The effect of the sheep dip pesticide diazinon on behavioural and molecular markers in the freshwater shrimp *Gammarus pulex*

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Declaration

I hereby certify that the work embodied in this thesis is the result of my own investigations except where reference has been made to published literature.

Abstract

The purpose of this research was to determine the effects of the organophosphorothioate pesticide diazinon, its metabolite diazinon oxon and diazinon in a commercial formulation on the freshwater amphipod *Gammarus pulex*. It was found that diazinon in the commercial formulation was significantly more toxic to *G. pulex* than diazinon oxon, which in turn was significantly more toxic than diazinon.

In non-lethal ecotoxicity assays, 24 h exposure to diazinon and diazinon in the commercial formulation significantly inhibited the vertical movement of *G. pulex*, whereas diazinon oxon had no significant effect. Inhibition of feeding was observed, with diazinon in the commercial formulation causing the most reduction, then diazinon to a lesser extent, whereas diazinon oxon had no significant effect.

Only diazinon oxon caused a significant inhibition of acetylcholinesterase (AChE) activity *in vitro*. However, only diazinon and diazinon in the commercial formulation caused a significant inhibition of AChE activity following *in vivo* exposure. The levels of small heat shock protein (sHSP) and HSP 90 in post-9,000g supernatant samples were significantly increased by exposure to diazinon and the commercial diazinon formulation. Only diazinon significantly increased the level of HSP 70 in the supernatant. In contrast, diazinon and diazinon in the commercial formulation significantly increased the level of HSP 70 in 9000g pellet samples. Only diazinon in the commercial formulation significantly increased level of HSP 90 the in pellet sample. There was no significant effect of these agents on HSP 60 levels.

Western blotting analysis of 10 % gels showed increased phosphotyrosine content in 51 and 75 kDa bands and increased phosphoserine and phosphothreonine content in the 75 kDa band following exposure to diazinon and diazinon in the commercial diazinon formulation. In contrast, a 51 kDa anti-phosphoserine reactive band only showed a significant increase after exposure to diazinon and

diazinon in the commercial diazinon. Analyses by 2D-PAGE found that a small numbers of low abundance proteins had altered levels in the 9000g supernatant sample after exposure to diazinon. However, the levels were too low to allow identification by mass spectrometry (MS). In conclusion, behavioural assays and biomarkers can be employed as useful and reliable bioindicators of environmental contamination.

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My completion of this thesis would not have been possible without their presence with me in the UK during the full period of my study.

Dedication

To the soul of my beloved father and beloved mother

To my wife and children

To all my brothers, sisters and dearest friends

without whom this thesis could never have been complete

Contents

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Abbreviation

2D-PAGE	Two-dimensional polyacrylamide gel electrophoresis
A. abdita	*Ampelisca abdita*
AChE	Acetylcholinesterase
AcP	Acid phosphatase
AIDA	Image Data Analyzer
AkP	Alkaline phosphatase
ALAT	Alanine aminotransferase
APS	Ammonium persulphate
ASAT	Aspartate aminotransferase
ATCh	Acetylthiocholine iodide
BCA	Bicinchoninic Acid
BSA	Bovine serum albumin
CHAPS	3-((3-Cholamidopropyl) dimethylammonium) -1-propanesulfonate
ChE	Cholinesterase
D. magna	*Daphnia magna*
DDVP	Dichlorvos
DEP	Diethyl phosphate
DETP	Diethyl thiophosphate
DMSO	Dimethyl sulfoxide
DOM	DOM Dissolved organic matter
DTT	5, 5 dithiobis 2-nitrobenzoic acid
EC_{50}	The half maximal effective concentration
ECL	Enhanced chemiluminescence reagent
EQS	Environmental Quality Standards
G. faciatus	*Gammarus faciatus*
G. fossarum	*Gammarus fossarum*
G. lawrencianus	*Gammarus lawrencianus*
G. pseudolimneau	*Gammarus pseudolimneaus*
G. pulex	*Gammarus pulex*
GST	Glutathione S-transferase
H. azteca	*Hyalella azteca*
HSP	Heat shock protein
HSP 60	Heat shock protein 60
HSP 70	Heat shock protein 70
HSP90	Heat shock protein 90
IC_{50}	Half maximal inhibitory concentration
IMP	2-isopropyl-6-methyl-4-pyrimidinol
kDa	Kilodalton
LC_{50}	Lethal concentration 50
LD_{50}	Median lethal dose
NaOH	Sodium hydroxide
NAWQA	National Water Quality Assessment Program
NOAEC	No Observable Adverse Effect Concentration

NOEC	No observable effect concentrations
OM	organic matter
OP	Organophosphate
PAGE	Polyacrylamide gel electrophoresis
PP1	protein phosphatase type 1
PP2	protein phosphatase type 2
PTKs	protein tyrosine kinases
PTPs	Protein tyrosine phosphatases
R. abronius	*Rhepoxynius abronius*
RPR-V	2-butenoic acid-3-(diethoxy phosphinothionyl) ethyl ester
SDS	Sodium dodecyl sulphate
sHSP	Small heat shock protein
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TEMED	(N,N,N',N'- tetramethylethylenediamine)
TEPP	(Tetraethlypyrophosphate)

CHAPETR ONE: General introduction

Water pollution is one of the major environmental concerns faced by the world today. Increasing industrialization has led to a rise in pollution. Water pollution can be caused by industrial waste products released into systems such as rivers, lakes and other water bodies. Water pollution can also occur as a result of chemicals leaching out of soils entering water bodies *via* run-off. This may arise from chemicals applied to fields by farmers to improve crop yields and for pest control. Alternatively, contamination may occur when chemicals are released from leaking underground storage tanks, which can result in heavy contamination of soil. Mining and drilling activity disturb the substrata in order to extract useful material. However, this results in spoil tips that may contain hazardous substances and the dumping of contaminated mine water into water bodies (Sharp and Bromley, 1979; Kanu and Achi, 2011).

Water pollutants might include heavy metals, pesticides, hydrocarbons, herbicides, chlorinated hydrocarbons and sewage. This can lead to local extinction of some animal species. Endocrine and reproductive damage have been reported in wildlife as a result of water pollution (Vos *et al*., 2000; Smolders *et al*., 2003). A current concern is the use of the contraceptive pill, which has resulted in contamination of many freshwater systems. This in turn has led to feminisation of freshwater fish and a corresponding reduction in fertility (Tarrant *et al*., 2005). Large scale deaths of aquatic and terrestrial animals, reduced reproductive activity and increased incidence of diseases have been described as effects of water pollution (Nebeker and Puglisi, 1974). Previous ecotoxicological studies have

reported several pollution-induced changes (Gray *et al*., 1979; Gray, 1981).

1.1 Ecotoxicology perspective

The essential goal of aquatic ecotoxicology is to establish how contaminants affect animals at the population level (Watts *et al*., 2002; Sanchez *et al*., 2008). Lethal toxicity testing is typically used to determine the risk potential of a single exposure to a chemical or product *via* oral, dermal or respiratory routes. The determination of lethality has usually relied on the *in vivo* measurement of the median lethal concentration (LC_{50}), which is the concentration of a test chemical that kills 50 % of the organisms in the experimental group. Many researchers have studied the basic toxicity of substances using simple response criteria at the individual level of biological organisation. The favoured method includes investigation of toxicity at the individual level, using survival and many sublethal endpoints (reproduction, growth, behaviour, etc.), which are then used in an effort to predict influences at the highest level of biological organization (Zbinden and Flury-Roversi, 1981; Mayer *et al*., 1986; Blockwell *et al*., 1999).

The use of relatively long-lived macro-invertebrates with complicated life histories (such as amphipod crustaceans) for toxicity testing has been described (Conradi and Depledge, 1999). Previous studies have examined parameters such as age profile, length of reproductive behaviour, sex ratio and fertility for the importance of population effects (Meliyan, 1991; Maund *et al*., 1992; Watts *et al*., 2002). The positive use of amphipods such as *Hyalella azteca, Corophium volutator* and *Gammarus pulex* to measure population level effects is associated with many factors. These include their ecological significance, sensitivity to contaminants, relatively short generation

interval and ease of laboratory culture (McCahon and Pascoe, 1988; Norwood *et al*., 1989; Watts *et al*., 2002).

In the Northern hemisphere, the genus *Gammarus* consists of more than 100 brackish, marine and freshwater species. They are frequently present in high abundance and are vital keystone species in aquatic ecosystems (Ashauer *et al*., 2006; Leroy *et al*., 2010; Gerhardt, 2011). Each species has its particular optimal conditions, especially in terms of salinity, incident light availability, temperature fluctuations and food resources. Their adaptability to various habitats contributes to the success of this genus. Thus, gammarids play a dominant role in the functioning of stream communities (Ahwaz, 2010).

Gammarus pulex is common in small, slow flowing lowland streams of Western Europe (McGrath *et al*., 2007). It has a lifespan of up to two years. Adults can achieve a size at maturity of 20 mm, and their population can reach a density of up to 30,000/m² in the field. Males carry the females during pre-copula until the female moults. The male then fertilizes the female's eggs and the pair separate. The female continues to carry the developing eggs in her brood pouch, and even for a time after hatching (McLoughlin *et al.*, 2000; Maltby *et al.*, 2002; Wallace and Estephan, 2004). The development period of the eggs depends on their size and on the water temperature. Small eggs develop faster than large eggs; for example, at 10 °C eggs of 0.41 mm diameter take around 17 days to develop. However, eggs of 0.69 mm diameter take approximately 35 days at 10°C. Hence, gammarids with small eggs can produce more broods each year than other gammarid species producing large eggs (Sutcliffe, 1992). Sheader and Chia (1970) reported that during embryonic development of *G. obtusatus,* which has a relatively small number of large eggs, the mortality was temperature dependent. The mortality was between 16 and 38% at 15-20 °C, and it increased to 63-84% at a lower temperature (9-12 °C). The developed oocyte of *G. duebeni* is approximately 400 µm in

diameter, which is similar to some other species. However, the biggest oocytes, which are around 650 µm in diameter, occur in *G. wilkitzkii*, a large circumpolar arctic marine organism (Steele and Steele, 1975; Sutcliffe, 1992). *G. pulex* is important to study because it is widespread and common throughout European streams and is sensitive to a range of pollutant stresses.

To be able to study the effect of environmental toxins such as organophosphate pesticides on freshwater animals, it is important to select a representative animal possessing the necessary characteristics with which such exposure studies can be conducted. *G. pulex* is an important part of the aquatic ecosystem. It is prey for birds, fish and amphibian species. *G. pulex* feeds on a wide range of food sources including plant material, small invertebrates and even the remains of larger animals. Gammarids also act as shredders, contributing to the aquatic detritus cycle (Kuhn and Streit, 1994, Satapornvanit, 2006 McGrath *et al*., 2007).

In the laboratory, gammarids are easily reared and manipulated (Taylor *et al*., 1994; Cold and Forbes, 2004; Lloyd Mills *et al*., 2006; Adam *et al*., 2009). These features make *G. pulex* an ideal model species for toxicity studies. Consequently, there are many studies in the literature using this organism. For example, Matthiessen *et al.* (1995) found that exposure to low concentrations of carbofuran (a carbamate insecticide) for 24 h decreased the feeding behaviour of *G. pulex*. Blockwell *et al.* (1996) showed that the growth of juvenile *G. pulex* was inhibited after exposure to 40 nM (6.11µg/l) of the insecticide lindane (gamma-hexachlorocyclohexane) for 14 days. Lindane, copper, and 3, 4-dichloroaniline also affected the feeding rate of *G. pulex* (Blockwell *et al*., 1998).

Several studies have used *G. pulex* as a model to investigate the impact of pesticides on acetylcholinesterase (AChE) activity (Crane *et*

al., 1995; Xuereb *et al*., 2007) and this species ~~*G. pulex*~~ has also been used as a model for other toxicity tests such as oxygen consumption (Kedwards *et al*., 1996), reproductive behaviour (Cold and Forbes, 2004) and precopula separation (Girling *et al*., 2000). Data from some of the many studies using G*. pulex* as a model species to investigate the effect of acute toxicity of pesticides are shown in Table 1.1

Table 1.1 LC_{50} of test pesticides for G. pulex

Toxicant	Life stage	LC_{50}	Time (h)	Source
Diazinon	Adult	0.014 µM (4.3 µg/l) 0.019 µM (5.9 µg/l) 0.028 µM (8.5 µg/l)	96 72 48	Ashauer *et al*. 2010
Propiconazole	Adult	13.7 µM (4703 µg/l)	96	Adam *et al*. 2009
Tebuconazole (triazoles fungicides)	Adult	5.3 µM (1643 µg/l)	96	
3-iodo-2-propinyl butyl carbamate	Adult	2.1µM (604 µg/l)	96	
Cypermethrin	Adult	0.0007 µM (0.3 µg/l)	24	Yordanova *et al*. 2009
Cypermethrin	Adult	0.00022 µM (0.1 µg/l)	96	Adam *et al*. 2009
Lindane	2–3 Moults	0.27 µM (0.079 mg/l)	96	Taylor *et al*. 1991
Atrazine	2–3 Moults	69 µM (14.9 mg/l)	96	
3,4-Dichloroaniline	2–3 Moults	0.107 µM (17.4 mg/l)	48	
Esfenvalerate	Adult Adult Adult	0.0008 µM (0.340 µg/l) 0.00034 µM (0.142 µg/l) 0.00031 µM (0.132 µg/l)	24 48 96	Cold and Forbes 2004

1.2 Pesticides

In general, pesticides are any chemicals used to control or kill pests such as fungi, rodents, weeds, bacteria or other pest species in agricultural and urban environments. Pesticides improve crop yields and may help to supply products at lower cost (EPA, 1997). The benefit of pesticides is considerable in developing nations. The cost of pests in these countries is billions of dollars of their national income in farm and post-harvest losses. These losses result in increased malnutrition, which kills millions of children each year. Pesticides are also important in stored goods, as they can extend the storage life of the produce by protecting against pests and diseases. For example, the marketable percentage reached 80-90% of produce by using pesticides by controlling the apple worm in Russia (Cooper and Dobson, 2007).

Pesticides are widely used throughout the world. It has been estimated that around 30 million kg of active pesticide components are applied to lawns each year (Zahm and Blair, 1992). However, only 0.1 % of applied pesticides reach the target pest, with the other 99.9% contributing to environmental pollution (Pimentel, 1995). Around 600 different kinds of pesticides are used in the United States with an annual cost of approximately $10 billion (Pimentel and Greiner, 1997; Pimentel *et al*., 2005). Pesticide applications have reached 2.6 million metric tons in the world, 85% of which is used in agriculture, with the majority applied in developing countries (Wilson and Tisdell, 2001).

The application of pesticides is continuing to increase in several countries. However, they are considered to be one of the major classes of environmental contaminants throughout the world (Pimentel *et al.*, 1992; Al-Saleh, 1994). The increasing use of pesticides is likely to result in residues in foods. This has led to extensive concern over the possible adverse effects of pesticides on human health (Al-Saleh,

1994). Diffuse contamination by pesticides has been documented as a problem for several years in the UK. A small amount of pesticide application in agriculture reach its target species, resulting in soil, water and air contamination. In addition, pesticide spillage into the environment affects local systems (Roast *et al.*, 1999a; Altinok *et al*., 2006). Wilson and Tisdell (2001) indicated that around 3 million people are affected and 200,000 die from the effects of pesticide each year, almost all of them in developing countries. For instance, approximately 1500 people a year died in Sri Lanka from pesticide poisoning between 1986 and 1996 (Wilson and Tisdell, 2001).

