure to light and temperature, available food, metabolic activity, and the life stage and the reproductive cycle phase (Dykens & Shick, 1982; Winston and Di Giulio, 1991; Shick et al., 1996; Abele et al., 1998b; Regoli et al., 2000; Vega and Pizarro, 2000; Lesser et al., 2001).

There are many kinds of environmental disturbances that enhance intracellular ROS generation, thus disrupting the balance between antioxidant defences and pro-oxidant factors. Such physiological alterations result in the oxidation of proteins, peroxidation of lipids, and disruption of the redox status of the cells (Di Giulio, 1991; Regoli, 1992; Storey, 1996; Regoli, 2000; Livingstone, 2001). These responses together, are known as oxidative stress, which is linked to several pathological and physiological conditions and is often observed in cases of hypoxia, hyperoxia, and exposure to UV radiation and pollutants (Storey, 1996; Lesser, 1997; Hermes-Lima et al., 1998; Livingstone, 2001).

As antioxidant defence systems exist in most animal species and in different animal tissue types, they also exist in most aquatic organisms. Several studies conducted on seawater organisms have indicated the significant role that antioxidant enzymes play in protecting the organisms' cellular systems from the damaging oxidative action that is induced by xenobiotics. Among these are SOD, which are metalloenzyme catalysts that convert the reactive $O_2\cdot^-$ into H_2O_2, which is also a significant ROS. Thereafter, H_2O_2 is detoxified and rendered harmless by CAT enzyme. In this way the two enzymes act together to neutralize ROS. The rate of dismutation of $O_2\cdot^-$ that is catalysed by SOD is nearest to the diffusion limit, thus becoming the most active enzyme as described by Zhou et al. (2004). When SOD and CAT and other antioxidant enzymes are exposed to different organic and metal contaminants, it has been observed that they respond by an increase in activity in order to alleviate the oxidative stress. However, such responses are often transient and vary in different species and enzymes, as well as toward single and mixed contaminants (Cossu et al., 2000; Livingstone, 2001; Valavanidisa et al., 2006).

The literature describes antioxidant enzymes in a few polychaetes, including *Heteromastus filiformis* (Claparède) (Abele et al., 1998), *Arenicola marina* (L.) (Buchner et al., 1996), *Laeonereis acuta* Treadwell (Geracitano et al., 2002), *Eurythoe complanata* (Pallas) (Nusetti et al., 2001), and lately, *Sabella spallanzanii* (Gmelin) (Bocchetti et al., 2004).

The results in this chapter show the DEHP accumulation increased in the treatment group worms at concentrations of 0.05, 2 and 10 μg/L respectively after long-term exposure (Figure 3.6) and 100 and 500 μg/L respectively after short-term exposure (Figure 3.9). The activity of CAT enzyme increases whenever DEHP concentration increases in both long-term exposure (Figure 4.2) and short-term exposure (Figure 4.3).

These results suggest that increased activity of CAT can be regarded as an indicator for the biochemical mechanisms used in the adaptation of the worm to overcome the stress of DEHP and to guard itself against its toxicity. It appears that DEHP is capable of causing oxidative damages to this worm, perhaps by creating a reactive oxygen stress in the worm's body, as is the case when ragworms are exposed to copper (Cu) (Ait Allaet al., 2006; Bouraoui et al., 2009) and in the same way as when other polychaetes are exposed to Cu (Nusetti et al., 2001; Geracitano et al., 2004b).

Moreover, CAT enzymes play a significant role in protecting the organisms' cellular systems from the damaging oxidative action that is induced by xenobiotics. Catalases convert hydrogen peroxide into oxygen and water. In the peroxisomes catalases cleanses the mitochondrial and cytosolic peroxides that form during urate oxidation (Oshino et al., 1977). Hence, catalase protect the cellular content from the damage that emanates from the creation of extremely reactive hydroxyl group by changing the superoxide ions into hydrogen peroxide (Miyazaki et al., 1991). Hydrogen peroxide is mainly generated by several peroxisomal oxidases such as acyl-CoA oxidase which shares β-oxidation with other fatty acids. Catalase and glutathione peroxidase decompose hydrogen peroxide or change it into hydroxyl radicals (·OH). These hydroxyl radicals can sever the peroxisomal membrane by peroxidating the unsaturated fatty acids. Hydroperoxides formed are broken down by glutathione-peroxidase and catalase.

This conclusion is in line with the conclusion of other studies about increases in Catalase activity. High Catalase activity is shown by *H. diversicolor* when the worm is exposed to copper at long-term exposure (Ait Alla et al., 2006) and short-term exposure (Bouraoui et al., 2009). The Catalase activity also increased when the worm is exposed to seawater acidification or to mercury contamination (Hg) (Freitas et al., 2017). When the worm was exposed to cadmium (Cd), a significant increase in the activity of CAT was observed (Banniet al., 2009). However, there were studies that contradicted all this the above finding, where there was a decrease in CAT activities in this worm when it was exposed to dissolved nano-silver and bulk-sized silver (Cozzari etal, 2015). Similar results were found in the olive flounder (*Paralichthys olivaceus*) when the flounders were exposed to diethyl phthalate (DEP) (Kang et al., 2010).

On the other hand, in the worms, the activity of SOD enzyme was almost stable wherever DEHP concentrations increased in both long-term exposure at low concentration for 0.05 µg/L, 2 µg/L and 10 µg/L respectively (Figure 4.4) and short-term exposure at high concentration for 100 µg/L and 500 µg/L respectively (Figure

4.5). SOD activity in numerous marine invertebrates is to a great extent temperature dependent (Buchner et al., 1996; Abele et al., 1998; Abele and Puntarulo, 2004; Moreira et al., 2006). For instance, the activity of SOD in the capetellid polychaete *H. filiformis* was found to be higher in summer than in winter, and such SOD activity in the case of summer worms was found to considerably decrease upon cooling (Abele et al., 1998). In the polychaete *Arenicola marina,* SOD activity showed almost the same seasonal variability than the levels of *H. filiformis* (Buchner et al., 1996). On the other hand, some abiotic factors have a considerable effect on the creation of ROS as well as the antioxidant biomarkers, such as salinity, pH, temperature, and oxygen concentration. The effect of these abiotic factors is well-documented in the capitellid worm *Heteromastus filiformis* and *Laeonereis acuta* (Polychaeta Nereididae) (Abele et al., 1998; Geracitano et al., 2004a)

This study is different to the studies that show increase in the activity of SOD. Moreira et al. (2006) found a considerable induction of the activity of SOD in *H. diversicolor* after exposure to contaminated sediments, relative to the pooled references. The activity of SOD in *H. diversicolor* also increased after exposure to Cd proportionally with exposure time (Sun & Zhou, 2007). Besides, the studies of Geracitano et al. (2002) confirmed the higher SOD activity in *Laeonereis acuta* populations that were exposed to Cu relative to the control group members (31.25 and 62:50 μg/L). However, there are other studies that found a decrease in the activity of SOD in *H. diversicolor* when the organism was exposed to dissolved nano-sized and bulk-sized silver (Cozzariet al., 2015).

Livingstone et al. (1995) demonstrated that the activity of SOD in the mussel *Mytilus galloprovincia* is quite stable during exposure to organic contaminants. Cossu et al. (1997) confirmed this, reporting that SOD shows a steady activity and was not affected when freshwater bivalves *Uniotumidus* is exposed to industrial sources of pollution, except in the most contaminated investigated sites. However, it was noted that the antioxidant systems activity increases or decreases depending on the intensity and duration of stress of the contaminants. Nevertheless, these two kinds of responses do not contradict each other, and mainly depend on the exact nature of the stress applied, the amount of stress as well as on the exposed species susceptibility (Cossu et al., 2000). Levels of the antioxidant enzyme also vary within and among species depending on the physiological conditions and seasonal factors, gametogenesis period and other sexual conditions, and behaviours (Cossus et al., 2000; Sun & Zhou, 2007).

In this chapter, we can conclude that the increase of the CAT enzyme activity in *H. diversicolor* is a sensitive antioxidant biomarker of the response to DEHP stress. The antioxidant systems' activity may increase or decrease under the contaminants stress. These two responses do not contradict each other, but emerge depending on the duration and intensity of the stress, as well as on the susceptibility of the living species exposed to the stress (Cossu et al., 2000). Moreover, the levels of the antioxidant enzyme may vary in the same species and among them depending on physiological and seasonal factors, gametogenesis period and other sexual conditions and behaviours (Sun & Zhou, 2007).

However, due to the nearby industrial, agricultural and domestic activities, the aquatic environment (hydrosphere) has become contaminated to the extent that it is now a global concern for the policy makers as well as for the general public. Assessment of environmental risks aims to measure the potential harmful effects that the ecosystems undergo due to these human activities (Allen and Moore, 2004; Moore et al., 2004). The eventual recipients of damage generated by pollutants are the biological systems. Fortunately, not all kinds of pollution lead to detrimental effects, in addition to the fact that such harmful effects usually depend on the species and life-stage thereof (Jha, 2004). Detection and measurement of environmental pollutants are carried out by means of analytical instruments, and is therefore, not sufficient to determine their accurate impacts on these systems (Jha et al., 2000; Jha, 2008). Moreover, as pollutants exist in the environment in the form of complex mixtures, accurate evaluation and consistent prediction of their toxic effects on the marine organisms is also difficult (Feron and Groten, 2002; Jha et al., 2000). Because of such difficulties, the use of subtle responses of organisms to stressors as biomarkers, such as biomarkers at the species, tissue, organ, cell, or molecule levels, now form a well-established part of ecotoxicology (e.g. Al-Subiai et al., 2011; Canty et al., 2009; Lewis et al., 1999; Moore et al., 2004). Such markers are in many ways advantageous relative to the usual analytical, chemical and ecological methods. Their advantages stem from their potential predictive and precautionary properties, as first hand effects markers at the biochemical level that always take place before populations are affected, especially in the case of long term low level exposure to contaminants; and from their broad range of applicability, as they are tailored to suit several cases that assess definite concerns; as well as from their practicality since many biological markers are so designed as to be compatible with general use laboratory instruments and minimum training (Cairns and Pratt, 1989; Lyons et al., 2010).

As human health is potentially linked to the environment (Jha, 2004; Moore et al., 2013), the phylogenetic groups that provide humans with ecosystem services by creating benefits for mankind, directly such as by providing food, or indirectly such as by boosting soil fertility, are especially important for the conservation of human health (Costanza et al., 2014; Simpson, 2014). In this respect one should appreciate the critical role of invertebrates that constitute 95% of the existing species in the ecosystems' functioning that serve the most important sources of food for humans. Furthermore, marine invertebrates have been found to provide a wide array of ecosystem services, and any threats against them, such as pollution and climatic changes should be earnestly heeded (Basset et al., 2013; Rife, 2014). Hence, several seawater and freshwater species are often used to evaluate the biological responses to relevant pollutants in the laboratory. This includes determination of the regulatory observance of existing and new chemicals in chronic and acute ecotoxicology studies (ASTM, 1992). Ecotoxicological tests need to be performed for a broad range of invertebrates that represent different phyla and at various trophic levels, as they have become imperative from both regulatory and scientific perspectives (Galloway and Depledge, 2001; Jha, 2004, 2008).

CHAPTER 5: THE EFFECTS OF DEHP ON THE STABILITY OF FUNCTIONAL TRAITS IN *H. DIVERSICOLOR* AFTER SHORT AND LONG-TERM EXPOSURE TO DEHP

5.1. INTRODUCTION

The description of the effects of DEHP exposure upon the behaviours of *H. diversicolor* in the presence and absence of predators first requires clarifying what the term behaviour means. Behaviour is defined as "the relationship of animals to their environment and to other animals" (Lawrence, 2008).

The burrowing behaviour of *H. diversicolor* is the most frequently used reliable quantifiable behavioural trait frequently used to measure the reactions of *H. diversicolor* to contaminants (Kalman et al., 2009; Bonnard et al., 2009). When *H. diversicolor* is exposed to contaminants, it has been observed that it becomes hypoactive, which indicates the inhibition of the locomotive activity behaviours (Bonnard et al., 2009). Among these, burrowing of *H. diversicolor* has been observed to suffer from severe impairment when the worms are taken from a relatively clean environment and then exposed to contaminants (Buffet et al., 2003). Burrowing behaviour impairment makes the worm more exposed to predator attacks and sweeping wave action, thus impairing its chances of survival (Kalman et al., 2009). However, such impairments in burrowing are much reduced when the worms develop tolerance behaviours.

A second behavioural trait observed in these worms, though less frequently mentioned in the literature, is their feeding behaviours which are measured by the amounts of ingested food. These traits are both behavioural and physiological in character. While the feeding trait is often described as behavioural, the processing and digestion of ingested food is classified as physiological. Poor feeding takes place in these worms as a response to contaminants (Kalman et al., 2009). In experiments by Kalman et al. (2009) conducted in France, *H. diversicolor* specimens were taken from the Loir estuary, which is highly contaminated, and others were taken from the Bay of Bourgneuf, which is relatively clean. Specimens from both worm populations were then taken to a source of food where they could feed at will. It was then observed that specimens from the contaminated Loir ate less food. This feeding depression is regarded as an avoidance behaviour by which the worms are responding to the present contaminants (Kalman et al., 2009).

5.1.1. PREDATORS AND BEHAVIOURS OF POLYCHAETES

Predation is considered as an important behavioural trait that affects evolution, life history and the visible traits of the prey animal (Ferrari et al., 2010; Abrams, 2000). Both aquatic and terrestrial animals have evolved mechanisms that help to evade and avoid predators, without having experienced direct attacks before (Schaum et al., 2013). Many marine polychaete worms engage in remarkable predator-prey interactions, especially when they emerge from their burrows, and expose themselves to relatively high predation risk levels (Davey & George, 1986). At such instances the costs of avoiding being eaten by predators outweigh the costs of losing energy by not emerging to feed (Watson et al., 2005). The animals' dependence on sound, shadowing and mechanical disturbance for detection of predators can be limited because of the higher frequency of non-predatory stimuli of abiotic nature in the seawater environment (Evans, 1969). Abiotic cues of this kind are known to orientate nereidid polychaete worms all the time, and there is a general belief (Clark, 1960) that most probably the worms would be under permanent influence of these stimuli.

Alternatively, detection can take place by means of the chemical cues that are carried via water columns by means of diffusion and brought into the worms' burrows during irrigation times (Riisgard, 1991). Predator avoidance behaviour, also known as alarm cues (Smith, 1986), by means of substance triggering (Ferrari et al., 2010), may come from various origins, such as the mucous of the predator (Forward & Rittschof, 2000), its skin cells (Chivers et al., 2007), or its faeces, especially when the predator's gut contains some ingested prey items (Brown, 2003; Nunes et al., 2013). There are also indirect alarm cues, that originate from other hetero-specific preys after being injured or just attacked (Rahman et al., 2000). Such cues have been described as having profound effects on the behaviour of preys in numerous invertebrate taxa, such as flatworms (Wisenden & Millard, 2001), zooplankton (Lass & Spaak, 2003; Pestana et al., 2013), brittle stars (Rosenberg and Selander, 2000) and gastropods (Mach & Bourdeau, 2011; Kicklighter et al., 2007).

Platynereis dumerilii, another closely related nereidid polychaete, has been shown to respond to chemical cues by modulating its out-of burrow activities (Gambi et al., 1996) and *Nereis virens,* the king ragworm, a third related polychaete, has been known to reduce its out-of-burrow activities when exposed to hetero-specific body homogenate e.g. by reducing its feeding events (Watson et al., 2005). Fish muscle extract has also been shown to reduce the worm's feeding events, but without considerable reduction of the activity levels, while extracts of *Arenicola marina,* the

sympatric lugworm, and *Hediste diversicolor,* did not induce any considerable responses (Schaum et al., 2013).

5.1.2. *H. DIVERSICOLOR* FEEDING BEHAVIOUR

The ragworms are omnivorous and use different feeding methods including carnivorous feeding, scavenging, feeding on filtered suspended matter, and feeding on deposited matter that exist on the sediment surface (Barnes, 1994). The worms feed using their eversible pharynx as well as the sensory appendages found on their heads that include the tentacles and palps. Unlike *N. virens,* its closely related polychaete, *H. diversicolor* has the distinctive ability to fulfill its metabolic requirements using a meal of phytoplankton, which forms an obligate filter feeding for the worms (Nielsen et al., 1995).

Harley (1950) described the mechanism of filter feeding, which starts with drawing across the burrow a net shaped like a funnel consisting of small mucous threads, thus driving a water current through the net, (Fauchald & Jumars, 1979), and by means of undulating body movements the food particles enter the net. After accumulating particles on the net, the worm consumes them together with the net. After some time, another net is generated (Fauchald & Jumars, 1979). Riisgård (1991) estimates that *H. diversicolor* has the ability to control the phytoplankton levels that exists in shallow brackish waters. It is unclear whether the worms can live exclusively on suspended matter particles, however worms always practice suspension feeding when there are sufficient amounts of algal cells in the water (Riisgård, 1991). 'Deposit feeding' by the worms was described by Esnault et al. (1990): in searching behaviour, the worm crawls on the substrate surface where food is prospected; when it finds some, it catches it by means of its jaws and ingests it immediately. In the second type, the worm deposits several mucous threads on the substrate surface on the side of its body to catch the prospected food particles. The worm then retreats to its burrow and brings the mucous threads back, thus making the particles into a pellet for immediate consumption or for storage to be consumed later on (Esnault et al., 1990).

Olivier et al. (1995) described the ability of young individuals of *H. diversicolor* to collect debris that exists on the sediment surface and take it to be stored in their burrows. The young worms then irrigate their burrows in order to create aerobic conditions that stimulate bacterial growth so as to aid in decaying the plant debris ('gardening'). Lucas and Bertru (1997) found some kind of bacteriolytic activity in the gut of these worms, which indicates their ability to feed on bacteria.

5.1.3. *H. DIVERSICOLOR* BURROWING BEHAVIOUR

The burrowing behaviour of *H. diversicolor* plays an important role for the worms, as it is a crucial component of their lifestyle, and constitutes a vital part of their strategies of survival. From a physiological standpoint, the burrowing behaviour of these worms is regarded as chain a reaction that emanates from instinctive reflexes (Bonnard et al., 2009). *H. diversicolor* burrowing must be effective as the worms utilize it to protect themselves from predation as well as from wave action (Kalman et al., 2009). In this way, the worms' burrowing ability increases their successful adaptation to the environment, and the overall successful burrowing behaviour of the entire population means higher survival rates and higher probability of reproductive success. High rates of burrowing of *H. diversicolor* can also impact the worms' success at the ecosystem level, because it does not only aid bioturbation, but it also increases the success of the species at the population level, and in this way may positively influence the whole species community (Lawrence & Soame, 2009; Scaps, 2002).

H. diversicolor lives in burrows shaped like a Y or U (Dales, 1950; Durou & Mouneyrac, 2007). The worms are territorial and will fight against members of their own species to defend their burrows. To perform their defending tasks, the worms make with each other contact on the sediment surface in order to settle the matter (Scaps, 2002).

5.2. AIMS AND OBJECTIVES

5.2.1. THE AIM OF THIS CHAPTER

To investigate and assess the effect of DEHP on *H. diversicolor*' behaviours (feeding response and burrowing time) in the presence and absence of predators and to examine whether DEHP exposure impacts upon the behaviours exhibited.

5.2.2. THE OBJECTIVE OF THIS CHAPTER IS TO ANSWER THE FOLLOWING QUESTIONS:

➢ Has DEHP any effect on the behaviours of *H. diversicolor*' in the case of long-term at low DEHP concentration and short-term at high DEHP concentration exposures?

➢ Did the recovered worms fail or succeed to respond in their feeding and burrowing times in the presence of the predator?

➢ Did the treatment worms fail or succeed to respond in their feeding and burrowing times in the presence of the predator?

Based on the above objective, it is hypothesised that:

- ➢ The behaviours will be negatively impacted on *H. diversicolor*' exposed to DEHP in the case of both long-term exposure at low DEHP concentration and short-term exposure at high DEHP concentration.
  - ➢ Treated worms will show reduced response levels towards the presence of the predator depending on the concentration of DEHP.
  - ➢ Untreated worms will succeed to detecting the presence of the predator and respond in feeding and burrowing time

5.3. MATERIALS AND METHODS

5.3.1. FEEDING RESPONSE IN THE ABSENCE AND PRESENCE OF PREDATORS

Mud crabs (*Rhithropanopeus harrisii)* were used as predators to examine the feeding behaviour of the worms in response to the absence and presence of the predator's odour. *R. Harrisii* feeds on *H. diversicolor* as a main source of food (Turoboyski, 1973). However, mud crabs leaves behind in the water a strong chemical cue and by doing so becomes easily detectable by *H. diversicolor* (Aarnio et al., 2015). Ten small mud crabs were placed in a 500 ml glass beaker filled with seawater and left for three hours in order to generate a sample of water conditioned for the crabs.

10 *H. diversicolor* worms were collected from each different concentrations of 0.05, 2 and 10 μg/L after long-term exposure at low DEHP concentration, 10 worms from different concentrations of 100 and 500 μg/L after short-term exposure at high DEHP concentration, and 10 untreated worms. The worms were then transferred one by one to crystallising dishes of about 67 mm diameter containing coral sand and filtered seawater. The worms were left for 48 hours without food prior to examination. They were then exposed to 200μl of supernatant of food in the form of fish food flakes placed in the water to stimulate the worms to eat, and the time of worms' response to eating was counted. This time represents the period the worms need to detect food. Thereafter, 500μl of crab conditioned water was added by injecting it into the water column to make up the predator environment in the water column. Five minutes later, a food source of fish food flakes was added in order to test the worms feeding response in the presence of the simulated predator, and the worms' response time to eat was counted (Figure 5.1.).
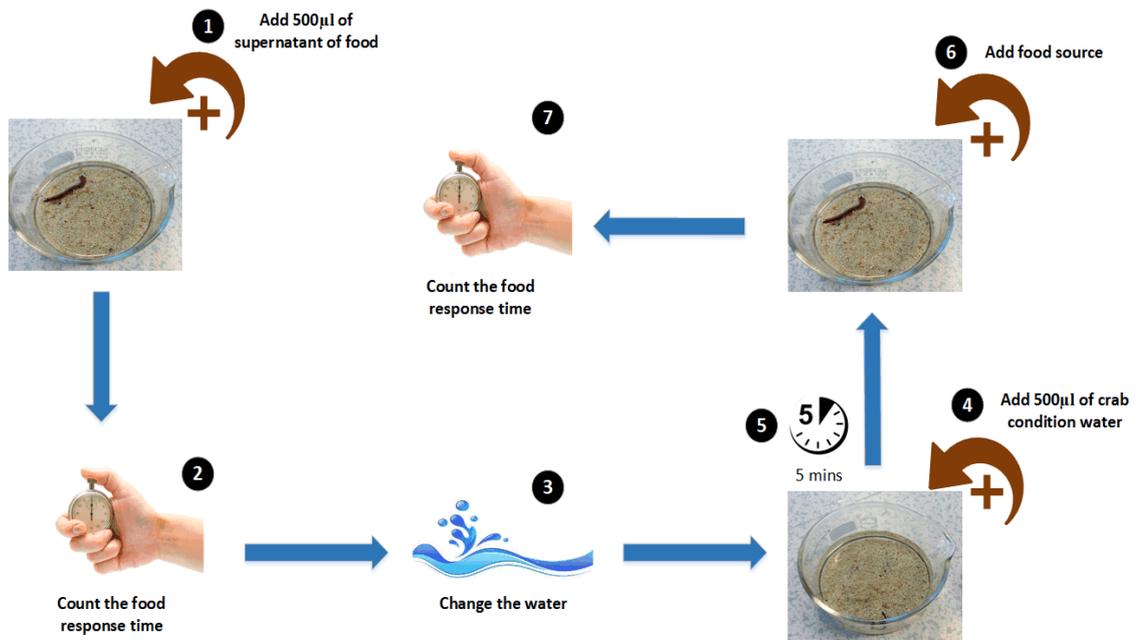
Figure 5.1: Shows feeding response time of exposed *H. diversicolor* to DEHP in the absence and presence of predator (Rhithropanopeus harrisii).

5.3.2. BURROWING TIME IN THE ABSENCE AND PRESENCE OF PREDATORS

Mud crabs (*Rhithropanopeus harrisii)* were used as predators to examine the burrowing time behaviour of the worms in response to predator odour. Ten small mud crabs were placed in a 500ml glass beaker filled with seawater and left for three hours in order to generate a sample of water conditioned for the crabs. Ten *H. diversicolor* worms were collected from different phthalate concentrations of 0.05, 2 and 10 μg/L after long-term exposure at low DEHP concentration, 10 worms from different phthalate concentrations of 100 and 500 μg/L after short-term exposure at high DEHP concentration, and 10 untreated worms. The worms were then transferred one by one to crystallising dishes of about 67 mm diameter containing coral sand and filtered seawater. Time was counted from when the worms were placed onto the coral sand; they immediately crawled with whole body undulations, and then rapidly disappeared from view as they went into the burrows. The same procedure was repeated with 500 μl of crab conditioned water added by injecting it into the water column (Figure 5.2.). This method was originally introduced by Bonnard et al (2009), but then variations, based on Kalman et al. (2009) and Buffet et al. (2011) and others, were introduced to the method.
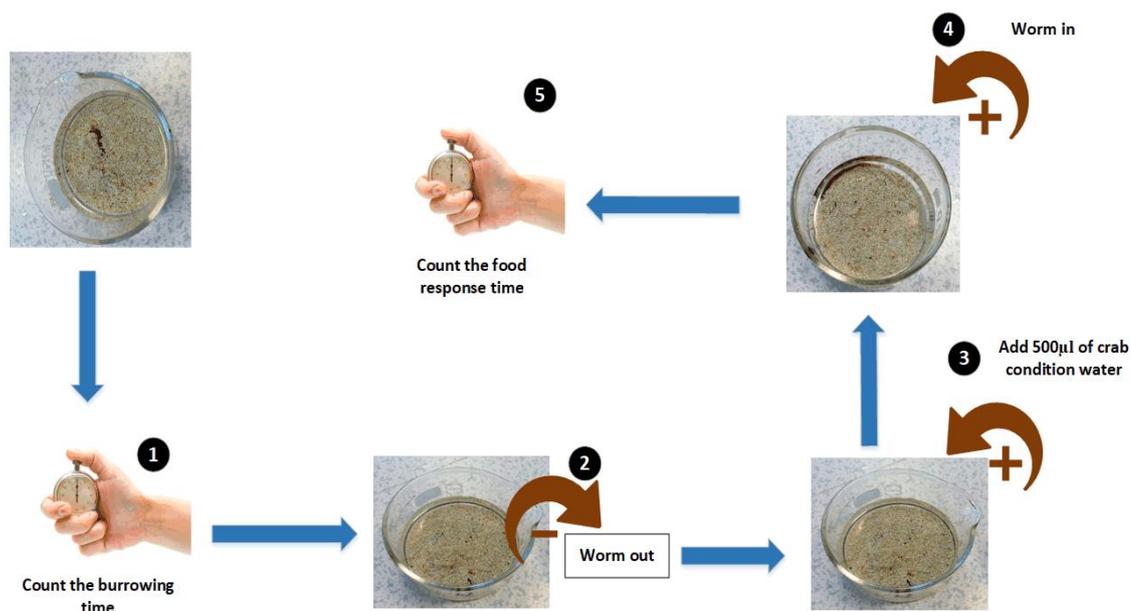
Figure 5.2: Shows burrowing time of exposed *H. diversicolor* to DEHP in the absence and presence of predator (Rhithropanopeus harrisii).

- **DATA ANALYSIS**

SPSS was used in the statistical analyses. First, descriptive statistics were performed. Then, Shapiro-Wilk's test was used to test the normality of data. Homogeneity of variance was checked by Levene's test. For data that fulfilled both conditions, multiple comparisons were assessed by one-way analysis of variance (ANOVA) with Tukey's test as a post-hoc test. A Dunnett's test performed to compare untreated group with other groups. However, Welch ANOVA was carried out with a Games-Howell post-hoc when the homogeneity of variances was violated. If data were not normally distributed, a log transformation was conducted to satisfy parametric conditions. However, when the log transformation failed normality test, a non-parametric Kruskal–Wallis test was performed with multiple Mann Whitney comparison tests as post hoc tests. A Bonferoni correction used with Mann Whitney comparison tests to minimise the error rate.

5.4. RESULTS

5.4.1. FEEDING RESPONSE TIME IN THE ABSENCE AND PRESENCE OF PREDATORS AT LONG-TERM OF EXPOSURE

Figure 5.3 below shows the mean feeding response time of long-term DEHP exposed *H. diversicolor* in the absence and presence of predators with low DEHP concentration (crab conditioned water). *H. diversicolor* were collected from tanks exposed to different DEHP concentrations of 0.05, 2, 10 µg/L and untreated worms as

controls. Worms were left for 48 hours without food prior to examination. They were then exposed to supernatant food in the water, and the time of worm's response to eating was counted. Thereafter crab conditioned water was added by injecting it into the water column. Five minutes later food source of fish food flakes was added, and the worms' response time to eat was counted. The mean feeding response time of *H. diversicolor* was lower for the worms taken from DEHP concentrations of 0.05 µg/L, 2 µg/L and 10 µg/L, (02:12 ±00:58, 01:20 ±00:49 and 00:54 ±00:37 mins) compared to the untreated group (02:39 ±02:41 mins) in the absence of predator odour, and (03:42 ±01:24, 02:33 ±01:29 and 01:58 ±00:50 mins) compared to the untreated group (04:50 ±03:37 mins) in the presence of predator odour.

Predator absence

When a Shapiro-Wilk's test (p=0.00) was used followed by data's histograms visual inspection, normal Q-Q plots and then box plots, it transpired that the data were not normal distribution. Therefore, after a log transformation was conducted, a Shapiro-Wilk's test (p=0.645) used, followed by data's histograms visual inspection, normal Q-Q plots and then box plots, it was then found that the data were approximately normally distributed. The Levene's test has verified the variances equality (variance homogeneity) in the samples (p>0.05).

Therefore, one-way ANOVA test was conducted, and it was evident that there was a statistically significant difference between groups (F (3,36) = 5.710, p = 0.003).

A Dunnett's test performed and a significant difference was found between untreated and 10 µg/L p=0.004.

In addition, a Tukey post hoc test found a significant difference between the 0.05µg/L and 10µg/L groups, p=0.008. However, no significant difference was found between other groups (p>0.05).

Predator Presence

When a Shapiro-Wilk's test (p=0.00) was used followed by data's histograms visual inspection, normal Q-Q plots and then box plots, it transpired that the data were not normal distribution. Therefore, after a log transformation was conducted, a Shapiro-Wilk's test (p=0.746) used, followed by data's histograms visual inspection, normal Q-Q plots and then box plots, it was then found that the data were approximately normally distributed. The Levene's test did not verify the equality of variances (homogeneity of variance) in the samples (p<0.05).

Therefore, a Welch ANOVA test was conducted, and it was evident that there was a statistically significant difference between groups (F (3,19.554) = 3.796, p = 0.027).

A Games-Howell test found a significant difference between the 0.05µg/L and 10µg/L groups, p=0.029. However, no significant difference was found between other groups (p>0.05).
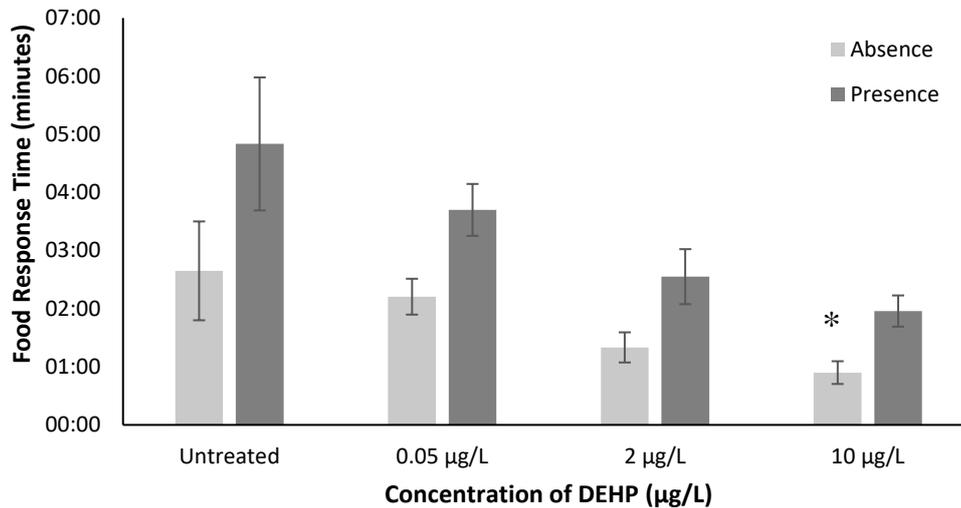
Figure 5.3: Mean feeding response time in the absence and presence of predators (± SE) of exposed _H. diversicolor_ to DEHP at long-term, n = 10, Asterisk indicates significant difference from untreated group (p<0.05).

5.4.2. FEEDING RESPONSE TIME IN THE ABSENCE AND PRESENCE OF PREDATORS UNDER SHORT-TERM EXPOSURE TO DEHP

Figure 5.4 below shows the mean feeding response time of short-term DEHP exposed _H. diversicolor_ in the absence and presence of predators (crab conditioned water, i.e. odour). Worms were collected from different DEHP concentration tanks (100,500 µg/L DEHP) and untreated worms as controls. Worms were left for 48 hours without food prior to examination. They were then exposed to supernatant water from food (tropical fish flakes), and the time noted the worms took to respond and to eat was counted. Thereafter crab conditioned water (predator odour) was added by injecting it into the water column. Five minutes later a food source of fish food flakes was added, and the worms' response time was again counted. The mean feeding response time of _H. diversicolor_ was lower for 100 µg/L and 500 µg/L worms (01:39 ±01:40 and 01:20 ±01:01 mins) compared to the untreated group worms (02:02 ±00:58 mins) in the absence of predator odour, and (02:51 ±02:22 and 02:27 ±01:42 mins) compared to untreated worms (03:28 ±02:01 mins) in the presence of predator odour.

Predator absence

When a Shapiro-Wilk's test (p=0.020) was used followed by data's histograms visual inspection, normal Q-Q plots and then box plots, it transpired that the data were not normal distribution. Therefore, after a log transformation was conducted, a Shapiro-Wilk's test (p=0.062) used, followed by data's histograms visual inspection, normal Q-Q plots and then box plots, it was then found that the data were approximately normally distributed. The Levene's test did not verify the equality of variances (homogeneity of variance) in the samples (p<0.05).

Therefore, a Welch ANOVA test was conducted, and it was evident that there was no a statistically significant difference between groups (F (2,17.010) = 2.008, p = 0.165).

Predator Presence

When a Shapiro-Wilk's test (p=0.019) was used followed by data's histograms visual inspection, normal Q-Q plots and then box plots, it transpired that the data were not normal distribution. Therefore, after a log transformation was conducted, a Shapiro-Wilk's test (p=0.043) used, followed by data's histograms visual inspection, normal Q-Q plots and then box plots, it was then found that the data were still not normally distributed.

Therefore, a Kruskal Wallis test was conducted, and it was evident that there was no significant difference between groups in the presence of predator, at $X^2(2)$ = 0.735, p=0.693. It was found that the highest mean rank was for untreated group followed by 100 µg/L and 500 µg/L groups, respectively.

Figure 5.4: Mean feeding response in the absence and presence of predators (± SE) of exposed *H. diversicolor* to DEHP at short-term, n = 10.

5.4.3. BURROWING TIME IN THE ABSENCE AND PRESENCE OF PREDATORS UNDER LONG-TERM EXPOSURE TO DEHP

Figure 5.5 below shows the mean burrowing time of DEHP exposed *H. diversicolor* in the absence and presence of predators (crab conditioned water, i.e. odour) after long-term culture with low DEHP concentration. *H. diversicolor* were collected from tanks with different DEHP concentrations (0.05, 2, 10 µg/L DEHP) and untreated worms as controls. The worms were then transferred one by one to crystallising dishes containing coral sand and filtered seawater. Time was counted from when the worms were placed onto the coral sand until they rapidly disappeared as they went into the sediment. The same procedure was repeated with crab conditioned water added by injecting it into the water column. The mean burrowing time of *H. diversicolor* increased for DEHP exposure cultured worms from 0.05 µg/L, 2 µg/L and 10 µg/L, (01:56 ±00:51, 02:07 ±01:09 and 02:23 ±00:58 mins) compared to worms from the untreated group (01:47 ±00:32 mins) in the absence of predator odour. Equally, differences where found when DEHP exposed worms were compared (01:10 ±00:26, 01:15 ±00:48 and 01:26 ±00:53 mins) to worms from the untreated group (01:07 ±00:39 mins) in the presence of predator odour.

Predator absence

When a Shapiro-Wilk's test (p=0.012) was used followed by data's histograms visual inspection, normal Q-Q plots and then box plots, it transpired that the data were not normal distribution. Therefore, after a log transformation was conducted, a Shapiro-Wilk's test (p=0.457) used, followed by data's histograms visual inspection, normal Q-Q plots and then box plots, it was then found that the data were approximately normally distributed. The Levene's test did not verify the equality of variances (homogeneity of variance) in the samples (p<0.05).

Therefore, a Welch ANOVA test was conducted, and it was evident that there was no statistically significant difference between groups (F (3,19.371) = 0.700, p = 0.564).

Predator Presence

When a Shapiro-Wilk's test (p=0.00) was used followed by data's histograms visual inspection, normal Q-Q plots and then box plots, it transpired that the data were not normal distribution. Therefore, after a log transformation was conducted, a Shapiro-Wilk's test (p=0.050) used, followed by data's histograms visual inspection, normal Q-

Q plots and then box plots, it was then found that the data were still not normally distributed.

Therefore, a Kruskal Wallis test was conducted, and it was evident that there was no significant difference between groups in the presence of predator odour, at $X^2(3)$ = 0.943, p=0.815. It was found that the highest mean rank was for the 10 µg/L group followed by the 0.05 µg/L, 2 µg/L and untreated groups, respectively.
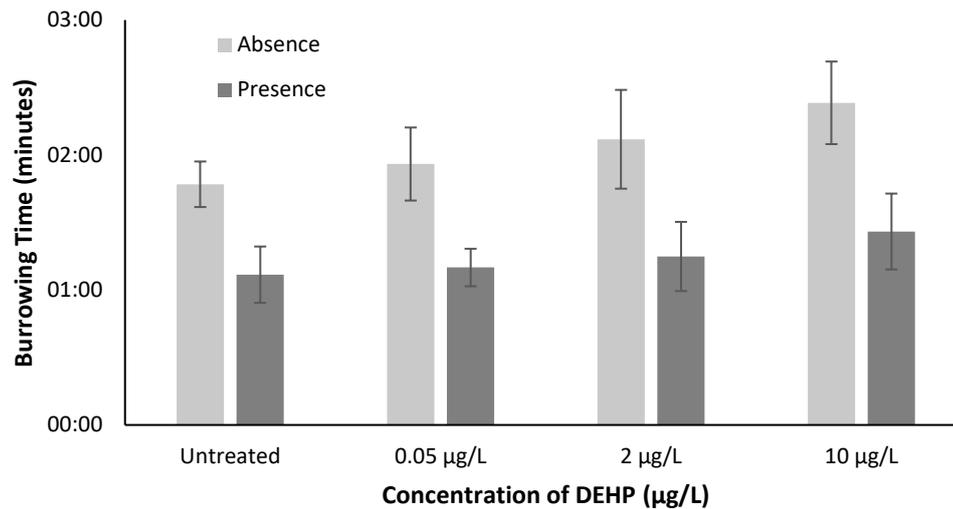
Figure 5.5: Mean burrowing time in the absence and presence of predators (± SE) of long-term DEHP exposed *H. diversicolor*, n = 10.

5.4.4. BURROWING TIME IN THE ABSENCE AND PRESENCE OF PREDATORS AFTER SHORT-TERM DEHP EXPOSURE

Figure 5.6 below shows the mean burrowing time of short-term, high concentration DEHP exposed *H. diversicolor* in the absence and presence of predators (crab conditioned water, i.e. odour). *H. diversicolor* were collected from different DEHP concentration tanks of 100,500 µg/L and untreated worms as controls. The worms were then transferred one by one to crystallising dishes containing coral sand and filtered seawater. Time was counted from when the worms were placed onto the coral sand until they rapidly disappeared as they burrowed into the sediment. The same procedure was repeated with crab conditioned water added by injecting it into the water column. The mean burrowing time of *H. diversicolor* increased for the 100 µg/L and 500 µg/L, (01:42 ±00:38 and 02:23 ±01:06 mins) groups compared to the untreated group (01:00 ±00:16 mins) in the absence of predator odour, and (00:59 ±00:21 and 01:15 ±00:41 mins) compared to the untreated group (00:44 ±00:17 mins) in the presence of predator odour.