Pesticides are designed to kill specific pests. Unfortunately, they also affect non-target organisms (Stark, 2005). Pesticide poisoning can cause major population effects, which may threaten the viability of rare organisms (Isenring, 2010). Bees are very sensitive to pesticides (Pimentel *et al.*, 1992). The mortality of these species is of particular concern to the agricultural industry. The population of bees can be affected by pesticides in two ways. Firstly, several pesticides used to protect the crops are highly toxic to bees. Secondly, by using herbicides to control weeds, there are less attractive plants for the bees to feed on. Bees might be directly poisoned by pesticides so they do not have enough strength to return to their hive. This may lead them to die in the field, or during their flight back (Decourtye *et al.*, 2002; Porrini *et al.*, 2003). Recently, a study in an Italian agricultural area observed that the number of wild bees detected was reduced after repeated application of the insecticide fenitrothion (Brittain *et al.*, 2010). US EPA (2006) has reported that pesticides are extremely toxic to bees and other beneficial insects on an acute contact basis. The honey bee is very important to crops that rely on insect pollinators, although the number of honey bee colonies has reduced by 60% during the past 60 years in the USA. Pesticide exposure (organochlorine, carbamate and organophosphate) has contributed to these honey bee losses (Johnson *et al.*, 2010).

The effect of lindane on non- target arthropods significantly decreased the number of spiders to 53% and ants to 64 % of the initial density in trials lasting around 3 weeks (Wiktelius *et al.*, 1999). Similarly, the numbers of Entognathous collembola significantly decreased by over 80% for an average of 6 weeks (Wiktelius *et al.*, 1999). The application of pesticides in agriculture can cause a decline in the abundance of insects and weeds, which are essential food sources for different organisms (Boatman *et al.*, 2004; Isenring, 2010). Organophosphate pesticides (OPs) such as chlorpyrifos, diazinon and malathion were identified as a cause of fatal bird poisoning (Isenring, 2010). In the United States around 672 million birds are exposed to pesticides each year from agricultural use alone. Around 10 % of these birds die, with 700 Atlantic Brent Geese killed after diazinon was applied on only three golf courses (Pimentel *et al*., 1992). The number of farmland birds in Western Europe is now only half that of 1980 (Isenring, 2010). Whilst this cannot be ascribed purely to pesticide use, as other roles such as loss of habitat may be involved, there is a strong link with pesticide application rates. Similarly, in Europe the average population of all common and forest birds was reduced by approximately 10% between 1980 and 2006. In the UK at least 60 birds in 2006 and 55 birds in 2007 were directly killed by pesticides (Isenring, 2010).

Pesticides can be transferred to the aquatic environment from a variety of sources such as industry, crop spraying and transport *via* surface water (Crane *et al*., 1999; Hoffman *et al*., 2000). Whilst the level of environmental contamination might be low, it can still have a significant effect on organisms such as fish and invertebrates. Although pesticides may not directly kill non-target organisms, they could reduce their fitness. The common contaminants in urban streams include chlorpyrifos and diazinon. These are highly toxic to aquatic animals and have affected many species that live in or near treated fields (Rush *et al*., 2010).

In the aquatic environment, pesticides affect fisheries in different ways. High pesticide levels in the aquatic environments directly kill organisms, whilst low concentrations kill highly sensitive species (Pimentel *et al.*, 1992). Naqvi and Vaishnavi (1993) indicated that, as a result of pesticide pollution, there were more than 50 incidences of fish kills. In addition, these authors suggested that fish might be more than 1000 times more sensitive than worms and snails. The EC_{50} values for *Daphnia magna* can be used as an upper threshold for fish tests, since daphnids are more sensitive to toxicants than fish (Zvinavashe *et al*., 2009). However, gammarids are more sensitive to different kinds of organophosphates such as azinphos, dichlorphos, dimethoate, parathion and phorate compared with *D. magna* (Gerhardt, 2011).

1.3 Organophosphate pesticides

Organophosphate (OP) pesticides were first produced in 1937 by German chemists. Tetraethlypyrophosphate (TEPP) was the first commercial OP insecticide used in the world. OP insecticides can be divided into two main forms; one group is organophosphates which are oxon (P=O) containing and the second group is organothiophosphates which are P=S containing (Ballesteros and Parrado, 2004; Johnstone, 2006). They are heavily used as they control pests, but are rapidly degraded and less likely to accumulate in the environment. However, OP insecticides are still of great concern in water pollution, as they are highly soluble in water and frequently used (Berger and Sultatos *et al*., 1997; Sparling and Fellers, 2007; Montagna and Collins, 2008). OP pesticides are replacements for organochlorine pesticides, because of their rapid decomposition and are less likely to accumulate in the environment (Ballesteros and Parrado, 2004). OP pesticides have been widely used against a broad spectrum of pests in agriculture and urban settings to control insects on food crops, plants, lawns, commercial buildings and to control mosquitoes (Huang and Huang, 2011). OPs

are the most heavily used class of pesticides (Hargreaves *et al.*, 2011). The most common pesticides found in the seawater were OPs such as diazinon, IBP (s-benzyl diisopropyl phosphorothionate) and fenitrothion (Kunio, 1984). Approximately 70% of the total pesticides used in the USA were OPs in the year 2001 (Singh *et al.,* 2009). Farmers in the UK extensively use OPs in sheep dips to control ectoparasites and around 200 million litres of spent sheep dip is produced every year. Spent sheep dip may be transferred to watercourses, where it can affect aquatic organisms (Jadhav and Rajini, 2009; Rush *et al.*, 2010). Data for some of the many OPs used in the world are shown in Table 1.2.

Table 1.2 Some of the common organophosphate pesticides

Pesticides (year introduced)	Use	Bird oral LD_{50} (mg/kg)	Fish LC_{50}	Rat oral LD_{50} (mg/kg)	Rabbit dermal LD_{50} (mg/kg)
Acephate 1971	To control of a wide range of biting and sucking insects, especially aphids	51 – 500	>100 ppm (>0.55 µM)	1,030–1,447	>10,250
Chlorpyrifos 1965	To control cutworms, corn rootworms, cockroaches, grubs	10 – 50	0.1 – 1 ppm (0.28-2.8 µM)	96 – 270	2,000
Diazinon 1950	Rice, fruit trees, sugarcane, corn, potatoes and to control lice in sheep. Used to control sucking and chewing insects of fruit trees and a wide range of crops including coffee, tea and cocoa plants	10-50	0.1-1 ppm (0.23-3.3 µM)	1,250	2,020
Malathion 1965	Fruits and vegetables, and is also used to control mosquitoes and fleas and ants. Used to control spider mites, leafhoppers and scale insects	51– 500	0.1– 1 ppm (0.3-3 µM)	5,500	>2,000
Methyl Parathion 1950	To control chewing and sucking insects in a wide range of crops	Not available	10 – 100 (0.34-343 µM)	6	45
Phosmet 1966	It is mainly used on apple trees and to control fleas, lice and hornflies. Used to control Codlin moth on apple trees	501–2,000	0.1 – 1 (0.32-320 µM)	147 – 316	>4,640

(Smith, 1987; Fishel, 2005; US EPA, 2006)

The contamination of aquatic environments by pesticides can cause acute toxicity to aquatic organisms. Pollution of aquatic ecosystems by OPs can lead to mass mortality of aquatic animals (Singh *et al.,* 2009). Several studies have investigated the acute toxicity of various pesticides on aquatic fauna. The mortality of the common carp *Cyprinus carpio*was was 100 % after exposure to 9.15 µM (2.1 mg/l) dimethoate for 96 h (Singh *et al.*, 2009), and the 96 h LC_{50} for nuvan

was 29.4 µM (6.5 mg/l) in the freshwater fish *Ctenopharyngodon idella* (Tilak and Kumari, 2009). The OP fonofos was highly toxic to three freshwater organisms, the 48 h LC_{50} was 10.96 and 158.3 nM (2.7 and 39 µg/L) for *Daphnia magna* and *Chironomous ripariusa*t, respectively, whilst the 96 h LC_{50} was 21.5 nM (5.3 µg/L) for the freshwater fish bluegill *Lepomis macrochirus* (Fairchild *et al.*, 1992). Roast *et al.* (1999b) found that the 96 h LC_{50} of a chlorpyrifos was 370.8 pM (0.13 µg/l) and was more toxic to *Neomysis integer* (crustacean: mysidacea) than dimethoate (96 h LC_{50} of 2.4 µM (540 µg/l).

1.4 Diazinon

Diazinon (O,O-Diethyl O-(2-isopropyl-6-methyl-4-pyrimidinyl), formula $C_{12}H_{21}N_2O_3PS$,is an organophosphorothioate pesticide. It is an acaricide developed in the 1950s (Moore and Waring, 1996; Burkepile *et al*., 2000; Üner *et al.*, 2006; Kretschmann *et al*., 2011b). It is commonly used to control insects in soil, plants, fruit and vegetable crops in agricultural and urban environments (Hamm *et al*., 2001; Jemec *et al*., 2007b). For example Central Valley in California, around 320 kg of diazinon was applied each year during 1992-1994 (Giddings *et al.*, 2002). It is also still extensively used in sheep dip to control ectoparasites (Boucard *et al.,* 2004; Gaworecki and Klaine, 2008; Jadhav and Rajini, 2009; Jemec *et al*., 2007b). Whilst diazinon has been banned as a crop pesticide in the UK, it is the only product licensed for use as a sheep dip in the UK (Veterinary Medicines Directorate, 2006). Diazinon can rapidly dissolve in aromatic solvents, aliphatic alcohols, and ketones. It is also soluble in water to 40 mg/l. Diazinon degrades quickly in plants with a half-time of persistence of around 14 days (Eisler, 1986). However, this period rises with low temperature and can be longer in crops with high oil content (Meier *et al.*, 1979; Jarvinen and Tanner, 1982). In soils, diazinon may be biologically stable for more than 6 months under conditions of low temperature, moisture and high alkalinity (Meier *et al*., 1979; Eisler,

1986). Due to the health and ecological hazards of diazinon, the US EPA phased out all residential applications of diazinon as of December 2004 (Whyatt *et al.*, 2005). Diazinon as an OP pesticide inhibits the enzyme AChE (Adedeji *et al*., 2010; Kretschmann *et al*., 2011b). Diazinon is comparatively water-soluble, and often comes into contact with aquatic animals. 2-isopropyl-6-methyl-4-pyrimidinol (IMP), diethyl phosphate (DEP) and diethyl thiophosphate (DETP) can be found in the environment due to the break down of diazinon (Morgan *et al*., 2010). Once inside these animals diazinon is metabolized to diazinon oxon, which is the acutely toxic form of this insecticide. Diazinon oxon in turn inactivates AChE (Girón-Pérez *et al.,* 2008). The structures of diazinon and diazinon oxon are shown in Figures 1.1. However, the commercial diazinon formulation contains 60% diazinon and the remaining 40% comprises excipient and trace impurities.

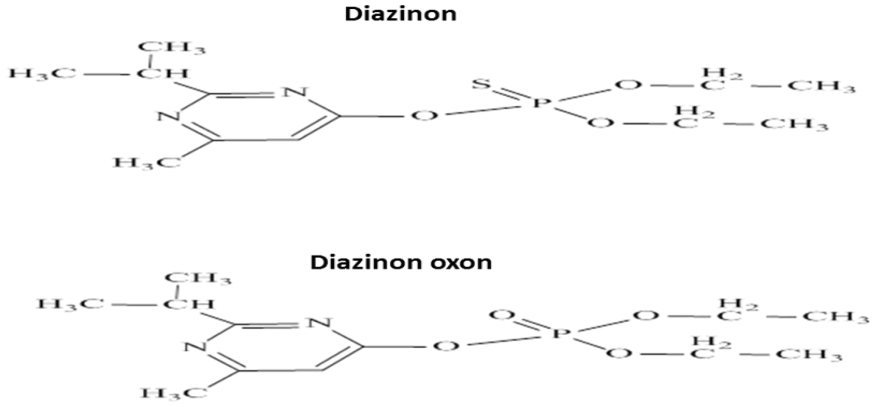


Figure 1.1 Structure of diazinon and diazinon oxon

(US EPA, 2006)

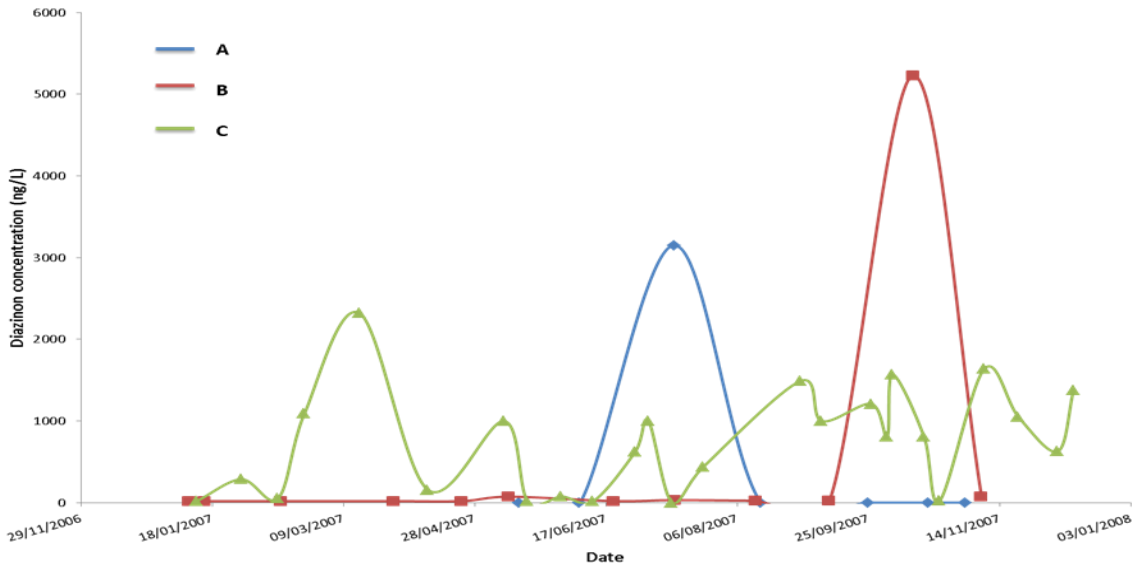
The extensive use of diazinon in some regions has led to contamination of water bodies, resulting in a significant environmental impact on aquatic fauna. Diazinon might become more toxic with storage due to transformation products. Jarvinen and Tanner (1982) reported that some diazinon formulations comprise 0.2 to 0.7% sulfotep (tetraethyl

dithiopyrophosphate) as an industrial impurity. It has been reported that sulfotep is at least 100 times more toxic than diazinon itself to some animals. Monosulfotep was 14,000 times more toxic than diazinon in a test of enzyme inhibition (US EPA, 2005). Sulfotep was observed to be more toxic than diazinon on different organisms such as the cladoceran *Daphina magna,* fathead minnows *Pimephales promelas* and bluegill *Lepomis macrochirus* (Meier *et al*., 1979; US EPA, 2005).

Several studies have detected diazinon in freshwater (Domagalski *et al.*, 1997; McConnell *et al.*, 1998; Bailey *et al.*, 2000). Kawai *et al*. (1984) observed that OP pesticides such as diazinon were found in almost all samples of seawater in Osaka city. For example, the concentration of diazinon was greater than 328.6 pM (0.1 µg/L) in the Osaka city harbour after diazinon was applied to rice paddy fields from June to August. Diazinon is also found in point source (wastewater treatment plant effluents) discharges (Villarosa *et al.*, 1994). Rainfall in the Sacramento–San Joaquin basin in the winter of 1992-1993 contained up to 0.006 pM (1.9 ng/l) diazinon and up to 0.7 pM (220 ng/l) diazinon oxon (Giddings *et al.*, 2002). Diazinon was also found in storm water runoff in both agricultural and urban zones (Domagalski *et al.*, 1997 and US EPA, 2005). The WHO (1998) indicated that the mobility of diazinon in soil was affected by the organic matter (OM) and carbonate levels of the soil. In soils, diazinon is only a slightly mobile with < 2% OM, but effectively immobile in those with > 2% OM (US EPA, 2005).

In 2005, over a third of all Environmental Quality Standards (EQS) failures in England and Wales in freshwater were attributed to sheep dips, and the main use of diazinon in the UK as the active element in sheep dips. Therefore, limitation of the level of aquatic contamination related with diazinon use is very important. Entry of diazinon into water will include spillage, disposal and run-off from treated

organisms. Recent strategies for the disposal of used sheep dip include spreading of diluted used sheep dip on to land. Evidently, the major source of water pollution will be from the soil, if such guidelines for use are followed, because there is a significant level of diazinon flowing into the soil (Fenlon *et al*., 2011; Kanda and Glendinning, 2011). In Scotland, diazinon was found in each of the 20 catchments sampled by the Tweed River Purification Board in 1990, with concentrations of 14-124 ng/L (46- 407.4 pM). In Wales, 75 % of the 107 water quality sampling sites revealed the presence of sheep dip insecticides; diazinon was present at 52 % of the sites in 1998 (Boucard *et al*., 2004). The time series data from three sites of diazinon from various locations in the UK are shown in Figure 1.2.



Environment Agency organics database (2007)

Figure 1.2 Time series data for diazinon from 3 sites in the UK. Location A in Meddanen Llanfechell Sewage Treated Water, Eastings 237747, Northings 392014. Location B in Huddersfield Colne Bridge Sewage Treated Water, Eastings 417559 Northings 420062. Location C

in Buckfastleigh (Kilbury) Effluent, Sewage Treated Water Eastings 274900, Northings 65900.

Although diazinon release into the environment tends to be episodic, uptake into animals may maintain cellular exposure for some time after the initial environmental pulse has subsided. Diazinon concentration in animals rapidly increased and reached a maximum after exposure of shrimp, leech, rainbow trout and carp to water containing 0.02 mg/l (66 nM) diazinon for 72 h, with food chain bioconcentration factors (BCF) of 3,26,63 and 120 for these organisms respectively (Seguchi and Asaka, 1981). The average of 7 days BCF values for diazinon were 20.9 in muscle, 32.2 in gall bladder, 60 in liver and 111 in kidney in the carp. The elimination rates for diazinon were 0.001-0.02 for liver, 0.0004-0.004 for kidney, 0.002-0.023 for gall bladder and 0.002-0.024 ng/g per h for muscle in the carp *Cyprinus carpio* (Tsuda *et al*., 1989). The BCF values were 775 in muscle and 1859 in liver of the European eel *Anguilla anguilla,* after being exposed to 137.99 nM (0.042 mg/l) diazinon for 96 h (Sancho *et al*., 1992). Hence, diazinon tends to bioaccumulate, with a higher uptake than elimination rate.

Diazinon is also toxic to fish. Aydın and Köprücü (2005) observed that its toxicity to the common carp *Cyprinus carpio* significantly increased with rising exposure time. The LC_{50} values for 24, 48, 72 and 96 h being 3.7, 2.9, 2.4, and 1.5 mg/l, respectively. Köprücü *et al*. (2006) demonstrated that the mortality of the catfish (*Silurus glanis*) significantly increased after exposure to 2 mg/l (6.6 mM) diazinon. Erythrocyte, leukocyte, haemoglobin and haematocrit counts were also significantly lower than those of control animals. The toxicity of diazinon towards freshwater fish was between moderately toxic to very highly toxic, with the 96 h LC_{50} ranging from 295.4 to 25.6 µM (90 to 7.8 ppb), whilst the 96 h LC_{50} for estuarine and marine invertebrates ranged from 13.8 µM to more than 3.3 mM (4.2 to 1000 ppb).

Gammarids are thought to be very sensitive to diazinon; the 96 h LC_{50} was 7 nM in *G. fasciatus* (Johnson and Finley, 1980), and 0.54 pM (165 ng/l) in *G. pseudolimnaeus* (Hall and Anderson, 2005). Arthur *et al.* (1983) assessed the toxicity of diazinon on macroinvertebrates for three outdoor experimental channels, two for low and high exposures and one as control. Results showed that the amphipod *Hyalella* was a more sensitive species than the amphipod *Crangonyx.* The population of *Hyalella* was sharply reduced at diazinon concentrations as low as 16.4 nM (5 µg/l). US EPA (2005) reported that the toxicity of diazinon to freshwater organisms had been estimated for different invertebrate animals and toxicity values ranged from 821.4 pM (0.25 µg/l) for the cladoceran *Ceriodaphnia dubia* to 38.2 nM (11.6 µg/L) for the planarian, *Dugesia tigrina.*

1.5 Sub-lethal indicators of toxicity

Sublethal toxicity may be a more suitable indicator of ecotoxicological risk than lethality toxicity, since mortality does not always occur in species exposed to stress under natural conditions. Behavioural parameters are useful to determine the potential influence of contaminants on non-target organisms. Behaviour is of ecological importance, as any disturbance of normal activity may interfere with movement, survival, feeding and reproduction, thus having the potential to impact at the population level (Roast *et al*., 1999b; Wallace and Estephan, 2004). Behavioural experiments are particularly useful since these endpoints are easy to measure. They are relevant to ecology. Behavioural endpoints can also link toxicity at the biochemical/cellular level to influences on populations. Therefore, they are considered suitable indicators of sublethal concentrations in the laboratory and in field studies (Villarroel *et al*., 1999; Roast *et al.*, 2001; Wallace and Estephan, 2004; Tu *et al*., 2010). Some behavioural activities could be more sensitive to chemical exposure than others. For instance, grass shrimp *Palaemonetes pugio* from

contaminated environments did not show effects with respect to predator avoidance, but reduced prey capture has been detected (Wallacea and Estephan, 1994; Perez and Wallace, 2004).

Exposure to pesticides such as diazinon may harm the ecological fitness of animals by changing behaviours such as reproduction, avoiding predators and movement. A decrease in individual fitness at the individual level, whilst not directly killing the organism could eventually affect the population. Studying behavioural alterations in animals exposed to pollutants could allow researchers to better understand the effect of biochemical changes as well as interpret potential consequences at the community level (Weis *et al.*, 2001; Gaworecki *et al.*, 2009). Different behavioural activities (movement, feeding and reproductive endpoints) have been investigated using a variety of techniques. In general, these are high sensitivity techniques with different toxicants and organism models including invertebrates, fish, and amphibians.

1.5.1 Movement behaviour

Movement is a typical feature of many species ~~and~~ as part of their natural behaviour, and is necessary for their survival. Alteration of movement of aquatic animals can be a useful indicator to help determine ecotoxicological risk. Several movement behaviours have been used to measure the effects of pollutants on various aquatic test organisms. Movement indices used include vertical movement, distance travelled, frequency of direction change, etc. Swimming speed is a common behaviour used as a surrogate of the physiological condition of the aquatic animal (Wall, 2000; Tahedl and Häder, 2001; Faimali *et al.*, 2006). A sublethal pollution-induced change in activity may have ecological consequences. Increased swimming activity may risk improved visibility to predators (Weis and Weis, 1995). Food searching patterns could be affected by inhibiting swimming activity or

decline in prey capture, which in turn may lead to decreased growth. Suboptimal growth rate can extend to time spent in a particular life stage more making individuals susceptible to predation (Kraus and Kraus, 1986; Weis and Weis, 1995).

In some studies, video tracking has been used to automatically detect the movement responses of organisms (Faimali *et al.*, 2006). A number of studies have recommended the measurement of phototactic behaviour to detect the effect of sublethal concentrations of other stresses on different organisms such as *Daphnia magna* and *Gammarus* (Hunte and Myers, 1984; Michels *et al.*, 1999). Phototactic behaviour is a highly heritable characteristic under standardized factors. However, it can be modified by environmental factors (Michels *et al.*, 2000).

Alterations in phototactic behaviour were observed in *G. lawrencinus* *G. lawrencinus*, *G. tigrinus* and *G. mucronatus* after exposure to water of salinity 16% (Hunte and Myers, 1994). Their result indicated that phototaxis is an evolutionary adaptation to minimise cannibalism by adults. Phototactic behaviour of *D. magna* significantly $_{by}$ increased when exposed to 1.6 mM (375 µg/l) dimethoate for 15 min. In contrast, this behaviour significantly reduced after exposure to 752.2 µM (218.8 µg/l) lindane for the same ~~exposure~~ period (Martins *et al*., 2007).

Other swimming behaviour changes have been observed. For example, vertical swimming of *G. lawrencinus* is more sensitive to cadmium than horizontal swimming (Wallace and Estephan, 2004).

Wall (2000) indicated that the effect of diazinon on swimming speeds of adult zebrafish (*Brachydanio rerio*) was dependent on the concentration of diazinon. Kwak *et al.* (2002) studied the rates of two types of movement, the shaking and smooth patterns of medaka

Oryzias latipes fish in scoto-phases (14 h) and photophase (10 h) before and after exposure to 328.5 nM (0.1 mg/l) diazinon over 2 days. The shaking pattern significantly increased, whilst the smooth movement pattern decreased after the exposure to pesticide. Roast *et al.* (2000) observed that chlorpyrifos affected the swimming movement of the hyperbenthic mysid *Neomysis integer.* The swimming activity increased when exposed to 108.4 nM (0.04 mg/l) chlorpyrifos, which is less than half the 7 days LC_{50}. The vertical distribution of this organism was also disturbed when compared to control mysids. This could impact on the organisms by altering the changes of predator contact and encounters into food items.

A sub-lethal chlorpyrifos concentration of 171 µM (60 mg/l: 20% of 7 days LC_{50}) decreased the locomotory behaviour of the mosquito fish *Gambusia affinis.* It reduced both distance travelled and swimming speed (Rao *et al.*, 2005). The duration of movement of zebrafish embryos was significantly reduced after being exposed to 513.4 nM (0.18 mg/l) chlorpyrifos (Selderslaghs *et al.,* 2010). The mean velocity of *Daphnia magna* was significantly higher after exposure to paraoxon-methyl for 24 h at 2.8 µM (0.7 mg/l) compared with the control. Following a 24 h recovery period, the velocity of previously exposed *D. magna* declined to the control level (Duquesne and Küster, 2010).

1.5.2 Feeding behaviour

One important behavioural endpoint is the feeding response, as any decrease in feeding rate may lead to significant reductions in growth rate and reproductive fitness. Feeding endpoint is a good indicator of common stress, being sensitive to environmental variation (Chaton *et al*., 2003). Feeding behaviour is a complex phenomenon that includes several responses related to food consumption. It includes modes of feeding and feeding habits, mechanisms of food detection, frequency

of feeding and food predilections (Maltby, 1999; Rinderhagen *et al*., 2000; Volkoff and Peter*,* 2006; Pestana *et al*., 2007).

Pesticides or other stressors may decrease the feeding activity due to the effect of these stresses on the senses (Satapornvanit, 2006). Observed decreases in feeding behaviour may be used to predict influences on individual growth and reproductive performance, which could be incorporated into population models in order to estimate influences on population dynamics (Williams, 2005). The inhibition of feeding activity of *G. pulex in situ* was linked with a decrease in the level of leaf decomposition and the diversity of the macro-invertebrate community (Maltby *et al*., 2002).

Feeding rate can be established by measuring food consumption and body weight change and several studies have determined the effects of sub-lethal contaminant concentrations on the feeding rate of aquatic animals (Matthiessen *et al.*, 1995; Mcloughlin *et al.*, 2000; Xuereb *et al.,* 2009). All these studies observed that feeding activity was a useful indicator to measure the effect of pesticides on amphipod behaviour, being a general and sensitive endpoint of chemical exposure.

The inhibition of feeding activity of *G. pulex* was between 30 and 70% of control after exposure of this organism to several concentrations of zinc, linear alkylbenzene sulphonate, lindane, pirimiphos-methyl or permethrin. Feeding in *G. pulex* was a more sensitive indicator of permethrin exposure than biochemical biomarkers such as glutathione *S*-transferase (GST) activity and cholinesterase (ChE) and (McLaughlin *et al*., 2000). However, feeding activity may be associated with different activities. Indeed, Heckmann *et al*. (2005) found that a reduction in AChE activity impaired both feeding and movement behaviour. The reduction of feeding rate of organisms may be dependent on the toxin and exposure period. For example, 2.9 nM chlorpyrifos did not alter this behaviour in *G. fossarum* after exposure

for 48 h, whilst it was significantly impaired after 96 h. However, there was no effect on the feeding behaviour of *G. pulex* after exposure to the OP insecticide Malathion 60 (Xuereb *et al.,* 2009; Crane *et al.,* 1995).

The feeding rate of the freshwater shrimp, *Macrobrachium nipponense*, when treated with 30.3 nM (0.01 mg/l) malathion was 82.5 % of control animals on the first day, but only 49.2 % after day 7 (Yuan *et al*., 2004). In addition, Satapornvanit (2006) examined feeding behaviour of the prawn *Macrobrachium rosenbergii* after exposure to the OP pesticides chlorpyrifos and dimethoate. Chlorpyrifos significantly inhibited feeding rates at 1.78 nM (0.625 µg/l) by 77.7 % after 24 h, whilst dimethoate did not significantly affect (50.4%) this behaviour at 1.4 µM (312.5 µg/l) for the same exposure period. Fernandez-Casalderrey *et al*. (1994) demonstrated that the feeding rate decreased in *D. magna* when exposed to as little as 1.5 nM (0.5 µg/l) diazinon.

1.6 Molecular markers of toxicity in aquatic organisms

In order to understand the adverse impacts of contamination detected at the higher level of biological organization, it is essential to develop assays at the molecular level. The changes at the molecular and biochemical levels are, in essence, a measure of an animal's attempt to maintain a homeostatic internal environment, and could therefore be observed as biological indicators of toxicity exposure (Rani and Sivaraj, 2010).

1.6.1 Acetylcholinesterase

Cholinesterase activity is usually divided into two parts: acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). They are distinguished mostly on the basis of their substrate specificity

(García-de la Parra *et al.*, 2006). BChE is an enzyme that contributes to cholinesterase activity and is found in many types of tissues. It is held to be involved in ~~the~~ lipid metabolism as the serum BChE has been found to be significantly related to triglyceride levels. Studies on organisms have shown that BChE may be a key in the regulation of lipoprotein metabolism (Scacchi *et al*., 2011). AChE is an enzyme that is responsible for the break down of acetylcholine in the synaptic cleft of the nervous system (Day and Scott, 1990; Ibrahim *et al*., 1998). AChE is commonly used as a biomarker to monitor environmental contamination, as it is very sensitive to certain chemical compounds (Day and Scott, 1990; Key, 1995; Mora *et al*., 1999; Dembélé *et al*., 2000; Roex *et al*., 2003; Adedeji, 2011).