Predator absence

When a Shapiro-Wilk's test (p=0.002) was used followed by data's histograms visual inspection, normal Q-Q plots and then box plots, it transpired that the data were not normal distribution. Therefore, after a log transformation was conducted, a Shapiro-Wilk's test (p=0.958) used, followed by data's histograms visual inspection, normal Q-Q plots and then box plots, it was then found that the data were approximately normally distributed. The Levene's test has verified the variances equality (variance homogeneity) in the samples (p>0.05).

Therefore, one-way ANOVA test was conducted, and it was evident that there was a statistically significant difference between groups (F (2,27) = 10.463, p = 0.000).

A Dunnett's test performed and a significant difference was found between untreated and both 100 µg/L p=0.026 and 500 µg/L p=0.00.

However, no significant difference was found between other groups (p>0.05).

Predator Presence

When a Shapiro-Wilk's test (p=0.00) was used followed by data's histograms visual inspection, normal Q-Q plots and then box plots, it transpired that the data were not normal distribution. Therefore, after a log transformation was conducted, a Shapiro-Wilk's test (p=0.191) used, followed by data's histograms visual inspection, normal Q-Q plots and then box plots, it was then found that the data were approximately normally distributed. The Levene's test has verified the variances equality (variance homogeneity) in the samples (p>0.05).

Therefore, one-way ANOVA test was conducted, and it was evident that there was no statistically significant difference between groups (F (2,27) = 3.119, p = 0.060).
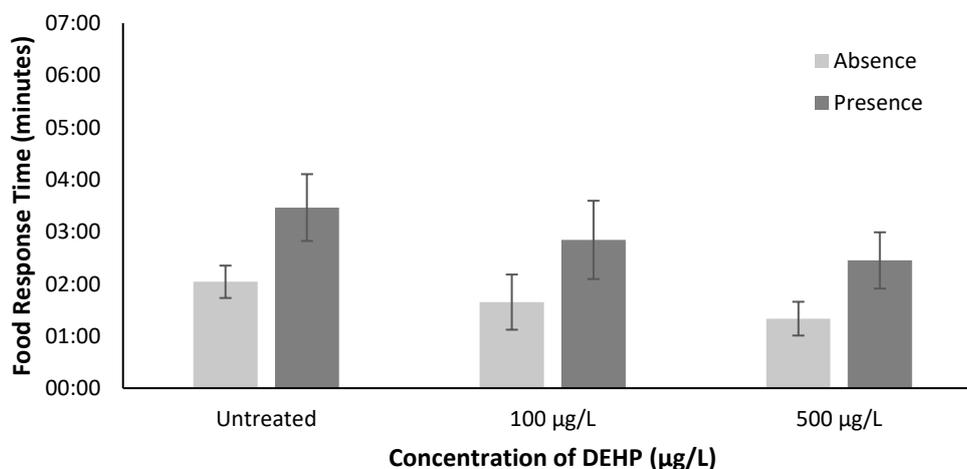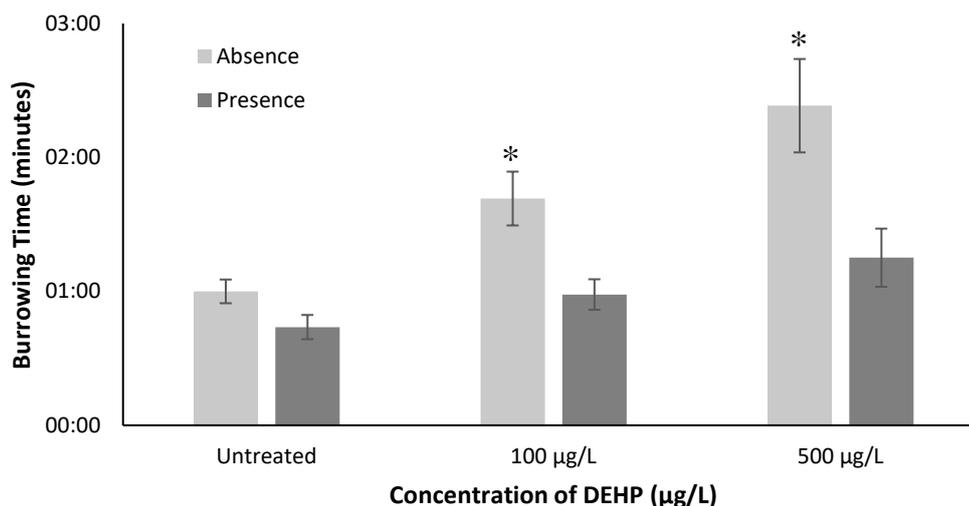
Figure 5.6: Mean burrowing time in the absence and presence of predators (± SE) of exposed *H. diversicolor* to DEHP at short-term, n = 10, Asterisk indicates significant difference from untreated group (p<0.05).

5.5. DISCUSSION

H. diversicolor represents a biological model species which was chosen because of their importance for estuarine ecosystem functioning. They constitute an important link in the food chains of these estuaries as prey for wading birds and flatfish. They have also an important role in the nutrients and contaminants biogeocycles through their bioturbation activities generated by their burrowing movements (Bonnard et al., 2009).

Numerous behavioural patterns have been found to be influenced or even controlled by environmental cues. Temperature and abundance of food are the most frequently studied environmental cues. Temperature has been found to affect the worms' life history characteristics (Fong, 1991; Olive et al., 1997), allocation of resources (Neuhoff, 1979; Qiu & Qian, 1998; Olive et al., 1998), feeding patterns (Yokoyama, 1988; Lambert et al., 1992), and ventilation (Kristensen, 1983b) in these polychaetes. Low temperature has been found to inhibit foraging in *H. diversicolor* (Lambert et al., 1992) and feeding in *Paraprionospio sp.* (Yokoyama, 1988), while temperature has been found to modulate ventilation and respiration in *H. diversicolor, N. succinea*, and *N. virens* (Kristensen, 1983a). As temperature is known to regulate the metabolic processes of ectotherms (Newell & Branch, 1980), its classification as an important environmental cue is not surprising.

Food availability and abundance affect all the metabolic processes of polychaetes including growth (Nielsen et al., 1995) and reproduction (Linton & Taghon, 2000a, b). Besides, food availability and abundance naturally regulate the feeding

behaviours of all intertidal organisms. For instance, *H. diversicolor* has been found to feed actively at high tide (Esselink & Zwarts, 1989) when food sources are available and more actively when they are abundant (Roman & Daiber, 1989).

As much as temperature and food availability affect the behaviours of *H. diversicolor,* stress such as exposure to DEHP likewise has been found to affect the behaviours of the treated worms in the case of both long-term at low DEHP concentration and short-term at high DEHP concentration exposures. DEHP was found to drastically change the behaviours of these worms by penetrating the body cells and chemically interacting with biologically important molecules (Browne et al., 2013). Because the mammalian cells in *H. diversicolor* which is exposed to DEHP, they produce reactive oxygen species and reduce the protein (Brown et al., 2001). In this case *H. diversicolor* uses antioxidants from its tissues in order to buffer the oxidative damage that is caused by hydrogen peroxide which has accumulated in its tissues. The capacity of the worm to counter oxidative stress may indicate that proteolysis is taking place, or reduction in the synthesis of its antioxidants, which then result in changes in behaviour (Browne et al., 2013). This has been confirmed by behavioural biomarkers, e.g. sub-organismal and supra-organismal avoid predators, which are sensitive tools that can assess the influence of the contaminating phthalates at concentrations under the lethal effect (Amiard-Triquet, 2009).

Successfully acquiring information is crucial for all the organisms of the animal kingdom to survive and be fit to adapt to their environments. Organisms gather information through visual, auditory, olfactory and tactile senses (Schmitt & Ache, 1979). The organism's olfactory sense is often intensified in the complex seawater environment, so as to make up for the poor visibility, which is sometimes due to increased turbidity (Schmidt et al., 2010). There are many studies that focus on feeding as they describe the chemical effect of elements in the seawater environment on the essential behaviours of the organisms (Mackie & Grant, 1974; Hara, 1975; Carr, 1982; Haye et al., 2009). However, most of these reports focus on feeding in crustaceans and fish.

Still there are many problems with the long-term experiments in which long-living species were used, such as fish. Such studies in most cases do not give enough time for the organisms to adapt (through multiple generations) (Barry et al., 2010). Short-living species, e.g. *H. diversicolor*, are perfect for the purpose of these experiments, because of their short lives, as well as the possibility of culturing multiple

generations in the laboratory, which also allows for adaptation in the long-term experiments.

The results of this chapter focused on the relationship between accumulation of different concentrations of DEHP and the worm's behaviour (feeding response time and burrowing time).

Feeding Response Time at Long and Short Term Exposure

The results of the feeding response time of *H. diversicolor* show the relationship between accumulation of different concentrations of DEHP and the worm's behaviour. DEHP accumulation increased in treated worms with long-term exposure at concentrations of 0.05, 2 and 10 µg/L (Figure 3.6), and short-term exposure at 100, and 500 µg/L (Figure 3.9), successively. The feeding response time of untreated worms was higher than that of treated worms in both absence and presence of predator *(Rhithropanopeus harrisi)* odour with long-term exposure at low DEHP concentration and short-term exposure at high DEHP concentration.

In the long-term exposure study (Figure 5.3), the feeding response time of *H. diversicolor* was decreased whenever DEHP concentration increased in the absence of predator odour, and in the presence of predator odour, for 0.05 µg/L, 2 µg/L and 10 µg/L, respectively, which indicates that the treated worms failed to detect the presence of the predator compared to the untreated group, leave them at a greater risk of predation.

Similarly, in the short-term exposure (high DEHP concentration) (Figure 5.4)*,* the feeding response time of *H. diversicolor* decreased whenever DEHP concentration increased in the absence of predator odour, and in the presence of predator odour, for 100 µg/L and 500 µg/L, respectively, which indicates that the treated worms failed to detect the presence of the predator compared to untreated group, leave them at a greater risk of predation.

Within an complex aquatic context where visual discovery of food is not normally feasible, a great many organisms are dependent on chemoreception, employing a multifarious combination of chemical cues to prompt suitable sensory organs in addition to finding food (Bronmark & Hansson, 2000). Additionally, chemoreception is essential to distinguish among options for food, for instance between dangerous or distasteful substances and nutritious ones (Wyatt, 2003).

Chemicals influencing feeding conduct are categorized as those that apply their influence from afar (repellents, arrestants and attractants) or those necessitating contact

(suppressants and incitants, deterrents and stimulants) (Bronmark & Hansson, 2012). Fish are capable of finding food from afar, both chemically and visually; in each of these circumstances gustation establishes the ultimate resolution of whether to consume food. Smaller organisms, for instance nereidid polychaetes, are capable of perceiving food visually at considerably smaller distances, and thus are greatly dependent on chemical tracing inside the water column (Bronmark & Hansson, 2012).

Invertebrates are able to deal with the availability of toxicants within their environment by stimulating several detoxication procedures, which result in a difference in their behaviour. The defence systems are nonetheless inadequate for the avoidance of behavioural weaknesses. In worms that were exposed, burrowing and feeding times were greatly weakened in contrast to controls within the research (Buffet et al., 2011). Feeding and burrowing activities were highlighted in this chapter as appropriate behavioural biomarkers to assist in the fitness appraisal of *H. diversicolor*.

Burrowing Time at Long and Short Term of Exposure

The results of burrowing time of *H. diversicolor* show the relationship between accumulation of different concentrations of DEHP and the *H. diversicolor* behaviour. DEHP accumulation increased in treated worms with long-term exposure at concentrations of 0.05, 2 and 10 µg/L (Figure 3.6), and short-term exposure at 100, and 500 µg/L (Figure 3.9), successively.

The burrowing time in untreated worms was lower than that of DEHP exposure treated worms in both absence and presence of predator *(Rhithropanopeus harrisi)* odour with low DEHP concentration at long-term exposure and high DEHP concentration at short-term exposure. In long-term exposure (Figure 5.5), the burrowing time of *H. diversicolor* was increased whenever DEHP concentration increased in the absence of predator odour, and in the presence of predator odour, for 0.05 µg/L, 2 µg/L and 10 µg/L, respectively, which indicates that the treated worms failed to detect the presence of the predator compared to the untreated group, which again leaves them at a greater risk of predation.

Similarly, at short-term, high DEHP exposure (Figure 5.6), the burrowing time of *H. diversicolor* increased whenever DEHP concentration increased in the absence of predator odour, and in the presence of predator odour, for 100 µg/L and 500 µg/L, respectively. This indicates that the treated worms failed to detect the presence of the predator compared to untreated group, which leaves them at a greater risk of predation.

Among the first studies to investigate in detail a polychaete's feeding behaviour, namely that of *Nereis virens*, were Copeland and Wieman (1924). *N. virens* emerges out of its tube when it senses the presence of crushed periwinkles or a similar type of animal food. Watson et al. (2005) utilized this feeding behaviour to expose *N. virens* to conspecifics full-body extracts as a prey for this polychaete, and flatfish muscle as a predator. They found that the foraging and feeding activities of *N. virens* were considerably reduced when it was exposed to conspecifics full-body extracts, the purpose of which was to use them as an alarm signal. Flatfish muscle extracts led to a decrease in the worms that were feeding, but did not reduce their numbers outside their burrows (Watson et al., 2005).

Mangum and Cox (1971) outlined the feeding conduct of the onuphid polychaete, *Diopatracuprea* (Bosc) as a reaction to extracts of 32 marine organisms as well as various chemical materials such as amino acids available in parts of bivalve flesh. The worms displayed reactions to parts of almost every organism, including itself. In addition, Haye et al. (2012) portrayed a decrease in feeding of the hermit crab *Pagurus bernhardus* in seawater with reduced pH levels. Furthermore, interruptions in feeding behaviour were observed among *H. diversicolor* from the greatly contaminated Loire estuary in contrast to the controls from a reference area (Bay of Bourgneuf). Other instances include reduced feeding among bluegill sunfish (*Lepomis machrochirus*) in contact with cadmium (Bryan et al., 1995) as well as largemouth bass in contact with pentachlorophenol (Mathers et al., 1985). A rise in the latency period of feeding was observed for *Gasterosteus aculeatus* (threespine stickleback) that had contact with increased intensities of 1-dichloroethylene (DDE), p,p'-2-2-bis(p-chlorophenyl)-1 as well as butylbenzylphthalate (BBP).

With poor burrowing behaviour, the worm becomes more exposed to predator attacks as well as to wave action, both of which reduce the organism's chances of survival (Kalman et al., 2009), as burrowing is among the organism's most important defence and survival strategies. *H. diversicolor* burrowing behaviour is a physiological activity composed of a series of events that emerge as a result of instinctive reflexes (Bonnard et al., 2009). When DEHP accumulates in the *H. diversicolor*, it becomes unable to detect and/or respond to the chemical cues that are brought by diffusion and water currents into the *H. diversicolor*'s burrow, similar to the effects of copper on *H. diversicolor* burrowing behaviour (Bonnard et al., 2009).

Comparisons of burrowing effectiveness and activity within *H. diversicolor* amid clean as well as chemically contaminated estuarine contexts as a possible

biomarker of ecosystem wellbeing were applied by Durou et al. (2008), demonstrating the appropriateness of employing burrowing operations to appraise the health of organisms when dealing with stressful conditions. Burrowing conduct was not greatly influenced when comparing *H. diversicolor* from the contaminated Loire estuary to controls from a reference area (Bay of Bourgneuf) (Fossi Tankoua et al., 2012). Furthermore, cross tests performed by Kalman et al. (2009) illustrated that *H. diversicolor* from the Loire had a higher level of burrowing if they were permitted to burrow within the Bay of Bourgneuf's cleaner sediment compared to their origin sediment. Disturbances in burrowing for *H. diversicolor* may only be disclosed if a broader contamination gradient is taken into account and these interruptions are attributable to avoidance of polluted sediments, as opposed to physical weaknesses (Kalman et al., 2009; Mouneyrac et al., 2010). Furthermore, the period of clam *Prototha castaminea* burrowing was raised with increasing concentrations of copper on sediment (Phelps et al., 1983).

In conclusion this chapter of the study shows that *H. diversicolor* fails to respond successfully to many chemical cues related to vital life processes under the stress, such as feeding and avoidance of predators. Moreover, *H. diversicolor* is less likely to detect predators in a DEHP environment which leave them at a greater risk of predation. If these laboratory results could be translated to the natural environment one can conclude that many behavioural patterns of this species will change when stressed by DEHP. Furthermore, *H. diversicolor* is inclined to spend more time searching and locating food, which leaves it at increased risk of predation.

Haye et al. (2009) proposed four mechanisms through which reception of chemical cues in the marine environment can be disrupted by means of environment stressors:a- Changes in the odour molecules charge distribution which disrupt the receptor-ligand interactions. b- changes in the odour receptors charge distribution which disrupt receptor-ligand interactions. c-Physical damage of the sensory organs. d-Reduced motivation accompanied by increased metabolic load. Chemical cues bind with receptors with specific structures (Hardege et al., 2011) usually made up of receptor proteins bound with the cell membrane either external or internal. When such receptor proteins bind with signal molecules, they trigger a chain of internal events that lead to a behavioural or developmental response (Wyatt, 2003). Any changes that take place in the receptor's structure or in the signal molecule may prevent the receptor from binding successfully with the ligand (Haye et al., 2009). This may block the behavioural response or alter it (Hardege et al., 2011).

Some studies (e.g. Bridle & Vines, 2006; Derry & Arnott, 2007) have shown that invertebrates can resist environmental stressors, thus raising hopes that marine species may likewise be capable of adapting to stressors. Moreover, the genetically based resistance to toxins of the marine oligochaete (Levinton et al., 2003) and aquatic organisms (Klerks & Weis, 1987) may be seen as stress tolerance evolution.

Finally, many of the compounds discharged into water interfere with the physiological processes of aquatic organism's endocrine systems, and as such are referred to as "endocrine disruption compounds" (EDCs). The American Environmental Protection Agency (USEPA) defines EDC as the "outer particle or substance that interferes with the transport, elimination, synthesis, binding, secretion, or action of naturally occurring endocrine hormones in the organisms which are capable to maintain many bio-physiological processes such as development, homeostasis, behaviours and reproduction". EDCs are known to interfere with the endocrine system physiology at various stages, and thereby affect many of the bio-physiological mechanisms of such organisms including general metabolism (Propper, 2005). EDCs include heavy metals, PCBs, insecticides, natural steroids, alkylphenols and industrial wastes that often contain different mixtures of chemicals (Krajniak, 2005).

Organisms are always in quest harmful effects on endocrine active substances that endanger populations of organisms can be shown in invertebrates (Höss et al., 2004). In this respect, a strong evidence of the impact of pollution, on the hormonal level, and sexual maturation of organisms as a result of pollutants that have harmful endocrine effects can be shown in *H. diversicolor* (Durou and Mouneyrac, 2007).
Among the EDCs, Xeno-estrogens are the ones that mimic estradiol, the natural hormone, in its effect in binding to specific receptors. Estrogens take part in human development and normal physiology, including lipid metabolism, maturation, sexual differentiation and oocyte development and growth. Among these Nonylphenol (NP) is a potent xenoestrogen (Aruwke et al., 1997) which is estimated to be three times as potent as the pesticide DDT in mimicking oestrogen (Soto et al., 1991). NP is among the EDCs, other than hormones, that have a considerable effect on mollusc populations as well as other annelids such as Polycheates, Brachiopoda, Bryozoa, Nemertea and Sipuncula. Furthermore, The most valid evidence of the growth rates of the organism's population as can be seen in the marine copepod *Tisbe battaglia* and the polycheate *Capitella* sp. (Matozzo et al., 2004). It can also affect the endocrine system in *P. dumerilii* via estrogenic signalling, thus feminizing the male by inhibiting its maturation (García-Alonso et al., 2011). Furthermore, concentrations of NP that are

environmentally relevant can disrupt the growth of the crustacean *Americamysis bahia* by delaying its molting processes (Hirano et al., 2009). Moreover, NP was found to induce the activity of GST in the polychaete *Nereissuccinea* (Ayoola et al., 2011). It shows similar effects like many other EDCs such as mercury, lead, cadmium, phthalates, dioxins, chlorotriazines, PAHs, PCDD, PCBs, PCDFs and various pesticides (Langston et al., 2005).