OP and carbamate (CB) pesticides are commonly used to control insects, as they are highly toxic, have a short-term environmental persistence and low accumulation in organisms. The main action of OP and CB pesticides is to inhibit AChE in exposed organisms (Keizer *et al*., 1995; Xuereb *et al*., 2009). Acetylcholine (ACh) is a neurotransmitter involved in controlling contraction of muscles, amongst other roles. After secretion of ACh into the synaptic cleft, AChE rapidly destroys it. The inhibition of AChE leads to the accumulation of ACh and over stimulation of nerves and muscles, causing weakness, convulsion and in the end respiratory failure and death (Fukuto, 1990; Printes and Callaghan, 2003; Johnstone, 2006; Xuereb *et al.,* 2007; Kretschmann *et al*., 2011a). Roex *et al.* (2003) suggested a strong correlation between AChE inhibition and other behavioural responses. For example, inhibition of AChE activity of the mysid *Neomysis integer* by OP pesticides affected swimming behaviour (Barron and Woodburn, 1995; Roast *et al.*, 2001).

The effect of substances on AChE actually can be determined *via in vivo* and *in vitro* assays. Therefore, it is important to measure these before the use of AChE activity is suggested as a sublethal inductor in

aquatic bioassays. Especially, the response to OP and carbamate pesticides has to be established if AChE reduction is to have a diagnostic value (Ibrahim *et al*., 1998).

Crustaceans are more sensitive to anti-cholinesterase compounds than vertebrate species (Xuereb *et al.,* 2007). Many studies have used *Gammarus* as a model species to determine the effect of OP on AChE activity (Kuhn and Streit, 1994; Xuereb *et al.,* 2007). For example, the AChE activity of *G. pulex* was significantly inhibited after exposure to 2.9 nM chlorpyrifos for 96 h (IC_{50}, 0.99 nM), whilst exposure to 3.8 nM (1 µg/l) parathion-methyl significantly inhibited (35-39 % inhibition) AChE activity after only 24 h exposure time. However, AChE activity in *G. tigrinus* was not significantly altered by less than 10.8 nM (3 µg/l) fenitrothion (Kuhn and Streit., 1994; Xuereb *et al.,* 2007). The AChE activity in whole body extracts of *G. fossarum* was inhibited after exposure to 0.7 and 1.4 nM chlorpyrifos for 96 h and the activities were 19.9 % and 53.2 %, with 96 h IC_{50} being 1.5 nM (Xuereb *et al*., 2009). Similarly, the AChE of *G. pulex* was significantly reduced after exposure to 6.3 and 2.5 nM (1.9 and 0.8 µg/l) pirimiphos-methyl following 24 and 48 h exposure, respectively. However, the activity of AChE in *G. pulex* was not changed by exposure to 1.5 and 76.7 nM (0.6 and 30 µg/l) permethrin and lindane, respectively (Mcloughlin *et al.,* 2000). At 5.9 µM (0.8 mg/L), methamidophos significantly inhibited AChE activity of the white shrimp *Litopenaeus vannamei* after exposure for 24 h (García-de La Parra *et al*., 2006). However, exposure to only 285.2 pM (0.1 µg/l) chlorpyrifos, 99.4 nM (20 µg/l) carbaryl and 8.7 µM (2000 µg/l) dimethoate led to 80-85% inhibition of AChE activity in the freshwater shrimp *Paratya australiensis* (Decapoda: Atyidae) after exposure for 96 h (Kumar *et al.*, 2010). By contrast, Jemec *et al*. (2007b) found no change in AChE activity of *Daphnia magna* exposure to 23 nM (7 µg/l) diazinon for 48 h.

AChE activity has also been used as a biomarker to investigate the effect of OP pesticides in fish. However, significant AChE reduction in fish can only be observed at relatively high concentrations, because fish are able to detoxify these compounds more easily than invertebrates, which makes invertebrates more suitable than fish. Edwards and Fisher 1991; Ibrahim *et al*., 1998).

Diazinon normally disappears from water and aquatic organisms within a few days (Adedeji, 2011). Despite this, diazinon can have harmful effects on the nervous system through a variety of mechanisms. The primary action of diazinon is the inhibition of AChE, this may lead to the death of the organism (Oh *et al*., 1991; Adedeji, 2011). Numerous studies have attempted to assess the action of diazinon on aquatic organisms (Pan and Dutta, 1998; Üner *et al.*, 2006; Jemec *et al*., 2007b; Gaworecki *et al.*, 2009; Kretschmann *et al*., 2011a). For example, diazinon caused significant inhibition of AChE activity for hybrid striped bass *Morone saxatilis x M. chrysops* (66 % after 72 h), *Oreochromis niloticus* (93 % after 168 h) and juvenile largemouth bass *Micropterus salmoides* (53 % after 24 h) (Pan and Dutta, 1998; Üner *et al*., 2006; Gaworecki *et al.,* 2009).

In vitro studies indicated that brain AChE activity of four species was very sensitive to diazinon oxon, with IC_{50} values of 0.2, 2.5, 7.5 and 20 µM for *Cyprinus carpio*, *Oncorhynchus mykiss*, *Poecilia reticulate* and *Brachydanio rerio* respectively (Keizer *et al*., 1995). Diazinon significantly inhibited AChE in different tissues, such as the alimentary tract, kidney, gill and muscle of the freshwater fish *Oreochromis niloticus* (Durmaz *et al.*, 2006).

The above studies suggest that the inhibition of AChE can be used as a bioindicator to investigate the relationship between AChE activity and ecologically relevant factors such as survival and behaviour. The relationship between ACh decrease and its following effects on the

fitness of organisms may thus offer a key for predicting ecotoxicological disadvantages of OPs (Kumar *et al*., 2010). Some studies have observed that significant inhibition of AChE activity was interrelated with influences on behaviour, which might harm subsequent survival of the exposed animals (Kumar and Chapman, 1998 and Kumar *et al*., 2010). Studies by Kuhn and Streit (1994) and Crane *et al*. (1995) observed that cholinesterase inhibition in *G. pulex* is a suitable bioassay for OPs in both the field and laboratory. Therefore, it was deemed important to investigate whether diazinon, diazinon oxon and the commercial diazinon formulation could affect AChE activity.

1.6.2 Heat shock proteins

Since organisms are frequently exposed to environmental pollution and other environmental stresses, they have mechanisms to help cope with these stresses. Heat shock proteins (HSPs) are a group of intracellular proteins found in all organisms from bacteria to mammals. Discovery of the heat shock response is usually attributed to Ferruccio Ritossa who reported chromosomal puffs in the salivary gland cells of the fruit fly *Drosophila busckii* following a heat shock (Ananthan *et al*., 1986; Meinhardt *et al*., 1995; Gupta *et al*., 2010). Stress effects leading to certain phenotypes have been reported since the mid-1930s. However the discovery of HSPs has led to research interests in this group of proteins (Mitchell and Peterson, 1982; Gupta *et al*., 2010).

The finding that specific HSPs are expressed from genes in the chromosome puffs came in the late 1970s. Tissiéres *et al*., (1974) noted that heat shock induced puffs in the *Drosophila* salivary gland chromosomes, which occurred within minutes of the heat shock and that this was accompanied by the expression of some novel proteins. It was demonstrated that purified mRNA, which hybridized to the heat

shock puffs, was translated into specific HSPs when added to an *in vitro* system (McKenzie *et al.*, 1975; Spradling *et al.*, 1975).

Pollution stresses induce a suite of intracellular responses such as HSPs, which are readily induced under a variety of stress conditions. HSPs are a group of highly conserved proteins that play vital roles in protein folding (Iwama *et al*., 1998; Eder *et al.,* 2004; Lee *et al.,* 2006; Planelló *et al*., 2010). HSPs can enhance survival by protecting vital cellular functions when they are induced in cells following to exposure of organisms to a variety of stressors (Paranko *et al*., 1996; Iwama *et al.*, 1998).

HSPs are classified according to their molecular weights in kilodaltons (kDa) as determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Planelló *et al.*, 2010; Lee *et al.*, 2006). The most common groups of HSPs are HSP 100, HSP 90, HSP 70, HSP 60 and the small HSPs (sHSP) 12-43 kDa. However, the molecular weight of sHSP in the amphipods *G. lacustris* and *G. pulex* was in the range 35-37 kDa (Protopopova *et al*., 2011). HSPs are present in different subcellular fractions such as the cytosol, mitochondria, endoplasmic reticulum and the nucleus (Sanders, 1993; Kiang and Tsokos, 1998; Iwama *et al.*, 1998; Plesofsky and Brambl, 2002; Malinovska, 2012). These proteins have been used as biomarkers of pollution induced stress. Increased expression of these proteins can be induced after exposure to some environmental stressors such as pesticides, temperature and heavy metals (Clayton *et al*., 2000; Köhler *et al*., 2001; Hallare *et al*., 2004).

Thus, HSPs are recognized as a family of stress proteins, which are induced in response to a range of stressors in animals (Shashikumar and Rajini, 2010). Increased levels of HSPs can be related to a decrease in individual fitness (Sørensen *et al*., 2003).

HSPs have been widely used as biomarkers to study the effect of pesticides on aquatic organisms in studies using whole animals, cell lines and primary cultures (Iwama *et al.*, 1998). Werner and Nagel (1997) investigated the effects of diazinon on three species of amphipods. They found that HSP 71 (HSP 70 protein family) and HSP 58 and 64 (HSP 60 protein family) were significantly increased in *Hyalella azteca* and *Rhepoxynius abronius* when exposed to 98.6 nM (30 µg/l) diazinon for 24 h. However, in the pellet fraction (7,000 g for 30 min at 4 ^{0}C) of *Ampelisca abdita*, HSP71 showed no response, whereas HSP 58 and 64 were significantly elevated at the same concentration of diazinon and exposure period. The level of HSP 70 of zebrafish increased after being exposed to diazinon, 3, 4 dichloroaniline and chlorpyrifos (Scheil *et al*., 2009; Scheil *et al.*, 2010). In addition, the level of HSP 70 and HSP 90 in muscle and gill tissue significantly increased after juvenile Chinook salmon *Oncorhynchus tshawytscha* were exposed to chlorpyrifos, whilst HSP 60 expression was not affected. Exposure of *Oncorhynchus tshawytscha* to chlorpyrifos caused a significant increase of HSPs in liver, with a three fold increase in HSP 60 and two fold for HSP 70 and HSP 90 (Eder *et al*., 2009). However, the expression of HSP 70 in gilthead sea bream *Sparus aurata* after acute exposure to dichlorvos for 24 h was not significantly different to the control (Varó *et al*., 2007). Thus, it was essential to evaluate the effect of diazinon, diazinon oxon and the commercial diazinon formulation on HSPs of *G. pulex* used as biomarkers in this thesis.

1.6.3 Protein phosphorylation

Proteins are intimately involved in vital cellular processes, including signal transduction, cell cycle control, transcriptional and translational regulation, membrane fusion and transport (Bykova *et al*., 2003; Iakoucheva *et al.*, 2004; Mullis, 2008). Phosphorylation of structural and regulatory proteins is well known as an intracellular control

mechanism in eukaryotes (Wera and Hemmings, 1995). Reversible phosphorylation results in a conformational alteration in the structure of many proteins, causing them to become activated or deactivated (Cohen, 2000). The process of protein phosphorylation is to transfer a phosphate group to an amino acid side chain that holds a hydroxyl group or a terminating –OH (Mullis, 2008). Protein phosphorylation on serine, threonine and tyrosine is a reversible post-translational modification that is extensively used to control signal transduction (Macek *et al.*, 2007). Reversible protein phosphorylation controls almost all parts of cell life; therefore, uncontrolled phosphorylation is a cause or consequence of several diseases (Cohen, 2000).

Protein kinases are a group of enzymes that phosphorylate proteins. All protein kinases so far characterized can be divided into tyrosine and serine/threonine specifics. These types of proteins contain similar catalytic domain structures. These domains can be used to predict whether a supposed protein kinase will phosphorylate serine/threonine or tyrosine (Hanks *et al*., 1988). Protein kinases are important to cellular functions such as proliferation and differentiation (Paul *et al*., 1997). Changes in the activity of protein kinases and phosphatases occur in a number of illnesses such as cancer, diabetes, rheumatoid arthritis, or hypertension, and several naturally occurring poisons and pathogens exert their effects by changing the phosphorylation status of proteins (Cohen, 2001; Ariño *et al*., 2011). Approximately a third of human proteins have covalently bound phosphate. Around 500 protein kinases and 30% of this amount of phosphatases are encoded by the human genome (Burlando *et al*., 2003).

The function of protein phosphatases is to remove phosphate groups from serine, threonine, or tyrosine residues, thus counteracting the regulatory action of protein kinases (Cohen and Cohen, 1989). Protein phosphatases (serine/threonine) are divided into two main groups, which are protein phosphatase type 1 (PP1) and protein phosphatase

type 2 (PP2). These phosphatase families are found in organisms from yeasts to mammals. They help reduce the incidence of tumours by opposing phosphorylation induced by uncontrolled protein kinase C activation. This is only one of the many functions of these proteins in the body. Harmful mutations of protein phosphatase can lead to the ending of mitosis in the cell (Mullis, 2008). PP1 phosphatases play an important part in processes ranging from glycogen metabolism to protein synthesis and intracellular transport (Bollen and Stalmans, 1992).

The PP2 enzymes can be divided into three types termed PP2A, B and C, which support cell cycle regulation, T-cell activation and deactivation, dephosphorylation of transcription factors and signal transduction pathways (Wera and Hemmings, 1995; Mullis, 2008). Protein tyrosine phosphatases (PTPs) are linked to the regulation of numerous physiological processes, such as cell proliferation, movement and adhesion in concert with protein tyrosine kinases (PTKs) (Soulsby and Bennett, 2009). These proteins include different families that share one or two common PTP domains carrying the PTP activity (Ono-Koyanagi *et al.*, 2000).

A number of studies have investigated the effect of pesticides and metals on protein phosphorylation of aquatic organisms (Sreedevi *et al.*, 1982; Matsumura *et al*., 1989; Miyazawa and Matsumura, 1990). The alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), acid phosphatase (AcP), and alkaline phosphatase (AkP) activities of liver of the euryhaline fish *Oreochromis mossambicus* significantly decreased following 30 days exposure to 60.2 nM (0.02 mg/l) OP 2-butenoic acid-3-(diethoxy phosphinothionyl) ethyl ester (RPR-V). However, the activities of ALAT, ASAT, AcP, and AkP increased in plasma and in the kidney (Rao, 2006; Liu *et al*., 2008).

In work by Luskova *et al*., (2002) the values of creatine kinase, alkaline and acid phosphatases of carp *Cyprinus carpio* were comparable in the experimental and control groups. However, lactate concentrations and total protein levels were significantly lower in the experimental organisms after exposure to Basudin 600 EW (containing 600 g/L diazinon as the toxic substance) for 96 h On the contrary, it was found that the alkaline phosphatase activity of *Cyprinus carpio* was significantly lower and the total protein was not significantly different after exposure to diazinon (Banaee *et al*., 2008). The levels of phosphoserine, phosphothreonine and phosphotyrosine were used as phosphorylation biomarkers for environmental monitoring to investigate the effect of diazinon, diazinon oxon and the commercial diazinon formulation on *G. pulex* in this study.

Molecular techniques that comprise proteomics have helped the fields of pathology, toxicology and molecular genetics. Proteomic studies are potentially sensitive and useful approaches that can improve understanding of the effects of toxins on aquatic animals (Sinha *et al.*, 2007; Leroy *et al*., 2010). Proteomic approaches make possible the study of complete proteome profiling to recognise the molecular mechanism of complex multi-factorial and toxicant borne diseases (Clarke *et al*., 2003).

Alterations in the complement of proteins present in an animal can be evaluated by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). 2D PAGE is the most frequently used multidimensional separation technique for the analysis of proteome mixtures (Janini and Veenstra, 2002). By this technique, proteins are separated in two ways, by their molecular weight and the pH gradient of each respective protein (Marko-Varga, 2004). 2D PAGE is a sensitive and mature system. It is able to resolve thousands of proteins, but only the most abundant proteins are visualized by staining and can be identified (Janini and Veenstra, 2002). Therefore, by applying proteomic

techniques, different protein forms from a specific species exposed to a variety of stresses can be compared (Melin, 2004). Thus, one aim of this thesis was to attempt to identify proteins whose abundance was altered on exposure of *G. pulex* to diazinon, diazinon oxon and the commercial diazinon formulation

1.7 Aims of the project

This project had the following three aims:

To determine the 96 hour LC_{50} of diazinon, diazinon oxon and diazinon in the commercial formulation in *G. pulex*.

To determine the effect of sub-lethal concentrations of these chemicals on behaviour including movement and feeding activities in *G. pulex.*

To determine the effect of sub-lethal concentrations of these chemicals on molecular markers including AChE activity, HSPs and phosphoprotein contents in *G. pulex.*

CHAPTER TWO: Materials and Methods

2.1 Safety considerations

Experiments described in this study comply with the health and safety codes of practice for Nottingham Trent University. All procedures and preparation of materials were assessed and completed on the appropriate COSHH forms.

2.2 Gammarus pulex

2.2.1 Animal collection and maintenance

Adult *G. pulex* were collected by hand net from a single population in a freshwater slow-running stream (approximate dimensions 3 m wide and 0.5 m deep) located at the Creswell Crags nature reserve, Derbyshire (Ordnance Survey grid reference SK533741). This stream (Millwood Brook) is a tributary of the River Idle. It has been previously sampled and classified as "moderate" with a BMWP score of 50. (Reynolds, 2001) *G. pulex* tend to hide under stones and rocks on the stream's bed. A hand net was placed next to the downstream side of a rock and stones and the rock was lifted up. This exposed *G. pulex*, which were then caught by the net. They were then transferred to a 25 litre collection bin. After field collection animals were transported to the laboratory by road and stored in water-filled plastic aquaria.

Figure 2.1 Sample collection from Creswell Crags nature reserve

The journey from Creswell Crags to the laboratory at the Clifton campus of Nottingham Trent University took around 1h by car. Only undamaged and active organisms were stored. Gammarids were removed from the general holding tank using a net. Around fifty organisms were placed in each 12L plastic aquarium containing 6 L of aerated dechlorinated Nottingham tap water at 15 ^{0}C, and were kept under a 12 h light: dark cycle. Tap water was aerated for 48 h before use to ensure removal of residual chlorine. The water was changed weekly and the gammarids were fed at the same time with wheat germ. They were acclimated for at least 7 days and starved for 3 days prior to the experiments. All experiments were conducted at 15 ^{0}C under a 12 h light: 12 h dark cycle and animals were not fed during the experiments, unless otherwise stated. Adult gammarids of around 30 mg wet weight were randomly chosen for experimental use.

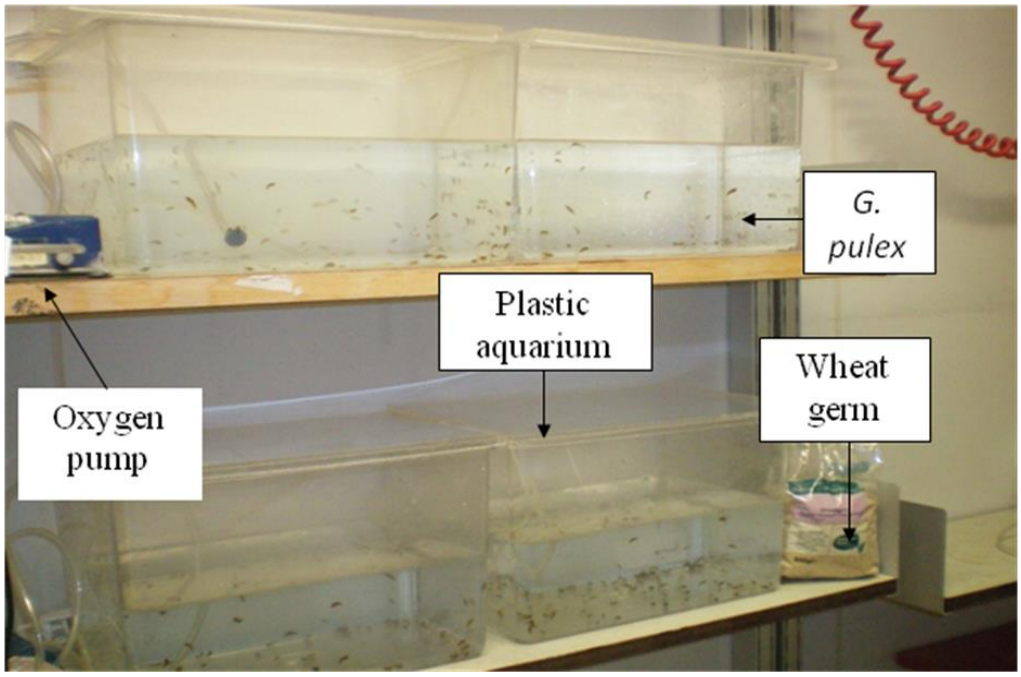


Figure 2.2 Aquaria in a temperature controlled room

2.3 Preparation of glass and plasticware

Before the running the experiments, all glassware and plasticware was washed with detergent. It was then rinsed and filled with 5% nitric acid and left for 24 h to minimize contamination from previous exposures. The tanks were then washed and filled with dechlorinated Nottingham tap water. Required items rinsed in distilled water and air dried before use.

2.4 Chemicals

Table 2.1 The list of chemicals with code number

Reagent	Source
3-((3-Cholamidopropyl) dimethylammonium)-1-	Sigma-Aldrich, code C9426

propanesulfonate (CHAPS)	
4x Protogel® Resolving buffer	Geneflow, code EC 892
5, 5 dithiobis 2-nitrobenzoic acid (DTNB)	Sigma-Aldrich, code D 8130
AccuGel™ 29:1 acrylamide	Geneflow, code EC852
Acetone	Fisher Scientific, code 121403/0031
Acetylthiocholine iodide (ATCh)	Sigma-Aldrich, code A5751
Ammonium persulphate (APS)	Sigma-Aldrich, code MW 228.21
Bicinchoninic Acid (BCA)	Sigma-Aldrich, code 23223
Blueye prestained protein ladder	GeneDirex, code PM 007-0500
Bovine serum albumin (BSA)	Melford, code A1302
Bromophenol blue	Fluka, code 18030
Carrier ampholytes	Bio-Rad, code 163-1112
Commercial diazinon formulation (Osmonds Gold Fleece)	Provided by a farmer in Nottinghamshire
Copper (II) phthalocyanine	Sigma-Aldrich, code C2284
Diazinon (99 %)	ChemService, code F2060
Diazinon oxon (99 %)	ChemService, code 1621A
Dimethyl sulfoxide (DMSO)	Fisher Scientific, code CHE 1854
Dithiothreitol (DTT)	Melford, code A21526
Electrophoresis running buffer (X10)	Geneflow, code EC870
Electrophoresis transfer buffer (X10)	Geneflow, code EC 880
Enhanced chemiluminescence reagent (ECL)	Thermo Scientific, code ME 157124

Ethanol	Fisher Scientificcode 101076H
Glacial acetic acid	Fisher Scientific, code 200.580-7
Glycerol	ACROS, code 15892001C
Iodoacetamide	Sigma-Aldrich, code 1149
Methanol	Fisher Scientific, code M400/17
Mineral oil	Sigma-Aldrich, code EC232-455-8
N,N,N',N'-tetramethylethylenediamine (TEMED)	National diagnostics, code EC503
phosphatase inhibitor cocktail	Calbiochem, code 78420
Precision Plus Protein™ Standards	Bio-Radcode 161-0374
Protease inhibitor cocktail	Calbiochem, code 539134
Protogel® Stacking buffer	Geneflow, code EC 893
Sodium azide	Fisher Scientific, code S2002
Sodium chloride	Sigma-Aldrich, code B 22297
Sodium dodecyl sulphate (SDS)	Melford, code 18299
Sodium hydroxide (NaOH)	Sigma-Aldrich, code 1823
Sodium phosphate dibasic	Fisher Scientific, code 94046
Sodium phosphate monobasic	Fisher Scientific, code 71505
Tris (hydroxymethyl) aminomethane	Sigma-Aldrich, code C 22561
Triton X-100	Sigma-Aldrich, code T8787
TWEEN® 20	Sigma-Aldrich, code P1379
Urea	Sigma-Aldrich, code U 6504
β-Mercaptoethano	Sigma-Aldrich, code M3148

For all experiments, Nottingham tap water was used after the residual chlorine had been removed. This was achieved by aerating the mains water for at least 24 h before use.

Table 2.2 Chemical composition of the dechlorinated Nottingham tap water

Parameter	Values
pH	8.51
$[Na^+]$	1.09 mM
$[Mg2^+]$	0.56 mM
$[Ca2^+]$	1.9 mM
Conductivity	651 µs

2.5 LC_{50} toxicity tests

Test organisms were exposed to different concentrations of diazinon, diazinon oxon and diazinon in the commercial formulation. Diazinon and diazinon oxon were prepared by dissolving in 0.01% v/v DMSO, whilst the commercial diazinon formulation was prepared in distilled water. Serial dilutions of stock solutions were prepared when required.

Small aliquots of the appropriate dilution were transferred to 1 L volumetric flasks and made up to 1L with dechlorinated Nottingham tap water. The pesticide solutions were replaced every 24 h with freshly prepared solutions of the same pesticide concentration. All controls and treatments received the same concentration of DMSO (0.01% v/v). This concentration of DMSO had previously been shown to have no effect on *G. pulex*. A semi-static acute toxicity bioassay was

performed to determine the 24, 48, 72 and 96 h LC_{50} values for diazinon, diazinon oxon and the commercial diazinon formulation. Three replicates were used for each of the pesticide and control solutions. For each replicate, 10 adult intermoult *G. pulex* were randomly chosen and removed from aquaria using a hand net. They were then placed in a 600 ml borosilicate glass beaker containing 500 ml of the required test solution. During the experiment *G. pulex* were not fed and the test solutions were renewed daily. The level of mortality was recorded and dead animals removed after 24, 48, 72 and 96 h of exposure. The data were then analysed with the use of the Trimmed Spearman-Karber method to estimate LC_{50} values and 95% confidence intervals (available from the US Environmental Protection Agency at www.epa.gov/nerleerd/stat2.htm). This is a nonparametric statistical test, which is less susceptible to artefacts when compared to the parametric logistic and probit analysis alternative methods. The trimmed Spearman-Karber method is suitable for actual and hypothetical bioassay and has good statistical properties It is easy to use, and is recommended for accurate and precise calculation of LC50 values and their 95% confidence interval end points (Hamilton *et al*., 1977).

2.6 Phototactic behaviour

Ten adult gammarids were randomly chosen and placed in a 500 ml glass beaker containing 500 ml of the required test concentration for 96 h. The concentrations of diazinon chosen were all below the 96 h LC_{50}. All test concentrations were renewed daily and organisms were not fed during the 96 h experiments. Horizontal clear acrylic tubes (18 cm long, 2.6 cm in diameter) had previously been painted black on the outside for half the length of the tube. There was a small opening in the middle of the top surface of the tube so that a single amphipod

could be placed into the tube. After the tubes were filled with dechlorinated Nottingham tap water, ten gammarids (one per tube) were placed in individual horizontal clear acrylic tubes in constant temperature room with normal light. After 0, 24, 48, 72 and 96 h of diazinon exposure, the location of each *G. pulex* was recorded using a Sony video camera (Sony DCR-HC37 Handycam) for ten minutes. The first five min was the acclimation period and was ignored. For the remaining five min the position of the gammarids was noted every 30 s and scored as 0 for the dark zone or 1 for the light zone. This gave a range in possible scores for an individual gammarid from 0 (always in the dark zone) to 10 (always in the light zone) during the final 5 min of the test.

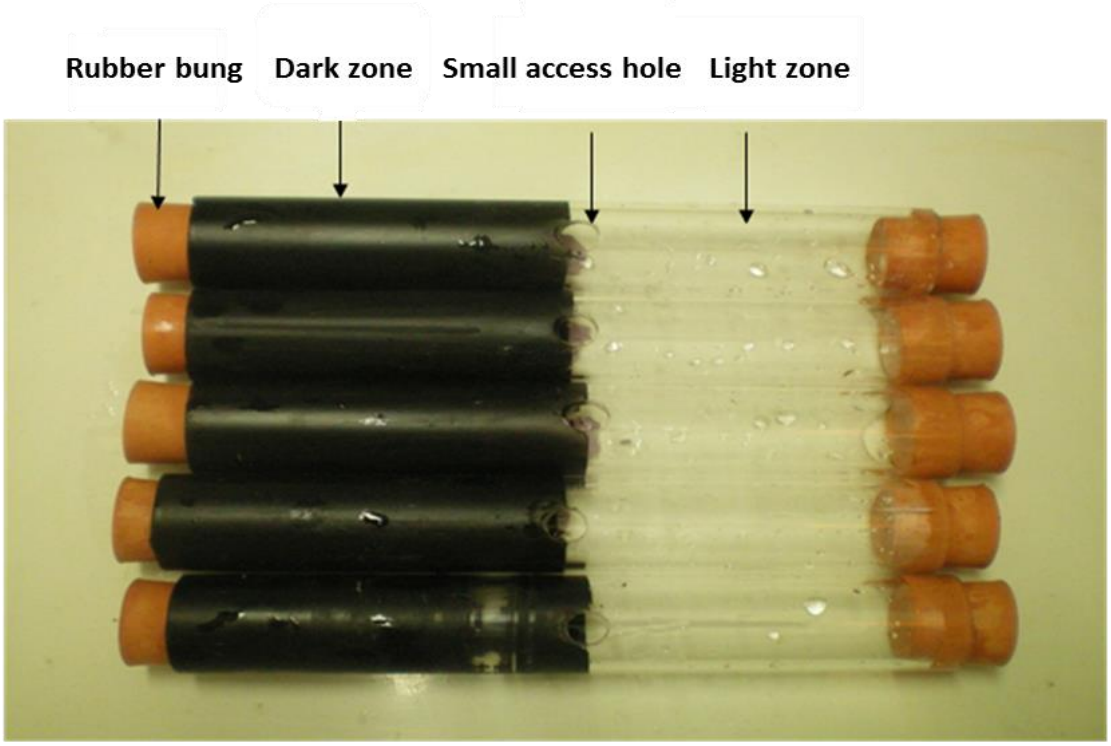


Figure 2.3 Phototactic apparatus

2.7 Geotactic behaviour

After 0, 24, 48, 72 and 96 h of diazinon exposure, ten gammarids were placed in individual vertical acrylic tubes containing dechlorinated water. The tubes were divided into five equal vertical zones and a small net was placed at the top of the water column, which the single *G. pulex* could hold onto. A black sheet was placed on the top and under the bottom of the tube to avoid any potential confounding influence of direct illumination. The experiment run in constant temperature room with normal light, the position of the individual gammarids was recorded with a Sony video camera (Sony DCR-HC37 Handycam) for 10 min. The first five minutes was an acclimation period and was ignored. For the next 5 min, the position of each animal was scored every 30 s from 1 in the bottom zone to 5 in the top zone of the acrylic columns. This gave a range in possible total score for an individual gammarids from 10 (always in the bottom zone 1) to 50 (always in the top zone) during the final 5 min of the test.