In this chapter, we can conclude that the behaviour of *H. diversicolor* shows a considerable effect when it is exposed to DEHP at long-term with low DEHP concentration and short-term with high DEHP concentration. The feeding response time in both the absence and presence of predator odour decreased whenever DEHP concentration increased. In contrast, the burrowing time in both the absence and presence of predator odour increased whenever DEHP concentration increased.

CHAPTER 6: SEASONALITY OF DEHP IN *HEDISTE DIVERSICOLOR*, IN SEAWATER AND IN SEDIMENT AT DIFFERENT SAMPLING SITES IN THE HUMBER ESTUARY

6.1. INTRODUCTION

6.1.1. THE HUMBER ESTUARY

The Humber catchment is the largest catchment in the British Isles, occupying an area of around 26,000 km^2, which is almost equal to one fifth of all England's land area (Figure 6.1). Its major tributaries include the rivers Aire, Ouse, Don, Trent, Derwent, Wharfe, Hull and Ancholme. The Humber estuary is characterized by low land and makes up the part of a system that extends for 62 km from Trent Falls, at the junction of Ouse and the Trent rivers, to Spurn Point and Donna Nook. The estuary receives waste water from the old industrial areas of south and west Yorkshire as well as from the midlands, and its tidal limits extend up to the Ouse and Trent rivers (creating tidal rivers that extend to Gainsborough dams on the River Trent and Naburn bridge on the River Ouse, thus doubling the Humber waters' length to 120 km and to 110 km respectively) (IECS, 2000). The catchment has a population of over 1.5 million that live in its floodplain, and the Humber catchment area has a population of 11 million people living in the metropolitan areas of the cities of Birmingham, Nottingham, Sheffield, Bradford, Leeds and Hull. Thus, for many decades, the waters of the Humber Estuary have been polluted by industrial and municipal waste water. With a density of 450 inhabitants per km^2, the Humber basin area with its intensive agricultural and industrial expanses is more densely populated than any other major European river basin (Kempe et al., 1991).

In order to predict the potential eutrophication symptoms that may develop in an estuary, its chemical and physical characteristics were investigated (McLusky & Elliott, 2004). Regarding the Humber estuary, its most important characteristic is its average flushing time, which is 40 days from Trent falls down to Spurn Point (with a winter average of about 20 days and a summer average of about 160 days) (Gameson, 1982). The Humber estuary is known for its well mixed macro-tidal features with an average tidal range of 5 m at Immingham. Due to its strong tidal currents, it is always highly turbid, shown by its distinctive brown colour, with a turbidity that often reaches 5 g/L^{-1} (compared to the background coastal seawater of 100-200 mg/l^{-1}) with a maximum turbidity zone of 14 g/L^{-1} (Marshall, 1995; LOIS/Anon, 1998). Turbidity attenuates

light, and enhances bacterial oxidation of organic matter in suspended particles (Maurice, 1994) and by doing so depresses oxygen levels of the water column, but plays a role in limiting and controlling the production of the primary water column. Estuaries' ecosystems are highly productive nurseries, and are important recruitment areas for numerous species, as they support dense invertebrate populations, that form a big part of the food chain as important prey species for higher animals (McLusky & Elliott, 2004; Dauvin, 2008). As they have fine grained sediments rich in organic matter, they absorb contaminants and by virtue of doing so they enjoy the identifying potential of bioindicator species and pollution biomarkers (Ducrotoy, 2010).

During the phase of production, phthalates enter the seawater environment in industrial wastewater, and as they are somewhat volatile, they are leached from plastic products while being used and after disposal (Brooke et al., 1991; Bauer & Herrmann, 1997). Phthalates show mild toxicity to seawater organisms, which usually decreases as the alkyl chain length increases (Giam et al., 1984; DeFoe et al., 1990). Their bioaccumulation in the seawater food chain depends on their biotransformation, which increases as trophic level increases (Staples et al., 1997). Applications and emissions of phthalates are presently of concern, however, because they are suspected of action akin to the action of estrogens (Jobling et al., 1995; Barton & Andersen, 1998; Tyler et al., 1998).
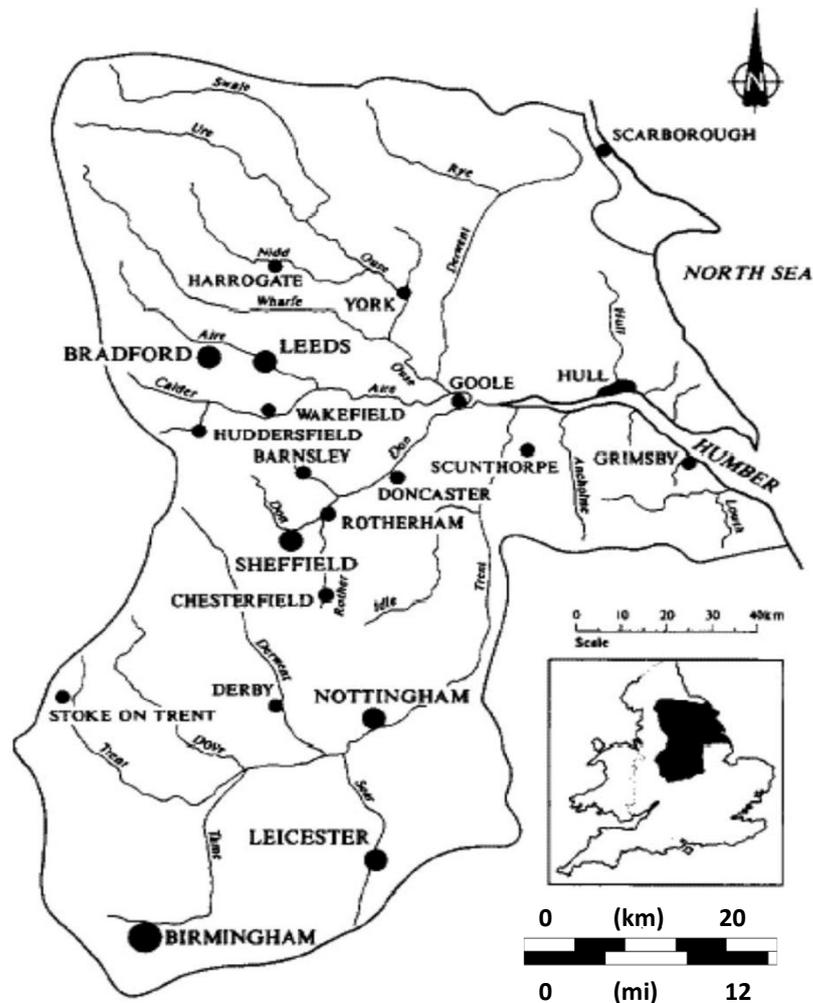
Figure 6.1: The Humber estuary catchment (adapted from Edwards et al., 1997).

Despite the large catchment, number of people and industries, the Humber estuary is clean relative to other British deep-water estuaries' (Shell U.K., 1987) but is nevertheless third in the UK in terms of pollution (Greenpeace, 1987). As early as the 18th century it has always acted as a key industrial point, and since then has been a key trade route (Cave et al., 2003). To date, traditional industries including iron and steel and textiles have declined in the Humber catchment, and there is a general shift toward chemicals, petrochemicals and power industry. The biggest number of power stations that generate electricity in the UK lies on the river Trent (Jarvie et al., 2000); these are mainly coal-fuelled, directly cooled stations. Some other directly cooled power stations lie along other tidal rivers that enter the estuary.

Forty-four power stations and other major plants are operated in the Humber catchment, all of which use the water of the Humber rivers for cooling and consequently release effluents into the Humber rivers (major effluent release points are shown in Figure 6.2) or their tributaries (Cave et al., 2003). Eighteen commercial phthalate esters

are produced in the environment, of which di(2-ethylhexyl) phthalate (DEHP) accounts for about 50%. As they are valued for their properties, and hence produced in large volumes, and used extensively, their concentrations in the environment are measurable and reflect a constant release of effluents into the environment (Peijnenburg & Struijs, 2006). DEHP are commonly reported as being the most frequently detected phthalates in the aquatic environment. Phthalates consumption can be estimated from per capita consumption rates, which may provide emission data for these regions based on the population densities (Peijnenburg & Struijs, 2006). Hence, this study aims to document potential DEHP levels that may be present in the Humber area relative to other UK aquatic environments.
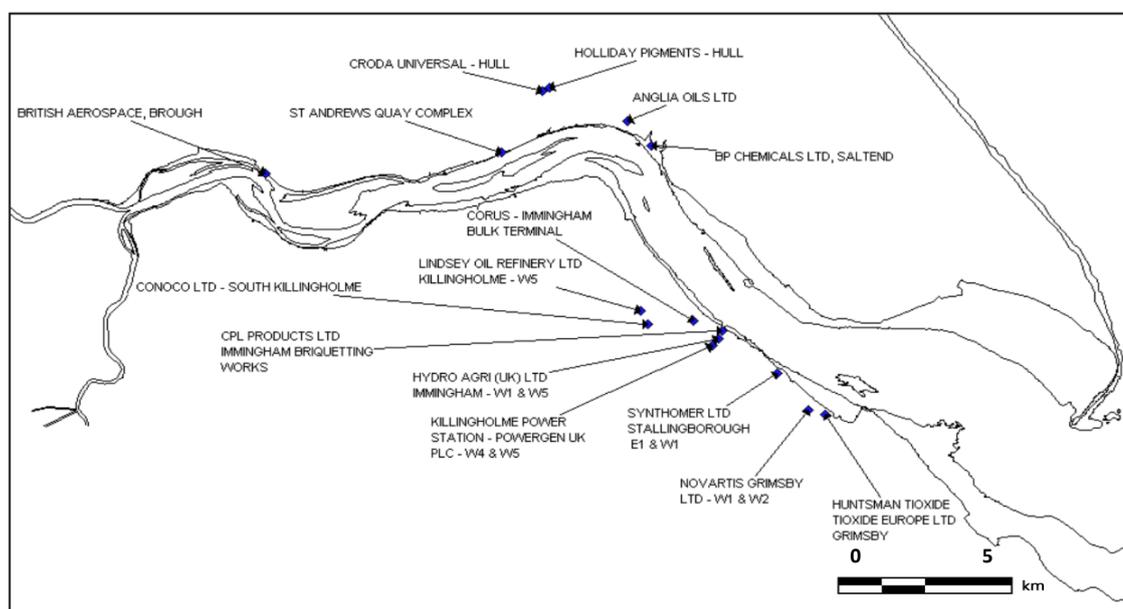
Figure 6.2: Some of the industrial discharge major points along the Humber (adapted from Elliott and Boyes 2002).

Several major industrial sources flourish in the Humber catchment, which include chemicals, petrochemicals and power plants. Compared to other UK areas, the Humber enjoys a more flourishing copper industry. A significant amount of literature has been written about the transport of trace metals and the interaction of metals with particulate matter suspended in the waters of the Humber (e.g. Millward & Glegg, 1997; Heijnis et al., 1987; Zwolsman et al., 1993; Croudace & Cundy, 1995; Lee & Cundy, 2001; Duquesne et al., 2006).

The coal-fuelled power stations that lie on the Ouse River can release annually into the atmosphere amounts of copper, lead and cadmium that may reach one tonne and another tonne of copper or so into the waters of river (Cave et al., 2003). These metals

have partial residence time of about 18 years, thus, allowing the metals that drain into the Humber catchment area to remain there for 18 years (Cave et al., 2003). Aquatic sediments have been found to play the ternary role of the sink, source and cycling centre for various types of contaminants (Moreira et al., 2006). The metals with greatest load in the Humber catchment have been found to be mostly of copper, lead, mercury, cadmium, nickel and arsenic origins. These metals are often in a perturbed condition, and such a condition is augmented by the natural interactions between the metals and dissolved particulate matter, which is also increased by anthropogenic activities (Cave et al., 2003).

6.2. AIMS AND OBJECTIVES

The aim of this study is to examine DEHP spatial and vertical distribution in worms, sediments and seawater, in the Humber estuary in eastern England (sampling points: Hessle, Paull, Immingham, Sunk Island, Grimsby and Spurn Point) (Figure 6.3). Previous studies conducted about estuarine DEHP concentrations in the UK (Table 6.1), were focused on water and the sediment only.
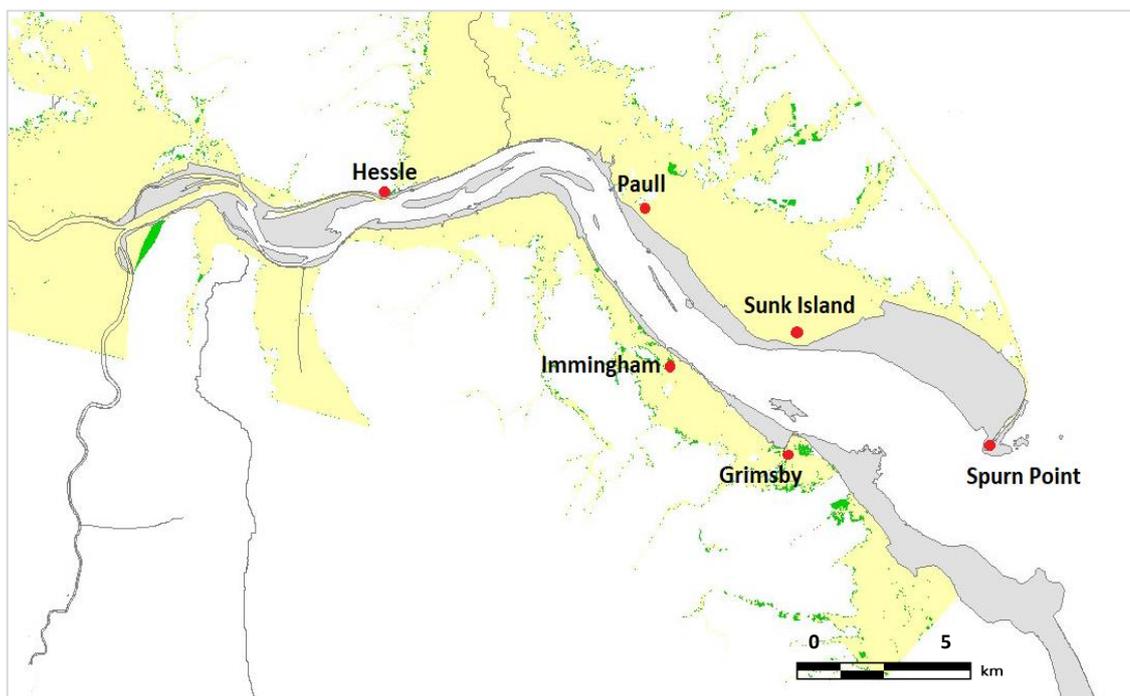
Figure 6.3: Samples Locations around the Humber Estuary, UK.

Table 6.1: DEHP concentrations in riverine and estuarine sediments and waters in UK.

Location	Water (µg/ml^1)	sediment (µg/ g^{-1})	Reference
River Aire	0.00036 – 0.021	7.89–115	Long et al. (1998)
Rivers Etherow and Irwell	0.0004 – 0.0019		Fatoki and Vernon (1990)
River Trent	0.00074 – 0.018	0.84–31.0	Long et al. (1998)
Mersey Estuary	0.00013 – 0.00069	1.20	Preston and Al-Omran(1989)

6.2.1. AIMS

➤ Firstly, to determine the level of DEHP in *H. diversicolor,* seawater and the sediment in one location in Humber estuary (Paull) at different times of the year.

➤ Secondly, to determine the levels of DEHP in *H. diversicolor,* seawater and the sediment at different locations around the Humber estuary.

6.2.2. THE OBJECTIVES OF THIS CHAPTER ARE TO ANSWER THE QUESTIONS:

➤ Is there difference in the concentration of DEHP in samples taken from the same location (Paull) at different times of the year?

➤ Is there difference in the concentration of DEHP in samples taken from different locations around the Humber estuary?

➤ Where is DEHP accumulation higher?

Based on the above objectives, it is hypothesised that:

➤ The concentration of DEHP will be different at different times of the year and locations.

➤ The DEHP levels will be higher in *H. diversicolor* than in sediment or seawater showing that worms accumulate DEHP.

6.3. MATERIALS AND METHODS

6.3.1. DETERMINE DEHP CONCENTRATIONS IN *H. DIVERSICOLOR*, SEAWATER AND SEDIMENT FROM ONE LOCATION IN HUMBER ESTUARY (PAULL) IN DIFFERENT TIMES OF THE YEAR

H. diversicolor and sediment samples, and depth of seawater were obtained at low tide. Sampling was carried out for a period of 10 months (different times of the year) (June - March) 2016-2017 from Paull village, Hull. The samples were self-collected and with the assistance of the Functional Ecology Research Group's members.

The samples were obtained from the same point and place, then brought to a laboratory in the University of Hull in containers.

6.3.2. DETERMINE DEHP CONCENTRATION IN *H. DIVERSICOLOR*, SEAWATER AND SEDIMENT FROM DIFFERENT LOCATIONS AROUND THE HUMBER ESTUARY

H. diversicolor worms and sediment samples, as well as seawater samples were obtained at low tide (October - November) 2016 from different locations around the Humber estuary. Sites were selected to include non-contaminated areas that were expected to show phthalate esters levels at ambient concentrations as well as areas expected to be exposed to possible sources of contamination. Samples were located on both the north and south banks of the estuary (Hessle, Paull, Immingham, Grimsby, Sunk Island and Spurn Point). The samples were self-collected and with the assistance of the Functional Ecology Research Group's members. The samples were obtained from the same point and place, then brought to a laboratory in the University of Hull in containers.

6.3.3. PREPARATION OF SAMPLES FOR GS/MS ANALYSIS

➢ *H. diversicolor Samples*

The *H. diversicolor* worms were washed using filtered seawater, which was run through a charcoal filter of 18-20 ppt salinity. Thereafter, they were divided into three categories based on worm size: large size (4-6 cm), medium size (2.5-4 cm) and small size (2-2.5 cm). Large and medium sizes were used to determine the concentration of DEHP at Paull. They were then left in Pyrex crystal dishes containing filter seawater for 6-8 hours so as to free their guts from any sediment. Worms were then kept at -20 ºC and dried by means of a freeze drying device for 48 hours and then homogenized with a carbide pestle and mortar. Then the worm tissue was analysed to extract DEHP by the method of phthalates liquid extraction from lipid rich substrates **(See chapter 2).**

➢ *Sediment Samples*

Sediment samples were taken from the same point of worms' collection. Then the sediment was analysed to extract DEHP by phthalates extraction from the sediment using the accelerated solvent extraction method **(See chapter 2).**

➢ *Seawater Samples*

Deep seawater samples were taken from the same point of worms' collection. Then the seawater was analysed to extract DEHP by the method of phthalates liquid extraction from water samples **(See chapter 2).**

- **DATA ANALYSIS**

SPSS was used in the statistical analyses. First, descriptive statistics were performed. Then, Shapiro-Wilk's test was used to test the normality of data. Homogeneity of variance was checked by Levene's test, For data that fulfilled both conditions, multiple comparisons were assessed by one-way analysis of variance (ANOVA) with Tukey's test as a post-hoc test. However, Welch ANOVA was carried out with a Games-Howell post-hoc when the homogeneity of variances was violated. If data were not normally distributed, a log transformation was conducted to satisfy parametric conditions. However, when the log transformation failed normality test, a non-parametric Kruskal–Wallis test was performed with multiple Mann Whitney comparison tests as post hoc tests. A Bonferoni correction used with Mann Whitney comparison tests to minimise the error rate.

6.4. RESULTS
6.4.1. DIFFERENT TIMES OF THE YEAR
➢ *H. diversicolor*

Figure 6.4 shows the mean level of DEHP in *H. diversicolor* from Paull (Humber Estuary) in June, July, August, September, October, November, December, January, February, and March was 0.064335846 ±0.042, 0.046588313 ±0.045, 0.056487628 ±0.015, 0.107975256 ±0.038, 0.046664911 ±0.013, 0.08899496 ±0.030, 0.060923289 ±0.033, 0.097662047 ±0.077, 0.055814357 ±0.022 and 0.12432845 ±0.045 µg/g respectively. The level of DEHP in the worm tissue was irregular.

When a Shapiro-Wilk's test (p=0.00) was used followed by data's histograms visual inspection, normal Q-Q plots and then box plots, it transpired that the data were not normal distribution. Therefore, after a log transformation was conducted, a Shapiro-Wilk's test (p=0.607) used, followed by data's histograms visual inspection, normal Q-Q plots and then box plots, it was then found that the data were approximately normally distributed. The Levene's test did not verify the equality of variances (homogeneity of variance) in the samples (p<0.05).

Therefore, a Welch ANOVA test was conducted, and it was evident that there was a statistically significant difference between groups (F (9,36.454) = 7.489, p = 0.00).

A Games-Howell test found a significant difference between July and both September p=0.041, and March p=0.013; between August and March p=0.002; between September and October p=0.007; between October and both November p=0.004 and March p=0.000, and between December and March p=0.036; and between February and March p=0.008. However, no significant difference was found with other groups.
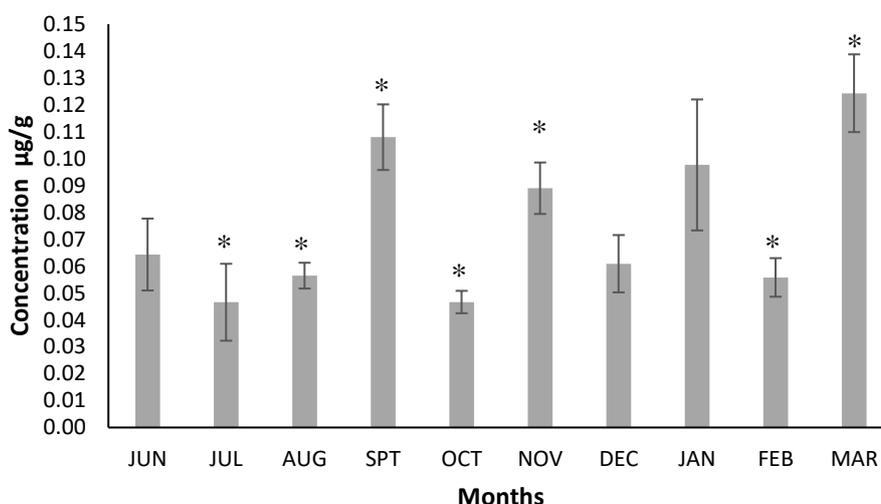
Figure 6.4: Mean levels of DEHP in *H. diversicolor* tissue (± SE) at different times of the year. n=10 samples of *H. diversicolor*, Asterisk indicates significant difference between groups p<0.05.

> **Sediment**

Figure 6.5 shows the mean level of DEHP in sediment from Paull (Humber Estuary) in June, July, August, September, October, November, December, January, February, and March was 0.001672784 ±0.00058, 0.00158053 ±0.00014, 0.001247501 ±0.00006, 0.001773954 ±0.00008, 0.001107291 ±0.00007, 0.001639919 ±0.0004, 0.002053455 ±0.00008, 0.002071971 ±0.00013, 0.001571132 ±0.00031, 0.001797694 ±0.00036 µg/g respectively. The level of DEHP in the sediment was irregular.

When a Shapiro-Wilk's test (p=0.037) was used followed by data's histograms visual inspection, normal Q-Q plots and then box plots, it transpired that the data were not normal distribution. Therefore, after a log transformation was conducted, a Shapiro-Wilk's test (p=0.020) used, followed by data's histograms visual inspection, normal Q-Q plots and then box plots, it was then found that the data were still not normally distributed.

Therefore, a Kruskal Wallis test was conducted, and it was evident that there was a significant difference between the groups, at $X^2(9) = 17.705$, p=0.039. It was found that the highest mean rank was for December followed by January, March, June, September, November, July, February, August and October, respectively.

Multiple Mann-Whitney U tests were conducted to measure differences between any two groups. Significant differences were found between October and both December and January p=0.002, and between October and March p=0.023, and between August and both December and January p=0.008. However, no significant difference was found between other groups. When a Bonferoni correction was applied, no significant difference was found between any groups (p>0.001).
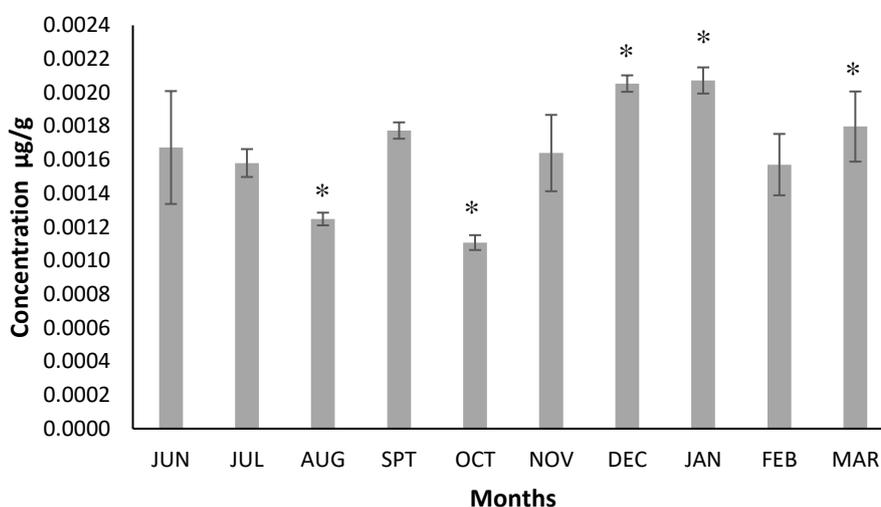
Figure 6.5: Mean level of DEHP in sediment (± SE) at different times of the year. n=3 samples of sediment (40g), Asterisk indicates significant difference between groups p<0.05.

> **Seawater**

Figure 6.6 shows the mean level of DEHP in seawater from Paull (Humber Estuary) in June, July, August, September, October, November, December, January, February, and March was 0.0000110654 ±0.0000066, 0.0000066448 ±0.0000046, 0.0000172787 ±0.000009, 0.0000104370 ±0.0000016, 0.0000063288 ±0.0000018, 0.0000162980 ±0.0000046, 0.0000112379 ±0.0000068, 0.0000080626 ±0.000005, 0.0000187694 ±0.0000077, 0.0000103360 ±0.0000071 µg/ml, respectively. The level of DEHP in the seawater was irregular.

When a Shapiro-Wilk's test (p=0.009) was used followed by data's histograms visual inspection, normal Q-Q plots and then box plots, it transpired that the data were not normal distribution. Therefore, after a log transformation was conducted, a Shapiro-

Wilk's test (p=0.438) used, followed by data's histograms visual inspection, normal Q-Q plots and then box plots, it was then found that the data were approximately normally distributed. The Levene's test has verified the variances equality (variance homogeneity) in the samples (p>0.05).

Therefore, one-way ANOVA test was conducted, and it was evident that there was no statistically significant difference between groups (F (9,20) = 2.216, p = 0.066).
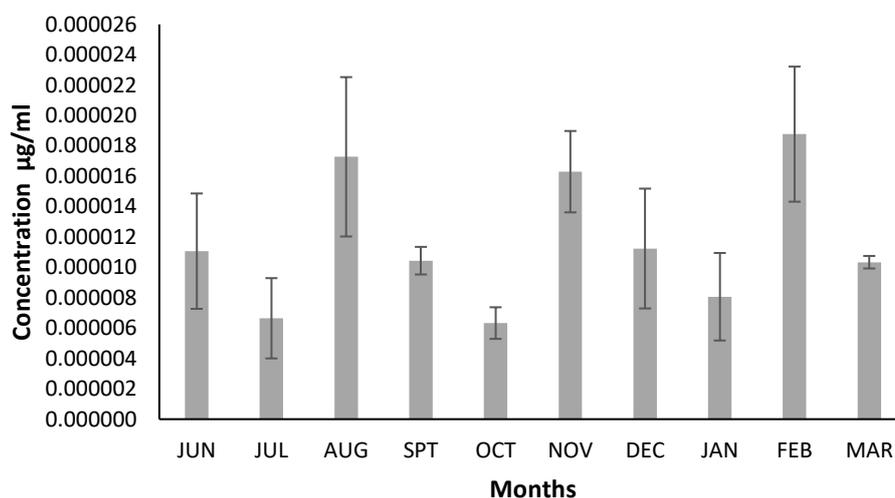
Figure 6.6: Mean level of DEHP in seawater (± SE) at different times of the year. n=3 samples of seawater (500ml).

6.4.2. DIFFERENT LOCATIONS AROUND THE HUMBER ESTUARY

➢ *H. diversicolor*

Figure 6.7 shows the level of DEHP in *H. diversicolor* from different locations around the Humber Estuary in Hessle, Sunk Island, Immingham, Spurn Point, Paull and Grimsby was 0.018930946 ±0.00697, 0.024890279 ±0.01028, 0.029054076 ±0.00864, 0.036045359 ±0.01521, 0.046664911 ±0.01317 and 0.058570866 ±0.04382 µg/g, respectively. The level of DEHP in *H. diversicolor* was increased from Hessle to Grimsby.

When a Shapiro-Wilk's test (p=0.00) was used followed by data's histograms visual inspection, normal Q-Q plots and then box plots, it transpired that the data were not normal distribution. Therefore, after a log transformation was conducted, a Shapiro-Wilk's test (p=0.358) used, followed by data's histograms visual inspection, normal Q-Q plots and then box plots, it was then found that the data were approximately normally distributed. The Levene's test did not verify the equality of variances (homogeneity of variance) in the samples (p<0.05).

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Therefore, a Welch ANOVA test was conducted, and it was evident that there was a statistically significant difference between groups ($F_{(5, 24.993)}$ = 9.354, p = 0.00).

A Games-Howell test found a significant difference between Hessle and Spurn Point p=0.018, Paull p=0.000 and Grimsby p=0.020; between Sunk Island and Paull p=0.005, and between Immingham and Paull p=0.023. However, no significant difference was found between other groups.
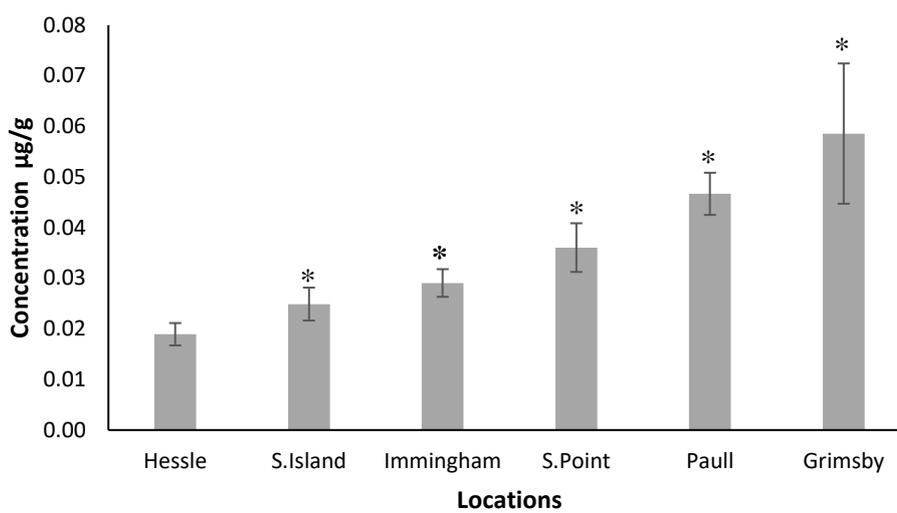
Figure 6.7: Mean level of DEHP in _H. diversicolor_ tissue (± SE) at different locations around the Humber Estuary. n=10 samples of _H. diversicolor_ per sampling point, Asterisk indicates significant difference between groups p<0.05.

> **Sediment**

Figure 6.8 shows the level of DEHP in sediment samples from different locations around the Humber Estuary in Hessle, Sunk Island, Immingham, Spurn Point, Paull and Grimsby was 0.001611749 ±0.000141, 0.001393763 ±0.000430, 0.001237683 ±0.000144, 0.001180283 ±0.000497, 0.001107291 ±0.000076 and 0.00020017 ±0.000046 µg/g, respectively. The level of DEHP in sediment was lowest in those from Hessle to Grimsby following the Humber estuary in the direction of the open North Sea.

When a Shapiro-Wilk's test (p=0.037) was used followed by data's histograms visual inspection, normal Q-Q plots and then box plots, it transpired that the data were not normal distribution. Therefore, after a log transformation was conducted, a Shapiro-Wilk's test (p=0.00) used, followed by data's histograms visual inspection, normal Q-Q plots and then box plots, it was then found that the data were still not normally distributed.

Therefore, a Kruskal Wallis test was conducted, and it was evident that there was no significant difference between the groups, at $X^2(5) = 10.965$, p=0.052. It was found that the highest mean rank was for Hessle, followed by Sunk Island, Spurn Point, Immingham, Paull and Grimsby, respectively.
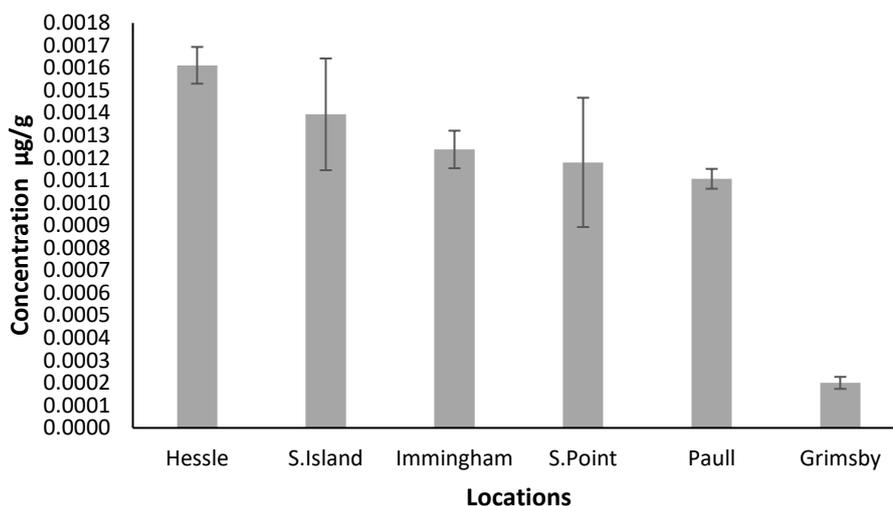
Figure 6.8: Mean level of DEHP in sediment (± SE) at different locations around the Humber Estuary. n=3 samples of sediment (40g) per sampling point.

➢ **Seawater**

Figure 6.9 shows the level of DEHP in seawater from different locations around the Humber Estuary in Hessle, Sunk Island, Immingham, Spurn Point, Paull and Grimsby was 0.000003938 ±0.00000111, 0.000002427 ±0.000000981, 0.000005835 ±0.00000259, 0.000002146 ±0.000000568, 0.000006329 ±0.00000180, 0.000001919 ±0.000000772 µg/ml, respectively. The level of DEHP in the seawater was irregular, with the two near industrial installation sites at Immingham and Paull showing the highest levels of DEHP.

When a Shapiro-Wilk's test (p=0.043) was used followed by data's histograms visual inspection, normal Q-Q plots and then box plots, it transpired that the data were not normal distribution. Therefore, after a log transformation was conducted, a Shapiro-Wilk's test (p=0.896) used, followed by data's histograms visual inspection, normal Q-Q plots and then box plots, it was then found that the data were approximately normally distributed. The Levene's test has verified the variances equality (variance homogeneity) in the samples (p>0.05).

Therefore, one-way ANOVA test was conducted, and it was evident that there was a statistically significant difference between groups ($F_{(5,12)} = 5.849$, $p = 0.006$).

A Tukey post hoc test found a significant difference between Immingham and Grimsby p=0.032; between Spurn Point and Paull p=0.038, and between Paull and Grimsby p=0.016. However, no significant difference was found between other groups (p>0.05).
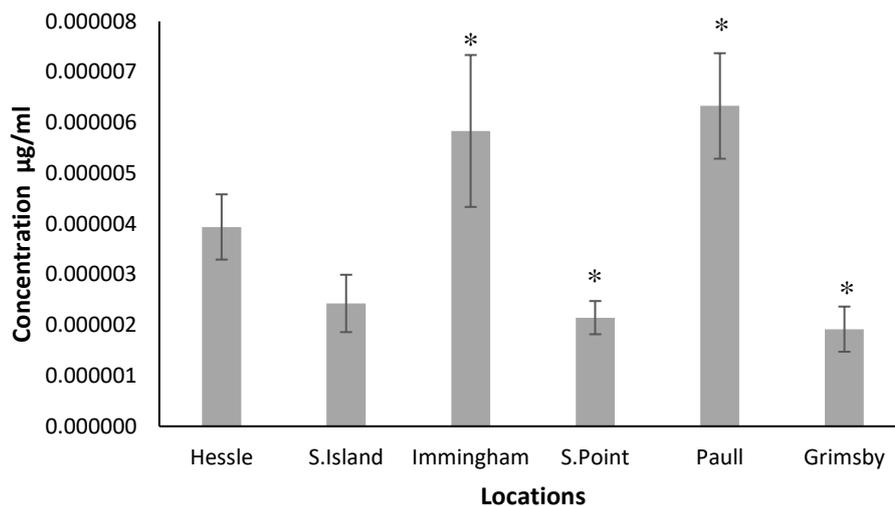
Figure 6.9: Mean level of DEHP in seawater (± SE) at different locations around the Humber Estuary. n=3 samples of seawater (500ml) per sampling point, Asterisk indicates significant difference between groups p<0.05.

6.5. DISCUSSION

For many centuries, cities and industries were developed on estuarine and other coastal areas in tandem with the development of natural resources. Such dual development has often damaged the aquatic habitats of these areas (McLusky & Elliott, 2004). Not only that, but the damage in one part of the aquatic ecosystem has often negatively affected the other parts of that ecosystem. The impacts of man-made structures in these areas not only disturb the physical components of the aquatic habitats, but are often followed by series of events that could compromise their future in an irreversible way, which is often the case in both industrialized and developing countries. In this respect, the deposition of toxic materials in these aquatic areas is increasing alarmingly due to the fast increases in populations and the resulting thriving economic activities (Chiffoleau et al., 1994). Such conditions are accompanied by land development as well as new industrial activities adjoining the coastal lines. Moreover, modern urbanization activities are now putting growing demands on the water resources of these coastal areas. Furthermore, environmental deterioration resulting from water

pollution is imposing ever increasing problems to future development (Suthar et al.,2009). The environmental consequences of water pollution include natural resources destruction which is leading in turn to depletion of biodiversity and demise of many aquatic species (Islam & Tanaka, 2004). The culprit in these cases is pollution from industrial and agricultural sources, which is the major cause behind high concentrations of harmful and toxic materials. These include wastes from oil refineries, chemical industry and fertilizers used in agricultural areas (Islam & Tanaka, 2004). Effluents coming from industrial sources are drained directly into the estuaries and rivers, as well as sewage systems. Some of these effluents are characterized by high biological oxygen demand (BOD), which is often compounded by effluents from sewage treatment works (STWs), thus, augmenting the threats to aquatic life by depleting the dissolved oxygen in tidal rivers and estuarine areas (Cave et al., 2003).

Most of the environmental studies in the Humber estuary have been conducted about the concentration of heavy metals such as copper in its waters because of their effluents (Cave et al., 2003), but no studies have been conducted about the concentrations of DEHP in the Humber estuary. In the past, high levels of lead and zinc from mining runoff have been found in the Humber as early as 1250 AD, but recently high levels of other metals have also been recorded due to recent activities of industrialization (Cave et al., 2003). However, as mentioned earlier (Cave et al., 2003), the main industrial sources in the Humber catchment include chemicals, petrochemicals and power plants. The coal-fuelled power stations that lie on the River Ouse, can release annually into the atmosphere amounts of copper, lead and cadmium that may amount to one tonne and another tonne of copper or so into the waters of rivers (Cave et al., 2003). The metals with greatest load in the Humber catchment have been found to be mostly of copper, lead, mercury, cadmium, nickel and arsenic origins (Cave et al., 2003). The study of Lee and Cundy (2001) found a range of metals in the Humber estuary with elevated concentrations, which included Cu, Fe, Al, Pb, Zn and Mn in the intertidal surface and near-surface sediments, while the study of García-Alonso (2001) found another range of metals with lower concentrations that included Al, Zn, Cu, Pb, Cr, Ni, Co, As and Cd in their sediment samples. These elements were similar to the ones found in other clean British estuaries, but were of lower concentration relative to historically polluted ones (Bryan et al., 1985). A third study in the Humber estuary by Kierkegaard et al., (2011) found cyclic volatile methylsiloxane bioaccumulation in ragworm and flounder.