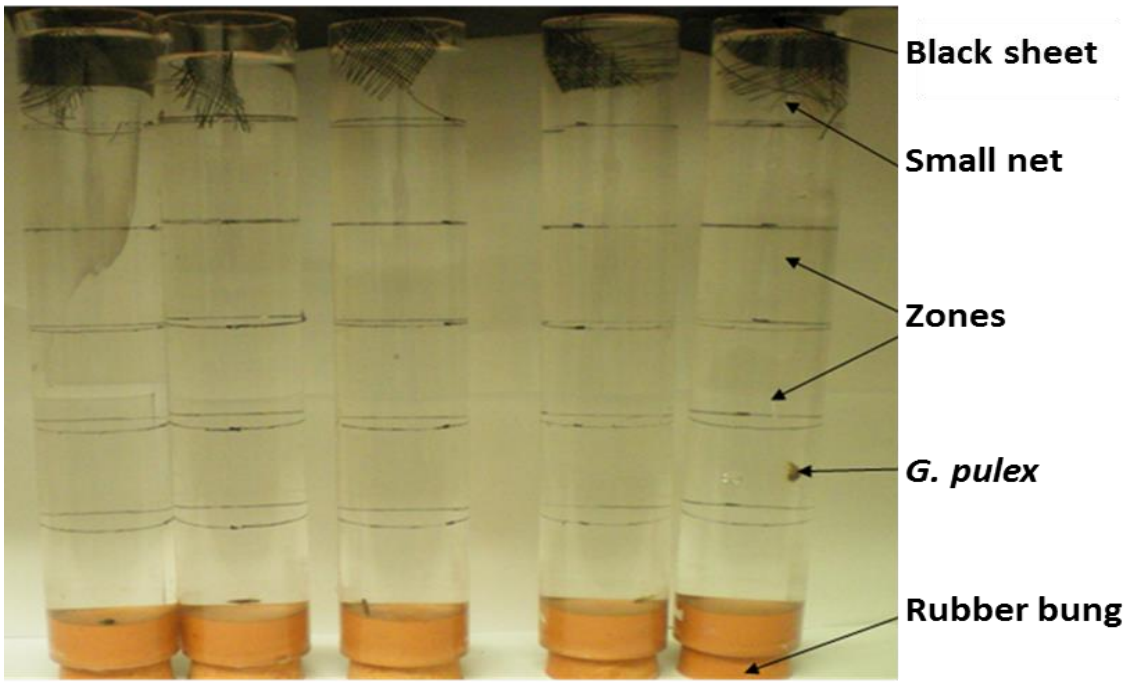


Figure 2.4 Geotactic apparatus

2.8 Horizontal movement

Ten adult *G. pulex* were randomly chosen and exposed to each concentration of diazinon. Each organism was placed in plastic petri dish containing 60 ml of the required diazinon concentration. White tape was placed on the outside vertical rim of the plastic petri dishes (petri dishes were 8.5 cm in diameter and 1.5 cm deep) to limit reflection of the organism in the camera. These plastic petri dishes were placed in LMS cooled incubator set to 15 ^{0}C. During the experiment, the movement of the gammarids was recorded with a webcam (MST Star cam 370i) for 30 min after 0, 2, 4, 6, 8 and 24 h of diazinon exposure. The movement of *G. pulex* was analysed *via* a video tracking system using Image J and the Mtrack2 software plugin.

2.9 Vertical movement assay

After exposure of adult *G. pulex* to the required concentrations of diazinon, diazinon oxon and the commercial diazinon formulation , ten randomly selected individuals from each group were transferred to individual clear plastic boxes (Tic-Tac container) containing 2.5ml of dechlorinated water. The dimensions of the Tic Tac containers were 60x35x12 mm. During the experiment, the movement of the gammarids from the bottom to the top of the water column was recorded with a Sony video camera (DCR-HC37 Hanycam) under infrared light (Scene®. IR, model: S-8030) for 40 min. The first ten min of recording was the acclimation period and was ignored during subsequent analysis. A single movement event was defined as movement from the bottom surface of the container to the top of the water column.

2.10 Feeding activity

Based on the 96 h $LC_{50,}$ a control and three concentrations of diazinon, diazinon oxon and the commercial diazinon formulation (5, 10 and 25 nM) were chosen to assess sub-lethal effects on feeding behaviour. Fresh green leaves were collected from alder trees at Nottingham Trent University, Clifton campus. Two centimetre diameter discs were cut with a cork borer, avoiding the major veins. The discs were then placed in dechlorinated tap water for 24 h prior to use. After 24 h the leaf discs were scanned with a Canon scanner (Canoscan 8800F) to measure their area. Nine adult animals around 30 mg wet weight (three per replicate) were exposed for 24 h to each test concentration, in 600ml glass beakers containing 500ml of the required test solution. One leaf disc was then added to each glass beaker containing three *G. pulex* and 500ml of the required test or control solution. Three discs were also individually placed in three glass beakers containing only Nottingham tap water, to control for any change in leaf area not due to feeding activity. After 24 and 48 h the leaf discs were again scanned. The pesticide solutions were changed every 24 h during the final 48 h of the experiments. The difference between the leaf disc areas before and after exposure to the gammarids was measured using Image J software.

2.11 Acetylcholinesterase activity

Three whole adult *G. pulex* were randomly chosen and homogenised in 4 ml of ice-cold phosphate buffer (100 mM, pH 7.8) containing 0.1% (v/v) Triton X-100, with an Ultra-Turrax® T25 Basic blender at 24,000 rpm for 10 s. The homogenate was then centrifuged for 15 min at 9000g at 4 ^{o}C. The clear supernatant was carefully removed and placed into clean 1.5 ml Eppendorf tubes. Samples were diluted 1:20 with the 10 % (w/v) sodium dodecyl sulphate (SDS) before protein

determination. All samples were kept on ice during the whole procedure.

Acetylcholinesterase (AChE) activity was determined in triplicate for each sample according to the method developed by Ellman *et al.* (1961). Acetylthiocholine iodide (ATCh) was used as the substrate. In brief, 16 µl of supernatant was added to 285 µl of reaction medium (100 mM phosphate buffer, pH 7.8 containing 0.2 mM 5, 5 dithiobis 2-nitrobenzoic acid [DTNB]). Spontaneous substrate hydrolysis was assessed using two controls, a blank without ATCh and a blank without the sample. Absorbance was read at 415 nm for 20 min at 25 ^{o}C (30 s intervals) using a microplate reader (Tecan Spectra Fluor).

In order to assess the *in vitro* inhibition of AChE activity, *G. pulex* that previously had not been exposed to pesticides were homogenised as previously described and the 9000g supernatant kept for measurement of AChE activity. The required amount of pesticide was then added to the gammarid supernatant extracts to obtain final concentrations of up to 10 µM. They were immediately vortex mixed and placed on ice for 15 min to allow interaction with the gammarid AChE. The enzyme activity was then determined as described above. For *in vivo* exposure gammarids were exposed to selected concentrations of diazinon, diazinon oxon and the commercial diazinon formulation for up to 24 h.

2.11.1 Protein assay

The protein concentration of samples was assayed by the method of Smith *et al*. (1985), using a bicinchoninic acid (BCA) assay kit from Sigma-Aldrich. One part of reagent B (4 % (w/v) copper (II) sulphate pentahydrate) was added to 50 parts of reagent A ((1 % (w/v) bicinchoninic acid, 2 % (w/v) sodium carbonate, 0.16 % (w/v) sodium tartrate and 0.95 % (w/v) sodium bicarbonate in 0.1 M sodium hydroxide at pH 11.5) then mixed well. Two hundred microlitres of this

solution was then added to 25 µl of all samples in a 96 well plate from Sarstedt (samples in triplicate). After 30 min incubation at 37 ^{0}C in an LEEC incubator (LSC 2933), the absorbance was measured at 570 nm. To prepare the standard curve, 20 mg of bovine serum albumin (BSA) was prepared in 20 ml of assay buffer to make a 1 mg/ml stock solution of BSA. Serial dilutions of BSA were prepared by adding assay buffer in Eppendorf tubes as indicated in Table 2.3

Table 2.3 Preparation of standard curve for protein determination

Volume of 1mg /ml BSA (µl)	Volume of assay buffer (µl)	Final concentration of BSA (mg/ml)
0	1000	0
200	800	0.2
400	600	0.4
600	400	0.6
800	200	0.8
1000	0	1

2.12 SDS Polyacrylaminde Gel Electrophoresis

2.12.1 Preparation of samples

Pilot experiments showed that it was necessary to use six whole gammarids per sample, in order to obtain sufficient protein for the antibodies to detect their respective proteins. *G. pulex* were homogenised by an Ultra-Turrax T25 basic® at 24,000 rpm for 5 s with in 4 ml ice cold phosphate Tris-buffered saline (TBS, Tris 50 µM and 200 µM sodium chloride NaCl, pH 7.4) containing 40 µL protease inhibitor cocktail from Calbiochem (catalogue no. 539134). The homogenate was then centrifuged at 1000g for 15 min at 4 ^{0}C. The

supernatant was collected and centrifuged at 9000g for 15 min at 4 ^{0}C. The supernatants were collected, boiled with 0.5% (w/v) SDS at 100 ^{0}C for 5 min. The 9000g pellets were resuspended in the same volume of homogenisation buffer and recentrifuged at 9000g for 15 min at 4 ^{0}C. Washed pellets were resuspended in 0.5 ml of 0.5% (w/v) SDS, vortex-mixed and incubated at 100 ^{0}C as described above. Each supernatant and pellet sample at the final stage was placed in a spin column (Dutscher Scientific, catalogue no. 789068) to remove any reaming DNA trace by centrifugation at 14000 g for 1 min at 4 ^{0}C.

2.12.2 Preparation of polyacrylamide gels

SDS-PAGE was carried out by the method described by Laemmli (1970) with some modifications, using the Bio-Rad Mini-PROTEAN III™ electrophoresis cell, which was assembled according to the manufacturer's instructions. Two glass plates (one with 1.5 mm spacers) were thoroughly cleaned with 70% (v/v) ethanol and dried before use. The plates were then clamped into position in the gel casting stand. Ten ml of resolving gel mix were prepared as shown in Table 2.4. TEMED was used as a polymerisation inducing agent and was added to the solution immediately before the gel was poured. Sufficient space was left above the resolving gel mixture to allow for a stacking gel. Distilled water was carefully added by a 1ml pipette above the resolving gel to create a smooth overaly. The gel mix was allowed to polymerise at room temperature for 30 min.

The objective of the stacking gel is to enhance the resolution of the protein bands that are made by electrophoresis. After polymerisation of the resolving gel, the distilled water overlay was removed. Then TEMED was added to the 4 % (w/v) acrylamide stacking gel mix (Table 2.4) immediately prior to pouring the gel mix on top of the resolving gel to the top of the glass plates. A 10 tooth comb was placed within the gel to make 10 individual wells. The gel was then

allowed to polymerise for 20 min at room temperature before the comb was carefully removed. The gel assembly was then transferred to an electrophoresis running chamber containing SDS-PAGE running buffer as in Table 2.4

Table 2.4 Preparation and reagents for SDS-PAGE and buffers

Resolving gels		
Reagent	10 % gel	12 % gel
Resolving Buffer (1.5M Tris–HCl , 0.4 % (w/v) SDS , pH 8.8)	2.5 ml	2.5 ml
40% (w/v) 29:1 Acrylamide Bis Acrylamide solution	2.5 ml	3 ml
Distilled water	4.88 ml	4.38 ml
10 %(w/v) ammonium persulphate (APS)	100 µl	100 µl
TEMED	20 µl	20 µl
Total volume	10 ml	10 ml

Stacking gels	
Reagent	volume
4% Stacking Buffer (0.5M Tris–HCl , 0.4 % SDS , pH 6.8)	2.5 ml
40% (w/v) 29:1 Acrylamide Bis Acrylamide solution	1 ml
Distilled water	6.4 ml
10 %(w/v) ammonium persulphate	100 ml
TEMED	20 µl
Total volume	10 ml

Electrophoresis running buffer
25 mMTris,192 mM glycine, 0.1% (w/v) SDS, pH 8.3

2.12.3 One dimensional gel electrophoresis of samples

Samples were mixed with Laemmli x4 buffer (see Table 2.5) in a 1:3 ratio and boiled for 5 min. Samples were allowed to cool down to room temperature prior to loading into the gel. 30 µg of protein was loaded into each well, to obtain even loading across the gel. After the gel was made up according to section 2.12.2, 2 µl of Blueye prestained protein ladder, (molecular weight range 245-11 kDa) was added to the well. Gels were then placed vertically in a Mini-PROTEAN III™electrophoresis chamber from Bio-Rad and run in electrode running buffer at 50 volts for 10 min using Bio-Radpower pac 300. This initial voltage allows the samples to enter the stacking gel. After that, the voltage was increased to 150 volts and run for around 1 h. As the dye front approached the base of the gel plates, the voltage was switched off. Gels were carefully removed and used for the western blotting.

Table 2.5 Laemmli x4 SDS – PAGE sample buffer

Reagent	Volume (ml)
Distilled water	0.8
1 M Tris HCl pH 6.8	2.4
Glycerol	4.0
10 % (w/v) SDS	1.6
β-mercaptoethanol	1.0
Bromophenol blue 0.05% (w/v)	0.2
Total volume	10.0

2.13 Western blotting

Western blotting was carried out as described by Towbin *et al.* (1979) with some modifications. Following separation of proteins by SDS-PAGE, the gels were carefully removed from gel plates and the stacking gels were cut off. The remaining resolving gel was used in western blotting. The nitrocellulose membrane filters (Hybond C, Amersham) and blotting papers (Whatman® filter paper, from Sigma-Aldrich) were placed in transfer buffer (Table 2.6) for 5 min. The gel, nitrocellulose membranes and blotting papers were arranged as a sandwich (see Figure 2.6).

Any bubbles were removed by rolling a glass roll across the components, because the air bubbles will prevent the transfer of proteins from the gel to the nitrocellulose membranes. The sandwich was placed into the blotting chamber (Mini Trans-Blot® Electrophoretic Transfer cell; Bio-Rad) containing transfer buffer, and the current was run at 30 volts overnight (16 h). The transfer process took place at room temperature.

Table 2.6 Transfer buffer

Reagent	Volume
10x Tris /Glycine (0.25 M Tris, 1.92 M Glycine)	100 ml
Methanol	200 ml
Distilled water	700 ml
Total volume	1000 ml

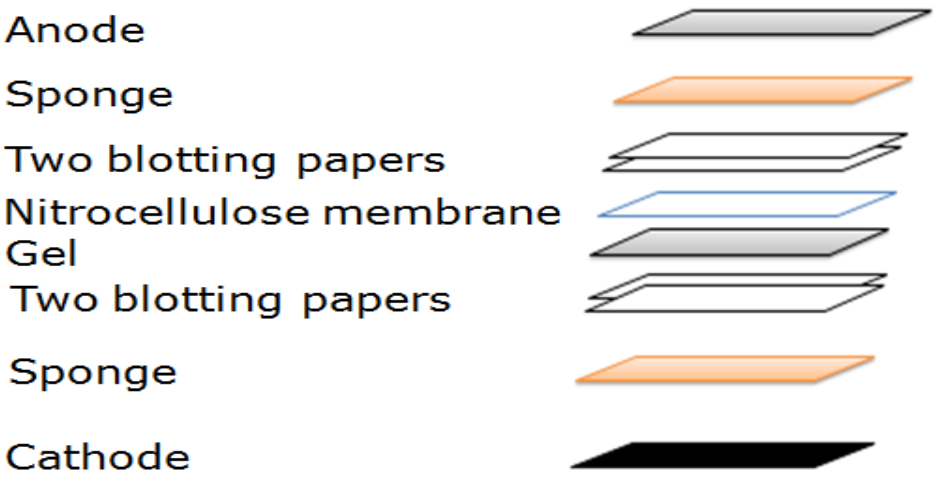


Figure 2.5 Arrangement of transfer cassette for western blotting

The sandwich was then removed from the blotting chamber, the nitrocellulose membranes were separated from the transfer cassette and placed in a plastic container. The nitrocellulose membranes were stained for 5 min with 0.5 % (w/v) copper pthalocyanine in 12.5 mM HCl to confirm efficient transfer to the membrane. Blots were destined by incubation with 12.5 mM sodium hydroxide (NaOH).

2.14 Immunodetection

After successful transfer of protein was confirmed, the nitrocellulose membranes were blocked for one hour in 3% (w/v) BSA in TBS containing 0.01% (w/v) sodium azide at room temperature. They were gently rocked on a shaker (Heidolph Unimax 1010) at 50 rpm, so that the BSA could eliminate non-specific protein binding. Following this, the membranes were incubated overnight at 4 ^{0}C with primary antibodies diluted as shown in Table 2.7. Then the membranes were washed five times for 15 min with 20 ml of 0.05 % (v/v) Tween 20 in TBSand once with TBS for 15 min. The membranes were incubated for

2 h at room temperature with secondary antibodies diluted in 3% (w/v) BSA in TBS containing 0.01% (w/v) sodium azide. Then the nitrocellulose membranes were washed again (five times for 15 min with TBS-Tween 20 and a final wash in TBS without Tween 20 for 15 min). Western blots were imaged using a G: BOX iChemi dark system from Syngene (Cambridge, UK). Equal volumes from each reagent (A and B) of the enhanced chemiluminescence (ECL) reagent (Thermo Scientific) were applied on the probed membranes to detect the bands of interest. Band intensities on developed blots were quantified using Advanced Image Data Analyzer (AIDA) software (Fuji).

Table 2.7 Primary antibodies

Primary antibody	Source	Dilution	Product number	Host species
Anti Phosphothreonine Clone:PTR-8	Sigma-Aldrich, UK	1:1000	P-3555	Mouse
Anti-HSP 60 Clone:LK2	Sigma-Aldrich, UK	1:1000	H3524	Mouse
Anti-HSP 70 Clone:BRM-22	Sigma-Aldrich, UK	1:1000	H5147	Mouse
Anti-HSP 90 Clone:AC-16	Sigma-Aldrich, UK	1:1000	H1775	Mouse
Anti-HSP~20	Assay Designs	1:500	ADI-SPA-224	Rabbit
Anti-Phosphoserine Clone:PSR-45	Sigma-Aldrich, UK	1:1000	P-3430	Mouse
Anti-Phosphotyrosine Clone:PT66	Sigma-Aldrich, UK	1:1000	P-3300	Mouse
Polyclonal anti ß actin	Sigma-Aldrich, UK	1:1000	AV40173	Rabbit

Table 2.8 Secondary antibodies

Secondary antibody	Dilution	Product number	Source	Host species
Anti-mouse IgG	1:1000	P0260	DAKOCytomation Ltd	Rabbit
Anti-rabbit IgG	1:1000	P0448	DAKOCytomation Ltd	Goat

2.15 Dot blot

Organisms were treated and samples prepared as described earlier (see Section 2.12.1). To quantify levels of phosphorylated proteins, serial dilutions were made for each sample using TBS. The nitrocellulose membrane was placed inside the manifold of a 96 well dot–blot system (Bio-Rad 84BR25150). Five microlitres of each sample was deposited onto the nitrocellulose membrane and then were allowed to dry for 2 h at room temperature.

The nitrocellulose membranes were then removed from the dot–blot apparatus and treated as previously described in Section 2.14. Thenanti-phosphoserine, anti-phosphothreonine and anti-HRP-conjugated phosphotyrosine were used as primary antibodies and anti-mouse Ig as secondary antibodies. The protein blots were developed and analysed with AIDA software as described in Section 2.14

2.16 Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

Post mitochondrial supernatants were extracted from *G. pulex* using the methods previously described in Section 2.12.1. The supernatants which were collected by centrifugation at 9000g for 15 min at 4 ^{0}C and were dialysed against ice cold TBS (1 litre) for 40 min. The buffer was

changed for another litre of ice cold TBS and the sample dialysed for a further 40 min. The dialysed supernatant was collected and then boiled with 0.5% (w/v) SDS at 100 ^{0}C for 5 min. SDS soluble supernatant proteins were placed in a spin column to remove any trace DNA by centrifugation at 14000 g for 1 min at room temperature. The protein samples were then treated to remove lipid and to concentrate the sample. For this step the required sample volume was transferred to an Eppendorf tube and vortex mixed with nine times their volume of ice-cold acetone and incubated overnight at -20 ^{0}C. The resultant protein precipitates were then centrifuged for 20 min at 14000 g. The supernatant was carefully removed and the pellet samples were left to dry for 1 h at room temperature. Pellet samples were then stored in a freezer at -20 ^{0}C until required.

2.16.1 First dimension: isoelectric focusing (IEF) in individual immobilized pH gradient (IPG) strips

The pellets from the control and treated samples were re-suspended in an appropriate volume of rehydration buffer (see Table 2.9). Samples containing 40 µg protein were made up to 125 µl with rehydration buffer and loaded into the strip holder channel. A 7 cm IPG strip gel (Bio-Rad, Ready Stripn^{TM} IPG strips 7 cm, pH 3-10 –catalogue no. 163-2000) was placed into a strip holder channel with the gel side facing down taking care not to introduce air bubbles. They were then left for at least 1 h until most of the liquid had been absorbed by the IPG strip. Each IPG strip was then covered with 2-3 ml of mineral oil by carefully moving the pipette along the length of the IPG strip to prevent evaporation during the IEF process. The rehydration tray was covered with the plastic lid provided. Isoelectic focusing was achieved using these conditions: start voltage 0 volts, end voltage 4000 volts, 8000 volt hours and the temperature was 20 ^{0}C for 15 h.

Table 2.9 Rehydration buffer

Component(final concentration)	Amount
8 M Urea	24 g dry urea dissolved in 25 ml of ultrapure water
4 % (w/v) CHAPS	2g
0.5 % (v/v) Carrier ampholytes	250 µl
0.002% (v/v) Bromophenol Blue	100 µl of 0.1% stock
*50 mM DTT	10 mg / ml rehydration buffer
Ultrapure water	Adjust to 50 ml

* DTT was added immediately before use.

2.16.2 IPG strip equilibration

Prior to running the second dimension gel, it was important to equilibrate the IPG strip to reduce cysteine and to alkylate residues, after the focusing step. The mineral oil was removed from IPG strips by placing them gel side up onto a piece of dry filter paper and blotting with a second piece of filter paper. IPG strips were then placed into the equilibration tray face side up and each IPG strip was covered with 2.5 ml of equilibration buffer 1 (see Table 2.10). IPG strips were then placed on the shaker (Heidolph unimax 2010) at 50 rpm /10 min to reduce sulfhydryl groups. After that equilibration buffer 1 was removed, after which 2.5 ml of equilibration buffer 2 (see Table 2.10) was added to each strip and IPG strips were placed on the shaker at 50 rpm for 10 min to alkylate the reduced sulfhydryl groups.

Table 2.10 Equilibration buffer solution for IPG gel strips

Component(final concentration)	Amount
6 M Urea	36 g urea
2 % (w/v) SDS	2 g
20 % (v/v) Glycerol	20 ml
0.05 MTris-HCl, pH 8.8	3.3 ml of 1.5 M buffer stock
Ultrapure water	Adjust to 100 ml

Equilibration buffer 1 consisted of 10ml of equilibration buffer mixed with 200 mg DTT.

Equilibration buffer 2 consisted of 10 ml of equilibration buffer mixed with 250 mg iodoacetamide.

2.16.3 Second dimensional gel electrophoresis: SDS-PAGE

For the second dimension, SDS-PAGE was performed with 12 % polyacrylamide gels (see Table 2.4) using the Mini-PROTEAN III® (Bio-Rad). Before IPG gel strips were ready to use for the next step, 5 µl of 1:20 diluted marker proteins (Protein ladder 10 to 245 kDa from Bio-Rad) were loaded on a piece of filter paper and left to dry. The equilibrated IPG gel strips and the piece of filter paper containing standards were placed on top of the SDS-PAGE gel and covered with 1 ml of molten agarose solution (0.5 % (v/v) agarose in Tris/Glycine/SDS). The SDS gel cassettes were inserted in the electrophoresis apparatus containing the electrode buffer (Table 2.4) and run with a voltage setting of 50 V for 10 min followed by 150 V until the dye front reached the bottom of the gel.

2.16.4 Visualization of proteins on polyacrylamide gels

After the second dimension run had finished, it was necessary to immobilize the separated proteins in the gel and to eliminate any non-protein components, as these may interfere with subsequent staining. The silver staining was carried out according to the Dodeca TM Silver Stain Kit protocol from Bio-Rad (Table 2.11). All steps in the silver staining protocol were performed on the shaker (Heidolph unimax 2010) at 55 rpm. After removing the gels from the glass plates, they were fixed in the ethanol/glacial acetic acid and ultrapure water for at least 1 h. The fixing solution was then removed and gels were placed in the sensitizing solution for 30 min, followed by washing 3 times in ultrapure water for 10 min each time. Gels were then soaked in staining solution for 30 min. After this, gels were rinsed with ultrapure water for 1 min, the rinsing water was then removed and gels soaked in developing solution for 10-30 min until the spots appeared. Development of spots was stopped with stopping solution for 10 min and the gels were washed with ultrapure water for 10 min. Gels were finally scanned with a Fujifilm scanner (FLA-7000) to obtain images for the analysis with Progenesis SameSpots software.

Table 2.11 Protocol for silver staining of gels using Dodeca TM Silver Stain kit

Step	Reagent	For 250 ml working solution ***	For 100 ml working solution **	procedure	Time
Fixing	Ethanol Glacial acetic acid Ultrapure water	100 ml 25 ml 125 ml	40 ml 10 ml 50 ml	Place the gel(s) in fixing solutions and shake for the indicated period of time.	1 h (overnight acceptable)
Sensitizing	Sensitizer concentrate Background reducer Ethanol Ultrapure water	25 ml 2.5 ml 75 ml 147.5 ml	10 ml 1 ml 30 ml 59 ml	Remove the fixing solution and shake gel(s) in the sensitizing solution	30 min
Washing 1	Ultrapure water	250 ml	100 ml	Remove sensitizing solution and wash 3 times	3x10 min
Staining	Silver reagent concentrate Ultrapure water	5 ml 245 ml	2 ml 98 ml	Remove water and shake in staining solution	30 min
Rinsing	Ultrapure water	250 ml	100 ml	Remove staining solution and rinse the gel(s)	1x1 min
Developing	Developing buffer concentrate . Image developer concentrate. Background reducer concentrate. Ultrapure water	25 ml 50 µl 12.5 µl 225 ml	10 ml 20 µl 5 µl 90 ml	Remove water and shake gel(s) in developing solution	10-30 min
Stopping	Glacial acetic acid Ultrapure water	12.5 ml 237.5 ml	5 ml 95 ml	Remove developing Solution and shake gel(s) in stopping solution	10 min
Washing 2	Ultrapure water	250 ml	100 ml	Wash gel(s)	10 min

*** 250 ml protocol gives enough reagent for 2 large gels

** 100 ml protocol gives enough reagent for 2 mini gels

2.17 Statistical analysis

Data is presented as mean ± one standard error of the mean (SEM). The data from the LC_{50} toxicity test was analysed by the Trimmed Spearman-Karber method to estimate LC_{50} values and 95% confidence intervals. This test is a nonparametric statistical test, which is less susceptible to artefacts when compared to the parametric logistic and probit analysis alternative methods. The values of other tests such as behavioural (movement and feeding activities) and molecular assays (AChE activity, HSPs and phosphoprotein content) were subjected to ANOVA, after which results were analysed using a *post hoc* Tukey test with 95% confidence limits to determine significance differences between the control and exposure groups.

CHAPTER THREE: Acute toxicity and potential behavioural markers of toxicity

3.1 Introduction

The application of chemicals to control a variety of pest species is frequently used in agriculture. These pesticides may inadvertently reach aquatic ecosystems and impact ~~the~~ non-target organisms (Adedeji *et al.,* 2009). Several methods have been established to determine the toxicity of pesticides and other chemicals on aquatic organisms (Boateng *et al*., 2009). The aim of toxicity assessment is to create data concerning the adverse influence of chemical agents on test animals (Stephan, 1977). Acute toxicity is a common test as it can rapidly assess relative toxicity using unambiguous endpoints (Hickey, 1989; Adedeji *et al*., 2010). These tests allow estimation of the concentrations of toxicants that induce harmful effects on the test animals during short–term exposure under controlled conditions (Cairns *et al*., 1978; Buikema *et al*., 1982). A common test is the 96 h median lethal concentration (96 h LC_{50}), which is defined as the concentration that causes 50% mortality of the exposed organisms over 96 h (Boateng *et al*., 2009; Kenneth and Willem, 2010).