DEHP at Different time of the year

H. *diversicolor*, sediment and seawater samples were collected at different times of the year and in different locations around the Humber estuary from the riverine to marine sites to be representative of the whole of the Humber estuary. Figure 6.4, 6.5 and 6.6, show the levels of DEHP found in *H. diversicolor*, sediment and seawater at different times of the year in Paull (Humber Estuary in UK). Paull is the base of a range of heavy industry such as the BP chemical works. Sampling was carried out in a period that spanned 10 months (different times of the year), and thereby allowed assessing whether the levels of DEHP depend on times of the year. The samples taken during different months in the summer, autumn, winter and spring were taken at temperatures of 19.6, 15.4, 6.2 and 8.2 °C respectively. These values represent the typical annual average temperatures in the Humber estuary (Uncles & Stephens, 2000). The sampling time of the season was carried out such as to make it relevant for the determination of DEHP concentrations in the Humber estuary, which was nearly consistent with Peijnenburg and Struijs (2004) in the Netherlands.

Some months of the year showed higher contaminations of DEHP relative to other months. DEHP accumulated more in worms, ranging between 0.046588313 and 0.12432845 µg/g, followed by sediment, ranging between 0.001107291 and 0.002071971 µg/g, and seawater, ranging between 0.0000063288 and 0.0000187694 µg/ml, respectively. In general, the level of DEHP was irregular in the worms, sediment and seawater, because sampling during different months seemed to be not a relevant determinant parameter. This is consistent with Peijnenburg and Struijs's (2004) study, in which data were reported in the Netherlands showing the levels of DEHP in freshwater, seawater, sediment, and fish spanning a period of 9 months (all seasons except winter).

DEHP at Different Locations around the Humber Estuary

Highly polluted locations are those locations that are considerably more polluted than other locations with all or many of the contaminants under investigation present. Contaminated locations usually require a wide characterization and a more general classification, such that the designation of such locations as highly contaminated will include most or many of the investigated compounds (Vethaak et al., 2000).

Figures 6.7, 6.8 and 6.9 show the levels of DEHP found in *H. diversicolor*, in sediment and in seawater in the Humber estuary in UK. Sampling spanned a period of 2 months (October and November) at different locations (Hessle, Paull, Immingham,

Sunk Island, Grimsby and Spurn Point) and allowed assessing whether DEHP levels depend on location or not. Some locations around the Humber Estuary showed higher DEHP contamination than others. DEHP accumulated more in worms, followed by sediment and seawater. DEHP contamination shows a successive increase from Grimsby to Hessle in worms and from Hessle to Grimsby in sediment, whereas it was irregular in seawater samples. This is because Hessle is considered relatively clean from industrial pollution, whereas Grimsby is the most polluted, and according to the environmental agencies, it is often exposed to industrial pollution from chemicals, metals, minerals and power fuels. Grimsby has major power stations lying close to the estuary along with one of Britain's busiest ports, handling over 30 million tonnes or so of energy related products (Humber Nature Partnership, 2016).

The DEHP accumulates more in worms in highly contaminated locations whereas it accumulates more in sediment in the less contaminated locations. This suggests that the detoxification mechanisms in the worms can only cope with a certain amount of DEHP and failed to cope with higher concentrations. However, DEHP accumulates more in sediment than seawater. It seems that sediment makes a good sink for DEHP in aquatic environments. DEHP is transported from seawater to sediment mainly as a result of the settlement of suspended material that carries the adsorbed chemicals with it (Staples et. al, 1997). The concentration of DEHP in the sediment depends on its rates of supply by means of sedimentation. On the other hand, the irregular concentration of DEHP in seawater might be due to the degradation conditions, for example, low temperature and low oxygen concentration (Andrady, 2011).

Published reports about DEHP concentrations in marine animal tissues are very few so that the results obtained in this study are difficult to compare to data reported in different locations of the world. The concentrations of DEHP in *H. diversicolor* worms at different locations around the Humber estuary range between 0.018930946 and 0.058570866 µg/g. However, DEHP concentrations in this study were higher than the concentrations in other marine animals in some studies. The study of Brown and Thompson (1982a) who found the range of the concentration of DEHP in the tissues of *Daphnia magna* between 0.00000053 µg/g and 0.0000267 µg/g, while these authors (1982b) found the concentration of DEHP in *Mytilus edulis* in the range of 0.0000097-0.0001106 µg/g. Furthermore Mackintosh et al. (2004) and Lin et al. (2003) found DEHP concentrations in marine organisms to amount 0.00407 µg/g.

However, the DEHP concentrations in this study were lower than the

concentrations in other studies, e.g. Vethaak et al. (2000) in the Netherlands found DEHP concentrations up to a maximum of 1.5 µg/g in fish and up to a maximum of 0.4 µg/g in mussels. Moreover, Chin Huang et al. (2008) found DEHP concentrations in fish samples in Taiwanese rivers to amount to 253.9 µg/g $^{-1}$dw in *Liza subviridis* and 129.5 µg/g $^{-1}$dw in *Oreochromis niloticus*.

In sediment, the range of DEHP concentrations in different locations around the Humber Estuary was between 0.001611749 and 0.00020017 µg/g. the DEHP concentrations of this study were lower than the concentrations found in some studies. For example, in the Netherlands, DEHP concentrations in the sediments were up to a maximum of 0.0076 µg/g (dry weight), while in Sweden (near big cities) they ranged between 0.05- 0.8 µg/g, and 0.01- 0.04 µg /g in the lakes (Remberger, 1999). Chin Huang et al. (2008) investigated the average and the range of DEHP concentrations in the sediments of 17 Taiwanese rivers and found that they were 4.1 (<0.05-46.5) µg/g^{-1}dw in the low-flow season, and 1.2 (<0.05-13.1) µg/g^{-1}dw) in the high-flow season (Magdouli et al., 2013). Finally, in Mersey Estuary (UK), Nueces Estuary (Texas), Lake Yssel (Rhine Estuary) and Mississippi Estuary were in the ranges of 1.20, 0.040-16.0, 12.0-25.0 and 0.069 µg/g^{-1}, respectively (Preston and Al-Omran, 1989; Ritsema et al., 1989; Ray et al., 1983; Giam et al., 1978).

In seawater, the range of DEHP concentrations in different locations around the Humber Estuary were between 0.000001919 and 0.000006329 µg/ml. However, DEHP concentrations of this study were consistent with the DEHP concentrations found in the North Atlantic (Open Ocean) in the range of 0.0000049 µg/ml $^{-1}$ (Giam et al. 1978). However, DEHP concentrations of this study were lower than the concentrations found in some studies; for example, in the Mersey Estuary (UK), Nueces Estuary (Texas), Lake Yssel (Rhine Estuary) and Mississippi Estuary, they were in the ranges of 0.00013-0.00069, 0.00021-0.00077, <0.00010 - 0.0003and 0.000070 µg/ml $^{-1}$ respectively (Preston & Al-Omran,1989; Ritsema et al., 1989; Ray et al., 1983; Giam et al., 1978). Furthermore, in Sweden and Denmark the DEHP concentrations in the surface water were in the range of LOD-0.0004µg/ml (Furtmann, 1999; Vikelsoe, 1999; Remberger, 1999), while the range in the Netherlands was between 0.002 µg/ml and 0.005 µg /ml (Vethaak et al.2000). In 2000, the European Community listed DEHP among the 33 hazardous substances that should be controlled and combated in surface water. In 2007 a European Directive recommended that DEHP concentrations in surface water should be controlled at 0.0013µg/ml^{-1}. Khan and Jung (2008) found that DEHP

concentrations in surface water were between 0.00033µg/ml[-1] and 0.09787µg/ml[-1]. Yuwatini et al. (2006) reported the range of DEHP concentration in river water between 0.008µg/ml[-1]- 0.025 µg/ml[-1], while in Germany, Greece and Croatia the concentrations were 0.00005 - 0.00006 µg/ml[-1], 0.00093µg/ml[-1] and 0.000247µg/ml[-1], respectively (Magdouli et al., 2013).

Finally, As human health is potentially linked to the environment (Jha, 2004; Moore et al., 2013), the phylogenetic groups that provide humans with ecosystem services by creating benefits for mankind, directly such as by providing food, or indirectly such as by boosting soil fertility, are important for the conservation of human health (Costanza et al., 2014; Simpson, 2014). In this respect one should appreciate the critical role of invertebrates that constitute 95% of the existing species in the ecosystems' functioning that serve the most important sources of food for humans. Furthermore, marine invertebrates have been found to provide a wide array of ecosystem services, and any threats against them, such as pollution and climatic change should be earnestly heeded (Basset et al., 2013; Rife, 2014). Hence, several seawater and freshwater species are used to evaluate the biological responses to relevant pollutants in the laboratory. This includes determination of the regulatory observance of existing and new chemicals in chronic and acute ecotoxicology studies (ASTM, 1992). Ecotoxicological tests need to be performed for a broad range of invertebrates that represent different phyla and at various trophic levels, as they have become imperative from both regulatory and scientific perspectives (Galloway and Depledge, 2001; Jha, 2004, 2008).

One can conclude from the above results that DEHP, the most abundant phthalates, exist in low concentrations in most environments. After taking fluctuations over months of the year and the differences between locations into account, DEHP concentrations in *H. diversicolor* are either higher or lower than the levels reported in the above studies, and below the reported levels in sediment, but it consistent or low in seawater.

An estuary's capacity to retain contaminants or decrease toxicant levels, is among the vital considerations for water quality managers (Meade et al., 1990; Brunk et al., 1997; Turner et al., 1999). In this respect, DEHP retention is controlled by two major effects: the first is the degree of adsorption of contaminants by suspended particles, which in turn depends on the concentration of such suspended particles and their distribution coefficient (K_D); and the second is the rate of decay of contaminants in

the aqueous phase, which in turn depends on the temperature and several water quality parameters, including dissolved oxygen and microbial density. The retention of such contaminants, in practice, depends on the water quality and its transit time from the region of discharge to the point of high turbidity, such as the turbidity maximum zone (TMZ), trapping of suspended particles in this zone, and the degree of bed sediment re-suspension (Turner & Rawling, 2000).

CHAPTER 7: GENERAL DISCUSSION

European Union common guidelines that classify environmental hazards due to chemicals, classify the chemicals in which the bioconcentration factor (BCF) is higher than 100 L/kg wet weight (w.w.), as having bioaccumulative potential. Bioaccumulation is defined as the process where compounds accumulate in aquatic organisms after being absorbed from the environment. Such absorption can take place directly from water, or by means of biomagnification from food. The BCF measures the compound's tendency to accumulate in an organism, and is expressed as the ratio of the compound's concentration in the organism's body relative to its concentration in water (Vethaak et al., 2000).

The test chemical used for this study was DEHP (di-2-ethylhexyl phthalate), which is a plasticizer generally used to increase the flexibility of plastics such as polyvinyl chloride, celluloses, polyvinyl acetate, and polyurethane (Graham, 1973; Giam et al., 1984). DEHP has been recognized by various regulatory agencies as a major pollutant due to its low solubility in water, frequent existence in the environment and high toxicity to the kidney, liver, thyroid, and immune system (Sarkar et al., 2013).

Furthermore, *H. diversicolor* (ragworm) was used as a bio-indicator species to monitor the level and effects of this pollution in a controlled laboratory environment and in field studies. Bioindicator species are applied to quantify contaminant concentrations in species that can store chemical compounds in their tissues (Durou et al., 2007a). However, there is no published information available about DEHP particles fate in the environment and their ecotoxicity in *H. diversicolor*. This study demonstrates that the rate of accumulation of DEHP in *H. diversicolor* is significantly higher than in seawater and sediment (Figures 3.6 – 3.11). This demonstrates that DEHP desorption in the tissues of *H. diversicolor* from sediment and seawater is a potentially important biomarker. The main route of DEHP transfer was found to be sorption via the body wall (Amiard-Triquet, 2009).

Numerous field studies have shown that using an approach of a set of biomarkers in invertebrates is often successful (Porte et al., 2001; Kopecka et al., 2006; Durou et al., 2007a; Lima et al., 2007; Martín-Díaz et al., 2008). Many such enzyme activity biomarker studies started with enzymes involved in the up and down regulation of oxidative stress such as catalase (CAT) and superoxide-dismutase (SOD) (Durou et al., 2007a). This study demonstrates an increase in the biomarker of oxidative-stress of CAT and stability in SOD (Figures 4.2- 4.5).

Behaviour was chosen as the second biomarker. Many different contaminants influence the behaviours of organisms, either by directly affecting the organism, or by affecting its ambient medium (Amiard-Triquet, 2009). Chemical contaminants influence the behaviour of the organism, either by acting directly on its senses, or by diffusion through its integument, and then reducing or modifying the information that reaches the brain. The response effects in this case emerge and the potential consequence will be a change in behaviour. In this study, it was found that DEHP negatively affected *H. diversicolor* in terms of fitness to cope with its environment, here by changing its feeding and burrowing abilities (Figures 5.3 - 5.6). DEHP effects on *H. diversicolor* also resulted in poor predator avoidance behaviour, which is a part of the organism's defence strategies against predators (Eisler, 2000).

DEHP adsorption in marine sediments is greater than its adsorption in freshwater sediments, owing to DEHP's reduced solubility in saltwater (ATSDR, 2002). Several degradation pathways are followed by phthalates; for example they are biodegradable by actinomycetes and bacteria, which may be the dominant mechanism of their loss in surface waters and sediments (Staples et al., 1997). DEHP degradation in seawater depends on the conditions of exposure, including its concentration. Seawater has the ability to degrade DEHP if it has sufficient oxygen supply and supports anaerobic conditions. Furthermore, the half-life of DEHP was found to depend on the concentration that was used, which was in the range of <1 day-2 weeks in marine waters (Amir et al., 2005, Staples et al., 1997).

The level of DEHP was high in *H. diversicolor,* which were collected from the polluted parts of the Humber Estuary (Paull). In the laboratory, the level of DEHP significantly decreased in the untreated worms after they were kept in a clean system (without added DEHP), and also when kept at a low exposure concentration of 0.05 µg/L (long-term). This means that the worms recovered from environmental DEHP exposure in the field by detoxification after they were kept in a clean environment or in one with low DEHP concentration (Figure 3.6). *H. diversicolor* has the potential ability of DEHP detoxification by means of active metabolism. On the other hand, the levels of DEHP body concentrations reached at the end of the exposure experiment were similar to those of the worms collected from the polluted estuaries in both the cultures at environmentally relevant estuarine levels of concentration of 2 and 10 µg/L in long term exposure conditions, and also the high DEHP exposure levels of 100 and 500 µg/L in short term exposures (Figures 3.6 and 3.9). The reason behind this might be the diffusion-limited exchange across the body surface. The level of DEHP in the treated

worms at the concentration of 500 µg/l was slightly higher than that of the worms collected from Paull, which indicates that the worms from Paull were exposed to either short-term bursts of a higher concentration of DEHP or higher chronic levels of DEHP. However, these results also confirm that decrease of active metabolism in the worms leads to a decrease in their ability of detoxification from DEHP as described by Wofford (1981).

Generally, in the *H. diversicolor* culture systems under both long and short term exposures (low and high concentration), DEHP accumulated more in the worms and sediment than in seawater, depending on concentration, whereas it degraded quickly in seawater (Figures 3.8 and 3.11), which indicates the presence of sufficient oxygen supply and anaerobic conditions in the culture systems. DEHP accumulates more in the sediment than in seawater because of its poor solubility in seawater and high vaporization (Figures 3.7 and 3.10). Several studies found that DEHP exists in different aquatic environments including river water, seawater, and industrial wastewater (Giam et al., 1998; Tan, 1995; Morris, 1970). This means that aquatic organisms tolerate DEHP and have pathways to tolerate exposure to DEHP. Conversely, this means DEHP poses potential risks to human health and environmental wellbeing.

Antioxidant defence systems exist in most animal species including most aquatic organisms. Several studies conducted on marine species have indicated the significant role that antioxidant enzymes play in protecting the organisms' cellular systems from the damaging oxidative action that is induced by xenobiotics. Among these are SOD which are metalloenzyme catalysts that convert the reactive $O_2^{\cdot-}$ into H_2O_2, which is also a significant ROS. Thereafter, H_2O_2 is detoxified and rendered harmless by the CAT enzyme. In this way the two enzymes act together to neutralize ROS. The rate of dismutation of $O_2^{\cdot-}$ which is catalysed by SOD, is nearest to the diffusion limit, thus becoming the most active enzyme, as described by Zhou et al. (2004). When SOD and CAT and other antioxidant enzymes are exposed to different organic and metal contaminants, they respond by an increase in activity in order to alleviate the oxidative stress. However, such responses are often transient and vary in different species and enzymes, as well as toward single and mixed contaminants (Cossu et al., 2000; Livingstone, 2001; Valavanidisa et al., 2006).

H. diversicolor exposure to DEHP increased the activity of antioxidant enzyme (CAT Figures 4.2 and 4.3) and stability in SOD enzyme (Figures 4.4 and 4.5) at both long-term (low concentration) and short-term (high concentration) exposures. This suggests an oxidative stress suffered by the organisms most probably depending on the

concentration of accumulated DEHP and the duration of exposure. It appears that DEHP is capable of causing oxidative damages to this worm, perhaps by creating a reactive oxygen stress in the worm's body, as is the case when the worm is exposed to copper (Cu) (Ait Allaet al., 2006; Bouraoui et al., 2009) and in the same way as when other polychaetes are exposed to Cu (Nusetti et al., 2001; Geracitano et al., 2004b). Increases in the activities of antioxidant enzymes have been reported by many other studies in cases of exposure to both metal and organic contaminants (Geracitano et al., 2002; Ait Allaet al., 2006; Moreira et al., 2006; Bouraoui et al., 2009; Banniet al., 2009) but not yet for DEHP.

The consequences of an organisms' exposure to DEHP include cellular oxidative stress and production of ROS. The cellular effects of ROS include production of energy, which is a cost to an organism that may be manifested the ability to sustain functional traits such as key stereotyped, energetically expensive behaviour. Changes in behaviour are of particular interest for researchers in their study of the sublethal effects of contaminants in seawater ecosystems, because of their relatively high sensitivity. Weis, et al. (2001) summed up behavioural responses as one type of response with clear connections to toxic concentrations at the individual's level, and clear links to their effects at the population levels. Impacted burrowing behaviour leads to longer exposure times and therefore greater availability of the worms to the predators of species that dwell in the sediment. Since interactions of prey with predator have special importance in controlling the population characteristics, and since contaminants availability in the sediments of aquatic environments is the highest, burrowing behaviour can be used as a relevant ecological and toxicological tool in the assessment of environmental risks (Bonnard et al., 2009).

Within an intricate aquatic context where visual discovery of food is not normally feasible, a great amount of organisms are dependent on chemoreception, employing a multifarious combination of chemical cues to prompt suitable sensory organs in addition to finding food (Bronmark & Hansson, 2000). Additionally, chemoreception is essential to distinguish amid options for food, for instance amid dangerous or distasteful substances or nutritious ones (Wyatt, 2003). Invertebrates are able to deal with the availability of toxicants within their context by stimulating several detoxication procedures, which result in a difference in their conduct. These biochemical defence systems were nonetheless inadequate for the avoidance of behavioural weaknesses. In worms that were exposed, burrowing and feeding times were greatly affected in contrast to controls (Buffet et al., 2011). As such. feeding and

burrowing activities can be highlighted as appropriate behavioural biomarkers to assist the fitness appraisal of *H. diversicolor*.

Effect of DEHP exposure and accumulation in the body upon the behaviours of *H. diversicolor* occurred in both the long-term (low concentration) and short-term (high concentration) DEHP exposures. DEHP was found to drastically change the behaviours of these worms most likely by penetrating the body cells (as it was added to the culture water) and then chemically interacting with biologically important molecules (Browne et al., 2013). This assumption has been supported by behavioural biomarker, which are sensitive tools that can assess the effects of phthalate contamination sub lethal concentrations in sub-organismal and supra-organismal (Amiard-Triquet, 2009). When *H. diversicolor* was exposed to DEHP, the activitiy of antioxidant enzyme (CAT, see Figures 4.2 and 4.3) increased, leading to a failure to detect/respond to the presence of the predator and decreased the feeding time, as well as increased the burrowing time. All of these effects are in agreement with Buffet, et al. (2011) who studied *H. diversicolor* exposure to metal stress, specifically copper. However, the SOD enzyme activity was stable, which might be the result of the removal of ROS by CAT enzymes (Sun & Zhou, 2007) or it could simply be due to the consumption of the pre-existing enzyme by means of the chemical's oxidative property (Kanget al., 2010).

The feeding response time in untreated worms was higher than in DEHP treated worms in both the absence and presence of predator odour (Figurs5.3 and 5.4). Furthermore, the feeding response time was decreased whenever DEHP concentration increased. On the other hand, the burrowing time in untreated worms was lower than in DEHP treated worms in both the absence and presence of predator odour (Figurs5.5 and 5.6). The burrowing time increased whenever DEHP concentration increased in both the absence and presence of predator odour. A reduction in burrowing behaviour leaves the worm more vulnerable to predator attacks and wave action, thereby reducing the worm's fitness and chances of survival (Kalman et al., 2009). 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). When DEHP accumulated in worms, they failed to detect and respond to chemical cues, which are diffusively carried in the water column and brought into polychaete burrows. However, Buffet et al. (2011) study indicated that feeding rates were not affected and burrowing rates decreased when *H. diversicolor* was exposed to copper, suggesting a different mode of action of the stressors DEHP and copper. Whilst copper impacts physiological fitness, DEHP seems to interfere more with the detection/response chain involved in chemical cue controlled

behaviours. A signal from the environment is received by interaction with a component in the cell, such as a receptor on the cell surface. The information about the signal is then changed to a chemical form. The signal often needs to be amplified before it evokes a response (Berg et al., 2002).

In the environment, most Nereids are capable of digging intricate burrows in the sediment columns, and such burrows endure wave action and sediment movement for different periods of time (Neuhoff, 1979). The worms are often busy ventilating and renewing oxygen in their burrows as well as clearing metabolites from them, which also significantly contributes to sediment oxygenation (Scaps, 2002). According to Kristensen (2001), both bioturbation of water columns and total water volume displaced by *H. diversicolor* are often in good quantity, which means that huge quantities of water are pumped into the sediment. Kristensen (2001) also estimated the water column passed by *H. diversicolor* into its burrow as in the range of 3-9 m daily. In this way the burrows of *H. diversicolor* will be flushed with excess of water on a continuous basis, thus creating very toxic and oxidized water in the burrows and the nearby sediments (Kristensen, 2001). Bioturbation is a physical process with which chemical changes are associated and is related to movement of sediment particles or water. It can change biogeochemical reactions by making resources available for microbes such as carbon and nutrients, or by altering the abiotic conditions that affect microbial reaction rates (e.g. redox and temperature) (Gutierrez & Jones 2006). Organisms use bioturbating activities, which involve active transport of sediment particles or water, to alter their microbial communities by making some chemical substances available or by changing their conditions. Changes in particle transport are generated by the bioturbator by reworking the current processes. By redistributing and biomixing the particles, the bioturbator can modify their chemical conditions, for instance, by means of redox changes, and can then make them available for other organisms. The bioturbator changes the conditions by ventilation and active in and out pumping of water through burrows in the sediment. The resulting bioirrigation may alter the chemical conditions of water solutes in the burrow and thereby change the microbial processes that occur inside the sediment (Kristensen et al., 2012).

The environmental deterioration resulting from water pollution by chemicals substances, is imposing increasing problems for future development (Suthar et al., 2009). The environmental consequences of water pollution include natural resources destruction, leading in turn to depletion of biodiversity and demise of many aquatic species (Islam & Tanaka, 2004). The culprit in these cases is pollution from industrial

and agricultural sources, which is the major cause behind high concentrations of harmful and toxic materials. These include wastes from oil refineries, chemical industry and fertilizers used in agricultural areas (Islam & Tanaka, 2004). Effluents coming from industrial sources are drained directly into estuaries and rivers, as well as to sewage systems. Some of these effluents are characterized by very high biological oxygen demand (BOD), which is often compounded by effluents from sewage treatment works (STWs), thus, augmenting the threats to aquatic life by depleting the dissolved oxygen in tidal rivers and estuarine areas.

Most of the studies in the Humber estuary have been conducted about the concentration of heavy metals such as copper in the environment because they are released in effluents. Metals can be taken up by organisms with ingested food, which may then become an important factor in their assimilation and bioaccumulation in their tissues and in the seawater food chain (Spencer et al., 1999; Wang and Fisher, 1999b). In most ecotoxicological bioassays marine invertebrates were exposed to chemical contaminants by the mechanism of dissolution, while absorption with ingested food is regarded as less important in metal uptake and toxicity (Spencer et al., 1999; Wang & Fisher, 1999b). However, metal accumulation may take place through all routes including food and water, which may depend on the species, type of metal and food source. Furthermore, metals in ionic form mostly come from food (Wang & Fisher, 1999a). large quantities of metals may be absorbed from water or from land effluents (Bernds et al., 1998), which may be an important metal accumulation factor in *Nereis(Hediste) diversicolor* and other Nereidid worms, because they are sediment dwellers, and often use large quantities of water to flush their burrows (McLeese & Burridge, 1987). Moreover, sediment ingestion is an important route of metal absorption, as it was found in the cells of the cuticle and mid-gut (Geffard et al., 2005), and in the epithelial cells of the intestine (Pirie & George, 1979).

As discussed in the chapter on the fate of DEHP in culture system of *H. diversicolor* in vivo, DEHP accumulated more in worms then in sediment and seawater, which is similar to the situation found in the environment (Chapter 3, Figures 3.6 – 3.11). DEHP, one of the most abundant phthalates, exists in low concentrations in most environments. After taking fluctuations over months of the year and the differences between locations into account (Figures 6.4 - 6.9), DEHP concentrations in *H. diversicolor* are either higher than the levels reported on other studies in marine animals (Brown & Thompson, 1982a ;1982b; Mackintosh et al., 2004; Lin et al., 2003) or lower than the levels of other studies (Vethaak et al., 2000; Chin Huang et al., 2008).

Furthermore, the levels of DEHP in sediment were below the reported levels in other studies (Remberger, 1999; Chin Huang et al., 2008; Magdouli et al., 2013; Preston & Al-Omran, 1989; Ritsema et al., 1989; Ray et al., 1983; Giam et al., 1978). However, in seawater it was consistent with Giam et al.'s (1978) study and lower than in other studies (Preston & Al-Omran,1989; Ritsema et al., 1989; Ray et al., 1983; Giam et al., 1978; Furtmann, 1999; Vikelsoe, 1999; Remberger, 1999; Vethaak et al.2000; Khan & Jung, 2008, Yuwatini et al., 2006; Magdouli et al., 2013).

An estuary's capacity to retain contaminants or to get rid, is among the vital considerations for water quality managers (Meade et al., 1990; Brunk et al., 1997; Turner et al., 1999). In this respect, DEHP retention is controlled by two major effects: the first is the degree of adsorption of contaminants by suspended particles, which in turn depends on the concentration of such suspended particles and their distribution coefficient (KD); and the second is the rate of decay of contaminants in the aqueous phase, which in turn depends on the temperature and several water quality parameters including dissolved oxygen and microbial density. The retention of such contaminants, in practice, depends on the water quality and its transit time from the region of discharge to the point of high turbidity, such as the turbidity maximum zone (TMZ), trapping of suspended particles in this zone, and the degree of bed sediment re-suspension (Turner & Rawling, 2000).

Finally, environmental pressures also play a role as a strong selective force that influences the morphology, physiology and behaviour of the organism (Pease et al., 2010). By environmental pressures we mean either natural ones such as salinity and temperature, or anthropogenic ones such as contaminants, which can threaten survival, or lead to more growth and booming (Pease et al., 2010). Organisms deal with contamination by means of two main strategies (Galletly et al., 2007); the first of which is avoidance, although many estuarine and open seawater species cannot swim far enough away or sufficiently fast to avoid environmental changes. The second strategy is adaption, where organisms try either to resist or tolerate the environmental change. However, resistance means less fitness (Galletly et al., 2007). Tolerance, on the other hand, in *H. diversicolor*, may be developed by decrease in the take up of the contaminant, increase in excretion thereof, or storage in a physiochemical non-toxic form (Berthet et al., 2003). In this case, energy invested in such processes will be at the expense of energy needed for other purposes such as growth and reproduction. Thus, tolerance can be rather expensive (Durou et al., 2007b). In *H. diversicolor* copper tolerance has its specific mechanisms including increase of upregulation of some genes

and decrease of others, changes in metal receptors, and increase in detoxifying enzyme production or in production of proteins (Pook et al., 2009). However, the most resource intensive process in the energy budget of the cell is protein synthesis and turnover (Pook et al., 2009).

Consumption of substantial resources and energy would put a lot of pressure on the organism's budget of resources and energy, thus restricting allocations for metabolism, growth and reproduction (Durou et al., 2008). As an example of this, *H. diversicolor* samples from the Seine estuary were found to have smaller sizes and produced fewer oocytes, compared to *H. diversicolor* samples from the Authie estuary which were larger, produced more oocytes; females were significantly bigger than males, and had a healthy appearance (Durou et al., 2008). In cleaner environments *H. diversicolor* invests about 70% of its energy budget on reproduction, while in contaminated waters it has to allocate some part of it to tolerance and maintenance, and another to growth and reproduction (Durou et al., 2008; Pook, 2009). Tolerance has a genetic element in it, because individuals acclimatized to clean conditions were found capable of tolerating contaminated conditions (Burlinson & Lawrence, 2007). The genetic element continues for generations in agreement with Darwinian natural selection (Nedwell, 1997). In this way, tolerant genotypes are better in survival and reproduction than the non-tolerant ones, and thereby their genomes will be passed to the next generations. However, as tolerance costs are high, it is used only in contaminated waters. As an example, *H. diversicolor* populations in contaminated waters were found capable of tolerating higher copper concentrations relative to other populations (Nedwell, 1997).

CHAPTER 8: CONCLUSION

8.1. SUMMARY

For the purpose of this study DEHP were used to expsoe ragworms in both long-term with low concentrations and short-term with high concentration aiming to expose worms to levels of DEHP that induce biological effects. However, it should be noted that the DEHP concentrations of 2, 10, 100 and 500 µg/L chosen here to do exist in some polluted environments (Lin et al., 2003; Mackintosh et al., 2004; Xie et al., 2005; Peijnenburg & Struijs, 2006; Mackintosh et al., 2006), but standard measurements or predictive data on DEHP concentrations are not available. DEHP accumulated in vivo in *H. diversicolor* more than in the sediment and significantly more than in seawater. DEHP activates oxidative stress biomarkers (CAT enzymes) in the worms. Exposure of *H. diversicolor* to DEHP results in behavioural impairments in terms of both burrowing and feeding behaviours. Finally, the selection of a marine invertebrate (*H. diversicolor*) in this work, as well as the set of biomarkers that were chosen, were found suitable for conducting ecotoxicity studies with DEHP in different UK sites.

8.2. LIMITATIONS

This research addressed the effects of phthalates on Nereididae polychaetes. Because the available time and resources to conduct this research were limited, Paull, as a part of Humber estuary, and *Hediste diversicolor*, as part of Nereididae polychaetes, were taken as model organisms for bioturbating marine invertebrates to investigate and analyse the marina life. It is hoped that the research will ultimately result in a good representation of the influence of phthalates also on other related Nereididae polychaetes and individuals from other ecosystems that may have a different level of DEHP pre-exposure or different detoxification capacities. In the addition, the study has lack of positive control, due to the limitation in the availability of systems and space of culture room.

The sample population in this research has limited representativeness. For instance, because the characteristics of the chemicals produced in the industrial areas in Paull are unknown, we cannot say with certainty that the findings of this research would apply to all industrial sites, nor that they would definitely apply to all Nereididae polychaetes species. However, the fate of DEHP in *H. diversicolor*, seawater and the

sediment has been analysed from five different locations in the Humber estuary and over different times of the year in Paull, in order to overcome this limitation.

8.3. FUTURE STUDIES

- In this study, *H. diversicolor* was examined to see whether it can reach homeostasis when exposed to high DEHP concentrations in short-term exposures (7 days). There is a need to conduct further studies on the ability of marine animals (*H. diversicolor*) to withstand DEHP different levels via homeostasis in their bodies as well as DEHP accumulation in their tissues in case of long-term exposure up to 12 months. Besides, levels of Glutathione-S-Transferase (GST) and of glutathione (GSH) could be used as bio-indicators to detect detoxification stress in *H. diversicolor*, as well as in different marine invertebrate species as these have been shown for endocrine stress in Nereids before (Ayoola et al., 2010; Garcia-Alonso et al., 2011).

- It would be useful to investigate whether the accumulation of DEHP in *H. diversicolor* at higher temperature i.e. climate change relevant scenarios or low pH to simulate ocean acidification scenarios will potentially impact its behavioural or biochemical responses.

- Research is needed to investigate whether the accumulation of DEHP in Nereis succinea will affect its fertilization rate in long-term exposure or affect its gametes in short-term exposure.

- A study that includes the expression, cloning and characterization of CAT and SOD in *H. diversicolor* should be considered in the future using CAT and SOD as applied in proteomics, genomics and other biomarker studies. Such a study can give us more insight into micro-RNA biomarkers, since we now know that cells' functionality is affected by the regulation of gene expression and mRNA degradation or translational inhibition, all of which can influence the development of the normal cells and make them develop into abnormal cells.

- The research findings for this study were gathered from the Humber estuary. As such, the research findings represent the characteristics of this particular site. Future research could repeat this study with a large sample size or in different locations with different Nereididae polychaetes species. This would offer further insight and create a more representative body of literature on this topic.

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APPENDICES
APPENDIX A

9.1. THE FATE OF DEHP IN THE LONG-TERM EXPOSURE CULTURE SYSTEMS
9.1.1. *H. DIVERSICOLOR*
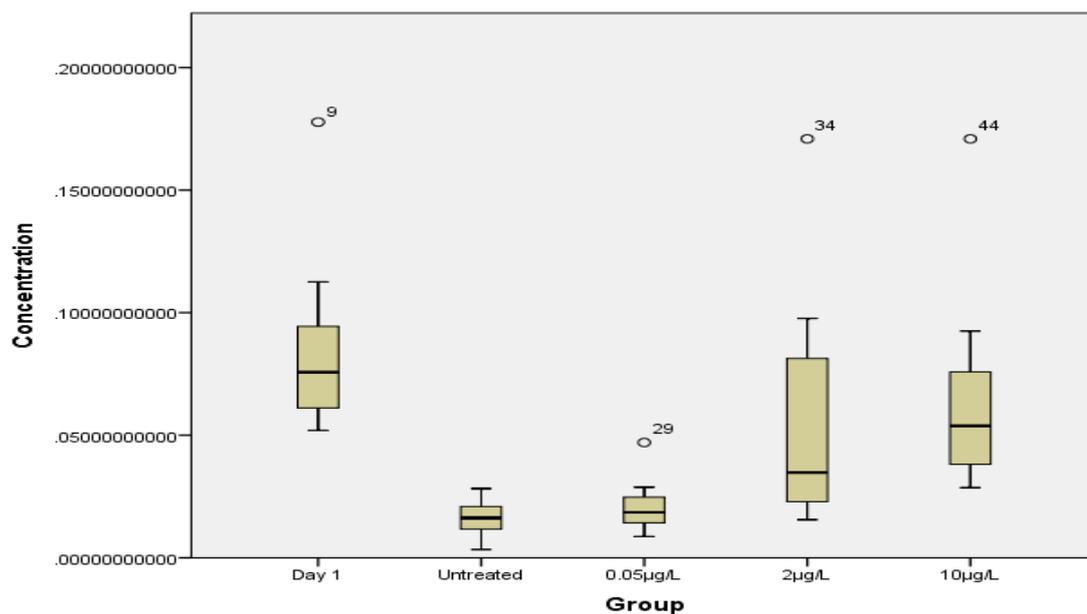
Figure 9.1: The box plot for mean level of DEHP for *H. diversicolor* tissue (± SE) in five culture systems that each contains 10 worms. Day 1 is worms that were analysed on the day of collection, whereas treated group expose to DEHP for 3 months at different concentrations (0.05, 2 and 10 μg/L).
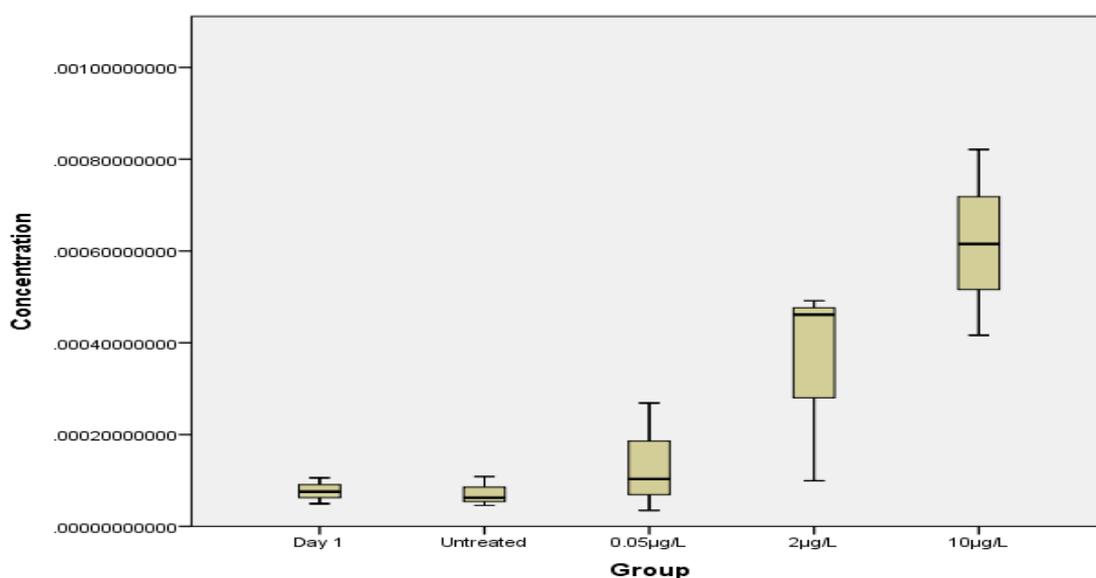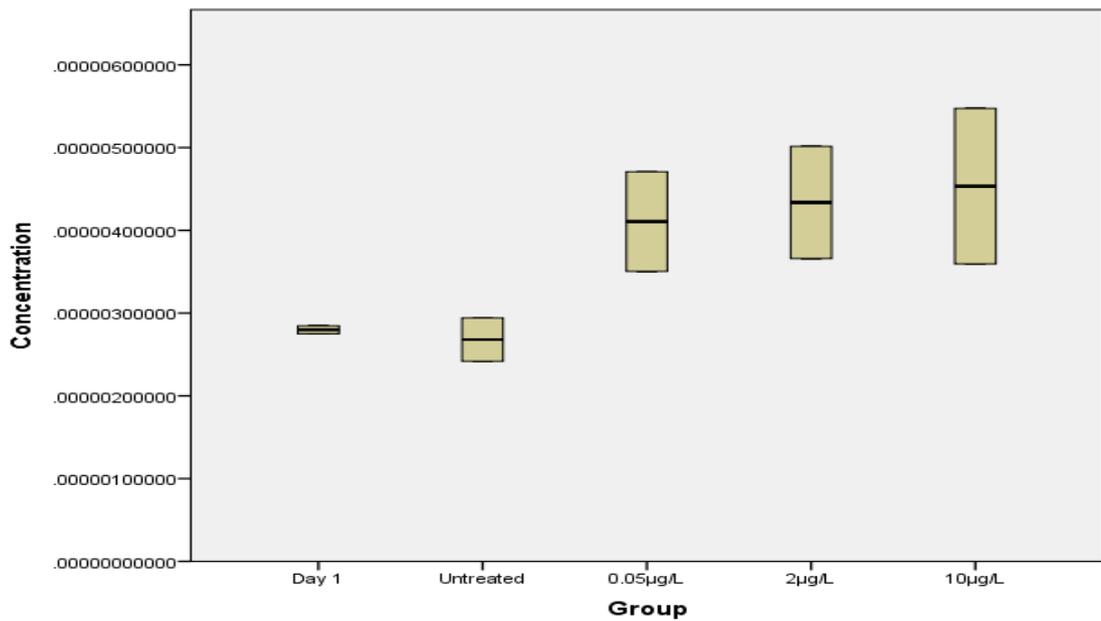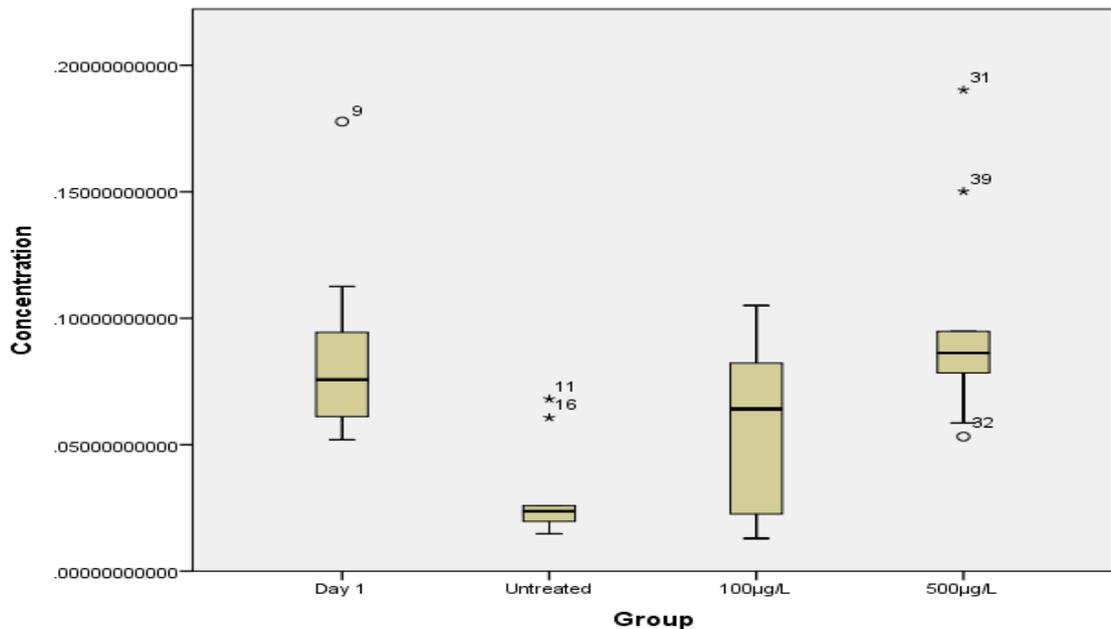
9.1.2. SEDIMENT

Figure 9.2: The box plot for mean level of DEHP for sediment (± SE) in the five culture systems. Three samples from each system have been analysed. Day 1 is sediment that was analysed on the day of collection, whereas treated group expose to DEHP for 3 months at different concentrations (0.05, 2 and 10 μg/L).

Figure 9.3: The box plot for mean level of DEHP for seawater (± SE) in the five culture systems. Three samples from each system have been analysed. Day 1 is seawater that was analysed on the day of collection, whereas treated group expose to DEHP for 3 months at different concentrations (0.05, 2 and 10 µg/L).

9.2. THE FATE OF DEHP IN THE SHORT-TERM EXPOSURE CULTURE SYSTEMS
9.2.1. _H. DIVERSICOLOR_

Figure 9.4: The box plot for mean level of DEHP for _H. diversicolor_ tissue (± SE) in four culture systems that each contains 10 worms. Day 1 is worms that were analysed on the day of collection, whereas treated group expose to DEHP for 7 days at different concentrations (100 and 500 µg/L).

9.2.2. SEDIMENT

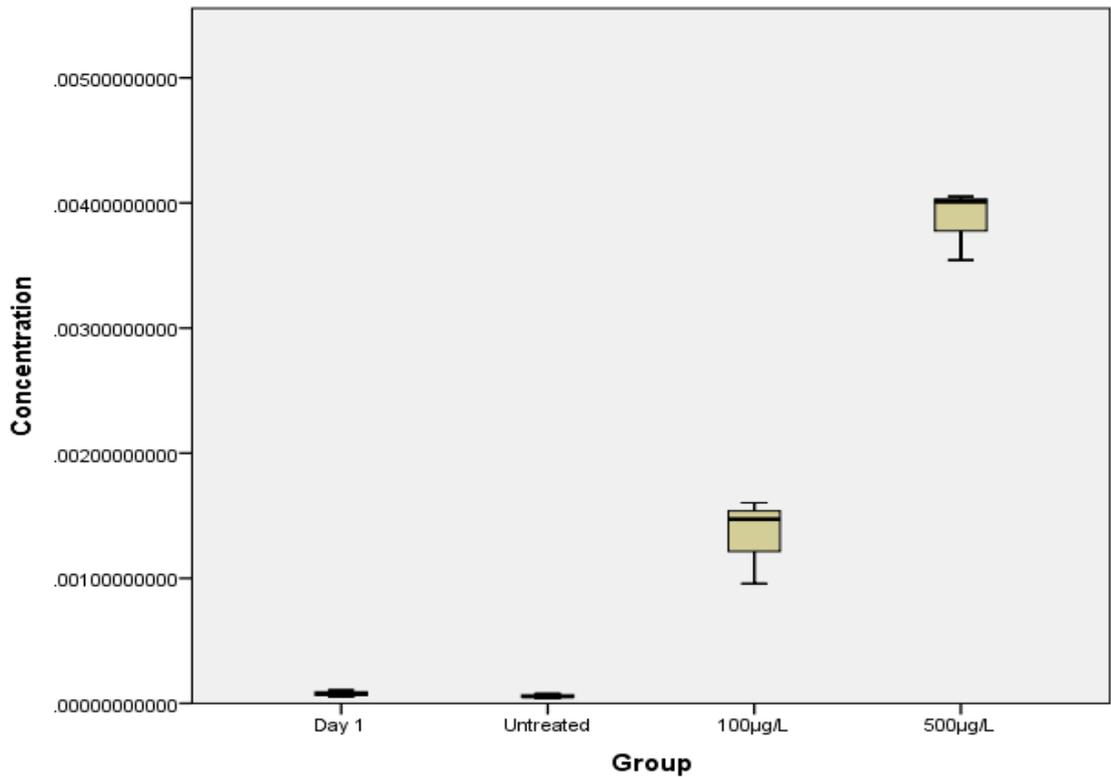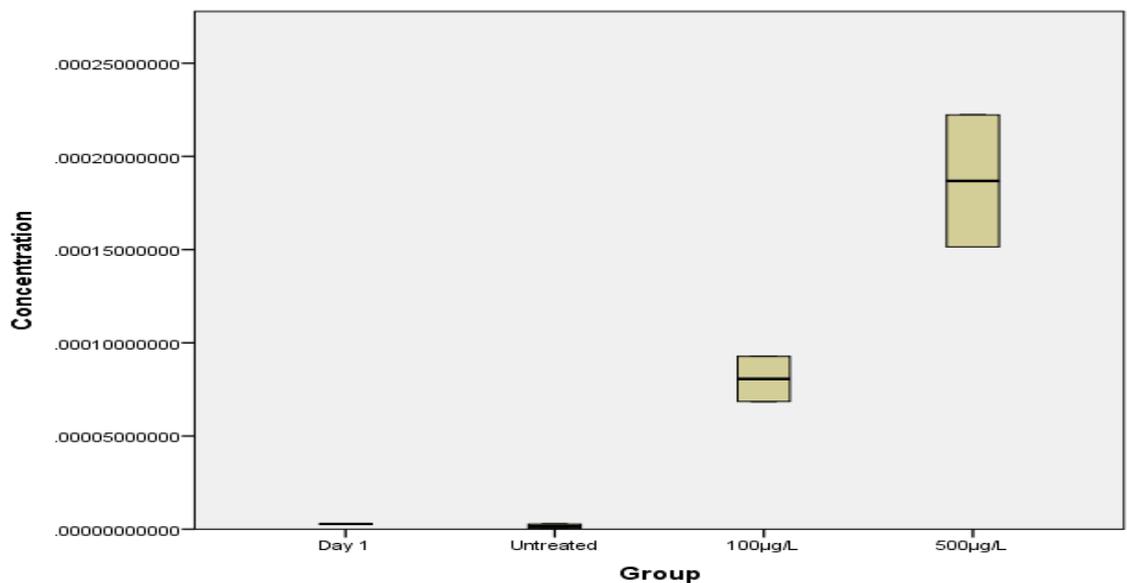**Figure 9.5: The box plot for mean level of DEHP for sediment (± SE) in four culture systems. . Three samples from each system have been analysed. Day 1 is sediment that were analysed on the day of collection, whereas treated group expose to DEHP for 7 days at different concentrations (100 and 500 µg/L).**

9.2.3. SEAWATER

Figure 9.6: The box plot for mean level of DEHP for seawater (± SE) in four culture systems. . Three samples from each system have been analysed. Day 1 is seawater that were analysed on the day of collection, whereas treated group expose to DEHP for 7 days at different concentrations (100 and 500 µg/L).

APPENDIX B

10.1. THE FATE OF DEHP IN THE LONG-TERM EXPOSURE CULTURE SYSTEMS

The following steps used to calculate the concentration of DHEP:

1. Relative response= (area of DHEP/area IS).

2. Concentration = (Slope M x Relative response) + Intercept C.

3. The concentration is divided by sample's weight.

10.1.1. *H. DIVERSICOLOR*

Table 10.1: Mean level of DEHP for *H. diversicolor* tissue (± SE) in five culture systems. Day 1 is *H. diversicolor* that was analysed on the day of collection, whereas treated group expose to DEHP for 3 months (chronic stress) at different concentrations (0.05, 2 and 10 µg/L).

Groups	Mean level of DEHP (µg/g)	Standard deviation (µg/g)
Day 1	0.08510	±0.03762
Untreated	0.01671	±0.00788
0.05 µg/L	0.02121	±0.01085
2 µg/L	0.05766	±0.04854
10 µg/L	0.06663	±0.04203

10.1.2. SEDIMENT

Table 10.2: Mean level of DEHP for sediment (± SE) in five culture systems. Day 1 is sediment that was analysed on the day of collection, whereas treated group expose to DEHP for 3 months (chronic stress) at different concentrations (0.05, 2 and 10 µg/L).

Groups	Mean level of DEHP (µg/g)	Standard deviation (µg/g)
Day 1	0.000077	±0.000028
Untreated	0.000072	±0.000032
0.05 µg/L	0.000136	±0.000120
2 µg/L	0.000386	±0.000218
10 µg/L	0.000614	±0.000202

10.1.3. SEAWATER

Table 10.3: Mean level of DEHP for seawater (± SE) in five culture systems. Day 1 is seawater that was analysed on the day of collection, whereas treated group expose to DEHP for 3 months (chronic stress) at different concentrations (0.05, 2 and 10 µg/L).

Groups	Mean level of DEHP (µg/ml)	Standard deviation (µg/ml)
Day 1	0.0000028	±0.00000006
Untreated	0.0000027	±0.00000036
0.05 µg/L	0.0000041	±0.00000085
2 µg/L	0.0000044	±0.00000095
10 µg/L	0.0000045	±0.00000132

10.2. THE FATE OF DEHP IN THE SHORT-TERM EXPOSURE CULTURE SYSTEMS

10.2.1. *H. DIVERSICOLOR*

Table 10.4: Mean level of DEHP for *H. diversicolor* tissue (± SE) in four culture systems th. Day 1 is worms that were analysed on the day of collection, whereas treated group expose to DEHP for 7 days (acute stress) at different concentrations (100 and 500 µg/L).

Groups	Mean level of DEHP (µg/g)	Standard deviation (µg/g)
Day 1	0.08510	±0.03762
Untreated	0.02994	±0.01863
100 µg/L	0.05593	±0.03454
500 µg/L	0.09733	±0.04178

10.2.2. SEDIMENT

Table 10.5: Mean level of DEHP for sediment (± SE) in four culture systems. Day 1 is sediment that were analysed on the day of collection, whereas treated group expose to DEHP for 7 days (acute stress) at different concentrations (100 and 500 µg/L).

Groups	Mean level of DEHP (µg/g)	Standard deviation (µg/g)
Day 1	0.000077	±0.000028
Untreated	0.000058	±0.000019
100 µg/L	0.001344	±0.000341
500 µg/L	0.003868	±0.000282

10.2.3. SEAWATER

Table 10.6: Mean level of DEHP for seawater (± SE) in four culture systems. Day 1 is seawater that were analysed on the day of collection, whereas treated group expose to DEHP for 7 days (acute stress) at different concentrations (100 and 500 µg/L).

Groups	Mean level of DEHP (µg/ml)	Standard deviation (µg/ml)
Day 1	0.0000028	±0.00000006
Untreated	0.0000016	±0.0000015
100 µg/L	0.0000219	±0.0000171
500 µg/L	0.0001934	±0.000050

10.3. THE EFFECT OF DEHP ON CATALASE ACTIVITY IN *H. DIVERSICOLOR*

10.3.1. <u>AFTER LONG-TERM EXPOSURE</u>

Table 10.7: Mean catalase activity level (± SE) of long term (3 months) DEHP exposed *H. diversicolor*.

Groups	Mean catalase activity level (mM)	Standard deviation (mM)
Untreated	836.17	±550.4
0.05 µg/L	940	±577.4
2 µg/L	1405	±233.6
10 µg/L	1545.87	±469.7

10.3.2. <u>AFTER SHORT-TERM EXPOSURE</u>

Table 10.8: Mean catalase activity level (± SE) of short term (7 days) DEHP exposed *H. diversicolor*.

Groups	Mean catalase activity level (mM)	Standard deviation (mM)
Untreated	1048.04	±489.8
100 µg/L	1336.78	±575
500 µg/L	1691.04	±486

10.4. THE EFFECT OF DEHP ON SUPEROXIDE DISMUTASE (SOD) ACTIVITY IN *H. DIVERSICOLOR*

10.4.1. <u>AFTER LONG-TERM EXPOSURE</u>

Table 10.9: Mean SOD activity level (± SE) of long term (3 months) DEHP exposed *H. diversicolor*.

Groups	Mean SOD activity level (mM)	Standard deviation (mM)
Untreated	64.02	±3.8
0.05 µg/L	65.25	±4.1
2 µg/L	65.38	±5.7
10 µg/L	65.61	±9.3

10.4.2. <u>AFTER SHORT-TERM EXPOSURE</u>

Table 10.10: Mean SOD activity level (± SE) of short term (7 days) DEHP exposed *H. diversicolor*.

Groups	Mean SOD activity level (mM)	Standard deviation (mM)
Untreated	63.73	±5.3
100 µg/L	64.92	±1.8
500 µg/L	66.07	±10.2

10.5. FEEDING RESPONSE TIME IN THE ABSENCE AND PRESENCE OF PREDATORS

10.5.1. AT LONG-TERM EXPOSURE

Table 10.11: Mean feeding response time in the absence and presence of predators (± SE) of exposed *H. diversicolor* to DEHP at long-term.

Groups	Mean feeding response time (mins)	Standard deviation (mins)
Absence of predator		
Untreated	02:39	±02:41
0.05 µg/L	02:12	±00:58
2 µg/L	01:20	±00:49
10 µg/L	00:54	±00:37
Presence of predator		
Untreated	04:50	±03:37
0.05 µg/L	03:42	±01:24
2 µg/L	02:33	±01:29
10 µg/L	01:58	±00:50

10.5.2. AT SHORT-TERM EXPOSURE

Table 10.12: Mean feeding response time in the absence and presence of predators (± SE) of exposed *H. diversicolor* to DEHP at short-term.

Groups	Mean feeding response time (mins)	Standard deviation (mins)
Absence of predator		
Untreated	02:02	±00:58
100 µg/L	01:39	±01:40
500 µg/L	01:20	±01:01
Presence of predator		
Untreated	03:28	±02:01
100 µg/L	02:51	±02:22
500 µg/L	02:27	±01:42

10.6. BURROWING TIME IN THE ABSENCE AND PRESENCE OF PREDATORS

10.6.1. AT LONG-TERM EXPOSURE

Table 10.13: Mean burrowing time in the absence and presence of predators (± SE) of exposed *H. diversicolor* to DEHP at long-term.

Groups	Mean burrowing time (mins)	Standard deviation (mins)
Absence of predator		
Untreated	01:47	±00:32
0.05 µg/L	01:56	±00:51
2 µg/L	02:07	±01:09
10 µg/L	02:23	±00:58
Presence of predator		
Untreated	01:07	±00:39
0.05 µg/L	01:10	±00:26
2 µg/L	01:15	±00:48
10 µg/L	01:26	±00:53

10.6.2. AT SHORT-TERM EXPOSURE

Table 10.14: Mean burrowing time in the absence and presence of predators (± SE) of exposed *H. diversicolor* to DEHP at short-term.

Groups	Mean burrowing time (mins)	Standard deviation (mins)
Absence of predator		
Untreated	01:00	±00:16
100 µg/L	01:42	±00:38
500 µg/L	02:23	±01:06
Presence of predator		
Untreated	00:44	±00:17
100 µg/L	00:59	±00:21
500 µg/L	01:15	±00:41

10.7. DETERMINE DEHP CONCENTRATIONS IN *H. DIVERSICOLOR*, SEAWATER AND SEDIMENT FROM ONE LOCATION IN HUMBER ESTUARY (PAULL) IN DIFFERENT TIMES OF THE YEAR

10.7.1. *H. DIVERSICOLOR*

Table 10.15: Mean levels of DEHP in *H. diversicolor* tissue (± SE) at different times of the year.

Groups	Mean level of DEHP (µg/g)	Standard deviation (µg/g)
June	0.064335846	±0.042
July	0.046588313	±0.045
August	0.056487628	±0.015
September	0.107975256	±0.038
October	0.046664911	±0.013
November	0.08899496	±0.030
December	0.060923289	±0.033
January	0.097662047	±0.077
February	0.055814357	±0.022
March	0.12432845	±0.045

10.7.2. SEDIMENT

Table 10.16: Mean levels of DEHP in sediment (± SE) at different times of the year

Groups	Mean level of DEHP (µg/g)	Standard deviation (µg/g)
June	0.001672784	±0.00058
July	0.00158053	±0.00014
August	0.001247501	±0.00006
September	0.001773954	±0.00008
October	0.001107291	±0.00007
November	0.001639919	±0.0004
December	0.002053455	±0.00008
January	0.002071971	±0.00013
February	0.001571132	±0.00031
March	0.001797694	±0.00036

10.7.3. <u>SEAWATER</u>

Table 10.17: Mean levels of DEHP in seawater (± SE) at different times of the year.

Groups	Mean level of DEHP (µg/ml)	Standard deviation (µg/ml)
June	0.0000110654	±0.0000066
July	0.0000066448	±0.0000046
August	0.0000172787	±0.000009
September	0.0000104370	±0.0000016
October	0.0000063288	±0.0000018
November	0.0000162980	±0.0000046
December	0.0000112379	±0.0000068
January	0.0000080626	±0.000005
February	0.0000187694	±0.0000077
March	0.0000103360	±0.0000071

10.8. DETERMINE DEHP CONCENTRATION IN *H. DIVERSICOLOR*, SEAWATER AND SEDIMENT FROM DIFFERENT LOCATIONS AROUND THE HUMBER ESTUARY

10.8.1. *H. DIVERSICOLOR*

Table 10.18: Mean level of DEHP in *H. diversicolor* tissue (± SE) at different locations around the Humber Estuary.

Groups	Mean level of DEHP (µg/g)	Standard deviation (µg/g)
Hessle	0.018930946	±0.00697
Sunk Island	0.024890279	±0.01028
Immingham	0.029054076	±0.00864
Spurn Point	0.036045359	±0.01521
Paull	0.046664911	±0.01317
Grimsby	0.058570866	±0.04382

10.8.2. <u>SEDIMENT</u>

Table 10.19: Mean level of DEHP in sediment (± SE) at different locations around the Humber Estuary.

Groups	Mean level of DEHP (µg/g)	Standard deviation (µg/g)
Hessle	0.001611749	±0.000141
Sunk Island	0.001393763	±0.000430
Immingham	0.001237683	±0.000144
Spurn Point	0.001180283	±0.000497
Paull	0.001107291	±0.000076
Grimsby	0.00020017	±0.000046

10.8.3. <u>SEAWATER</u>

Table 10.20: Mean level of DEHP in seawater (± SE) at different locations around the Humber Estuary.

Groups	Mean level of DEHP (µg/ml)	Standard deviation (µg/ml)
Hessle	0.000003938	±0.00000111
Sunk Island	0.000002427	±0.000000981
Immingham	0.000005835	±0.00000259
Spurn Point	0.000002146	±0.000000568
Paull	0.000006329	±0.00000180
Grimsby	0.000001919	±0.000000772