A statistical estimation process is then used to offer the best estimate of the LC_{50} from the concentration versus death data for each length of exposure (Stephan, 1977). Sucahyo *et al*. (2008) have shown that the toxicity of any chemical varies both between species and within class sizes, where different life stages may be more sensitive than others. Several methods of dispersion might be used. These include the estimated standard deviation and estimated standard error of $\log_{10}$ LC_{50} and the upper and lower 95% confidence limits. Confidence limits

are generally favoured as they have the same units as the LC_{50}, and they are used and understood by applied toxicologists (Stephan, 1977). Toxicological information is required at many levels of biological organization to estimate the potential hazard of pollutants on aquatic resources. Initial acute toxicity testing is cost effective and the unequivocal end points allow easy estimation of mortality in a short term test. From an ecological point of view, short term survival only gives limited information. Ecotoxicity tests that investigate the effect of pollutants on survival, growth, reproduction, spawning hatching success and foraging ability, *etc.*, are more environmentally relevant. However, classical toxicity testing takes longer to conduct, is substantially more expensive and can be labour intensive. There is a need to manually process each test chamber at suitable times for acute and chronic tests, as any manual inspection is subject to operator error (Girling *et al.*, 2000; Lloyd Mills *et al*., 2006).

The acute toxicity of diazinon has been determined for many freshwater invertebrates, but there are differences in the data recorded for its acute toxicity (Tsuda *et al*., 1997; Leight and Van Dolah, 1999; Office of Pesticide Programs, 2000; Aydın and Köprücü, 2005). The 96 h LC_{50} of diazinon ranged from 821 pM (0.25 µg/L) for the cladoceran *Ceriodaphnia dubia,* to 38 nM (11.6 µg/L) for the planarian, *Dugesia tigrina*. It was found that the invertebrates that were the most sensitive to diazinon were from the Class Crustacea (US EPA, 2005). The 96 h LC_{50} of diazinon on three species of amphipods (*G. faciatus, G. pseudolimneaus* and *Hyallela azteca*) ranged from 6.70 to 55.3 nM (2 to 16.8 µg/L). The 96 h LC_{50} of diazinon on the juvenile freshwater shrimp *Caridina laevis* was 1.9 µM (0.6 mg/L). In contrast, that of adult *C. laevis* was 4.8 µM (1.5 mg/L) indicating that adults were less sensitive than juveniles (Sucahyo *et al*., 2008).

Mortality under classic test conditions is generally defined as failure of an organism to respond to a suitable stimulus. It is also a poor environmental indicator, as animal fitness may be reduced by sublethal rather than lethal concentrations (Girling *et al*., 2000; Lloyd Mills *et al*., 2006). Environmental monitoring is an important tool for evaluating the effect of contaminants on natural ecosystems and for measuring the efficiency of pollution control measures (Maltby *et al*., 2002).

Both chemical and biological methods have been used to monitor the quality of aquatic environments. Whilst chemical analysis will give a snapshot of chemical concentration, biological monitoring offers the opportunity to assess environmental quality over the longer term. Exposure of aquatic organisms to pesticides such as diazinon could harm their ecological fitness, for example by affecting movement and feeding behaviour. Reduced fitness at the individual level may in turn influence population phenomena such as survival, growth and reproductive success. (Gaworecki *et al.*, 2009).

The use of sublethal concentrations to study the effect of chemicals on the behaviour of aquatic organisms may be more useful than mortality, since death does not always occur in species exposed to contaminations under natural settings (Roast *et al*., 2001; Christensen *et al*., 2005). Sublethal concentrations can inhibit feeding and growth, disturb the behaviour of exposed organisms and have an impact on their biochemistry (Satapornvanit, 2006). Several studies have demonstrated that behavioural endpoints are sensitive to sublethal concentrations of various chemical pollutants at levels significantly below their LC_{50} (Lemly and Smith, 1986; Dutta *et al*., 1992; Chon *et al*., 2005; Lloyd Mills *et al*., 2006).

In order to be adopted for routine use, toxicity tests should preferably be sensitive, easy to perform, rapid, cheap and robust (Lloyd Mills *et*

al., 2006). Movement is an important behaviour that can be disturbed by environmental pollutants. This behaviour is fundamental to feeding, competitive interaction, predator avoidance, and reproduction (Gaworecki and Klaine 2008). Several mobility endpoints have been utilized to evaluate toxicant influences on different test organisms *(G. duebeni, Balanus amphitrite***,** *G. pulex* and *G. lawrencianus*) such as swimming speed, phototactic behaviour and vertical and horizontal swimming (Lawrence and Poulter, 1998; Wallace and Estephan, 2004; Faimali *et al*., 2006; Tain *el al*., 2007; Ashauer *et al*., 2011).

Swimming endpoints have been widely used to evaluate the effect of various chemicals on teleost fish and some aquatic crustaceans (Boudrias, 1991; Baillieul and Blust, 1999; Roast *et al*., 2001; Wallace and Estephan, 2004). *D. magna* has been used as model to detect the effect of sublethal exposure on phototactic behaviour using the toxicants naphthalene (Whitman and Miller, 1982), copper, pentachlorophenol (Michels *et al*., 1999) and cadmium (Michels *et al*., 2000). For example, phototactic behaviour of *D. magna* was totally inhibited when exposed to 15.6 µM (2 mg/l) naphthalene (Whitman and Miller, 1982).

Phototatic behaviour in these short-term exposure assays has been found to be suitable for detecting sublethal effects of some metals, chlorinated phenols and petroleum derivatives. However, they did not apply to other substance groups counting several that usually occur in freshwater and marine environments (Martins *et al*., 2007). Alonso and Camargo (2011) have claimed that the use of videotaped bioassays for behavioural monitoring of organisms is an improvement, because they are simply and cheaply applied. Baganz *et al*. (1998) reported differences in the motility of zebra fish movement using videotaping after exposure to the cyanobacteria toxin, microcystin-LR. Wallace and Estephan (2004) observed that cadmium affected the horizontal and vertical swimming activity of *G. lawrencianus.*

Feeding behaviour is of high ecological significance, as it permits survival, growth, and reproduction. Feeding responses have been used in various ecotoxicological assays in different animals. Reduced feeding activity necessarily leads to a decreased energy consumption, which could have far reaching effects on reproduction, growth, and population level (De Lange *et al*., 2006). Amphipods have been widely used as model systems to investigate the effects of toxicants on feeding rate (Naylor *et al*., 1989; Maltby *et al*., 1990; Maltby and Naylor, 1990; Alonso *et al*., 2009). These organisms are a vital link in aquatic food chains and they are easily cultured in the laboratory (Oakden *et al*., 1984; Wallace and Estephan, 2004).

Several studies indicate that feeding behaviour is a relativity sensitive assay (Alonso and Camargo, 2004; Pestana *et al*., 2007; Felten *et al*., 2008). The freshwater organism *G. pulex* has been frequently used as a test organism to investigate the effect of pollutants on feeding responses (McCahon *et al*., 1991; Maltby *et al*., 2002; Alonso *et al*., 2009). Alonso *et al.* (2009) found that 444.8 nM (0.05 mg/l) cadmium reduced the feeding behaviour of *G. pulex.* A study by Scholz *et al***.** (2000) indicated that swimming and feeding behaviour of chinook salmon *Oncorhynchus tshawytscha* was reduced after exposure to 32.9 nM (10 µg/L) diazinon for 24 h compared to control fish. Measuring the movement and feeding behaviour of *G. pulex* might therefore provide a sensitive ecologically relevant response to environmental diazinon contamination.

Acute toxicity tests were conducted in this study to determine the 96 h LC_{50} values for diazinon, diazinon oxon and the diazinon in the commercial formulation. This allowed subsequent analysis of behavioural and molecular biomarkers of sublethal pesticide exposure. The latter part of this chapter will examine the effect of diazinon, diazinon oxon and diazinon in the commercial diazinon formulation on movement and feeding behaviours of *G. pulex*.

3.2 Results

Observed general behavioural changes included a reduction in general activity, loss of equilibrium and abnormal swimming. In addition, the gills of *G. pulex* tended to swell and became darkly coloured before death.

3.2.1 Acute toxicity

The effect of diazinon on *G. pulex* survival

The effect of diazinon on the survival of *G. pulex* is shown in Figure 3.1 and 3.2. No mortality was observed in control, 1 and 10 nM for 24 h and the mortality was less than 4% at other concentrations (75,100,250 and 1000 nM) for 24 h. In the control and at the lower concentrations (1 and 10 nM) mortality was less than 20% over 96 h. Exposure of *G. pulex* to 75, 100 and 250 nM caused 27%, 43% and 84% mortality, respectively at 96 h. At 1000 nM mortality dramatically increased with increased exposure period. The LC_{50} values for diazinon on *G. pulex* are shown in Table 3.1.

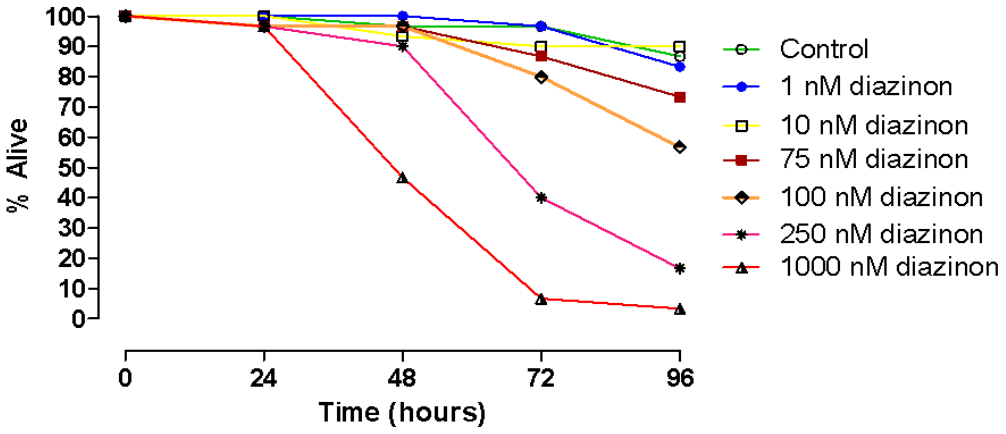


Figure 3.1. The time dependent survival of G. pulex exposed to different concentrations of diazinon (n=30, 10 organisms each replicate).

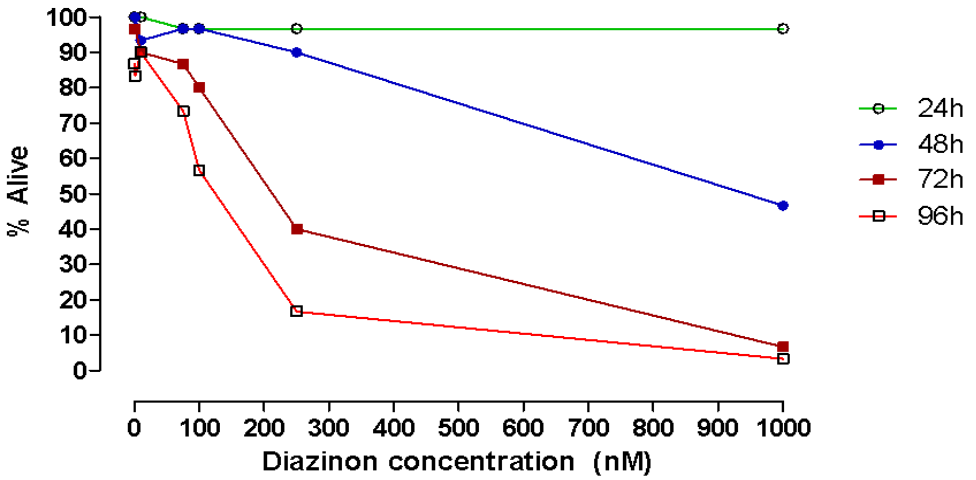


Figure 3.2 Survival of G. pulex after exposure to various concentrations of diazinon for different time intervals (n=30, 10 organisms each replicate).

The effect of diazinon oxon on *G. pulex* survival

The effect of diazinon oxon on the survival of *G. pulex* is shown in Figures 3.3 and 3.4. In control *G. pulex* 13% died by 96 h and % mortality increased with concentration of diazinon oxon. At 50 nM, 60% died, whilst it was 93% at 75 nM and 100 % at 100 nM for 96 h. The LC_{50} values of diazinon oxon for *G. pulex* are shown in Table 3.1.

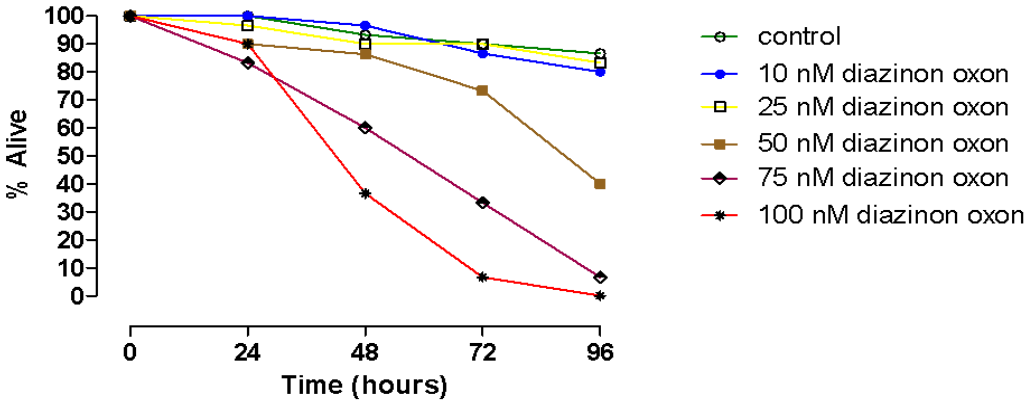


Figure 3.3 The time dependent survival of G. pulex exposed to different concentrations of diazinon oxon (n=30, 10 organisms each replicate).

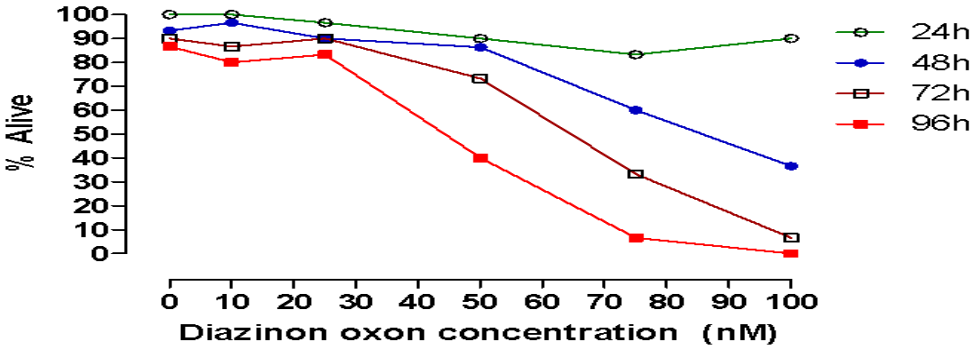


Figure 3.4 Survival of G. pulex after exposure to various concentrations of diazinon oxon for different time intervals (n=30, 10 organisms each replicate).

The effect of diazinon in the commercial formulation on the survival of *G. pulex*

The effect of diazinon in the commercial formulation on the survival of *G. pulex* is shown in Figures 3.5 and 3.6. Low mortality was observed in *G. pulex* in the control group (20% at 96 h). At 7.5 nM the mortality was increased to 30 % at 96 h. The toxicity of the diazinon in the commercial formulation on *G. pulex* strongly increased with concentration and exposure time. The mortality was 57% at 10 nM, 87% at 25 nM and 100% at 50 nM for 96 h exposure. The LC_{50} of diazinon in the commercial formulation are given in Table 3.1. The 24 h LC_{50} and 48 h LC_{50} values could not be calculated because mortality did not exceed 50 %.

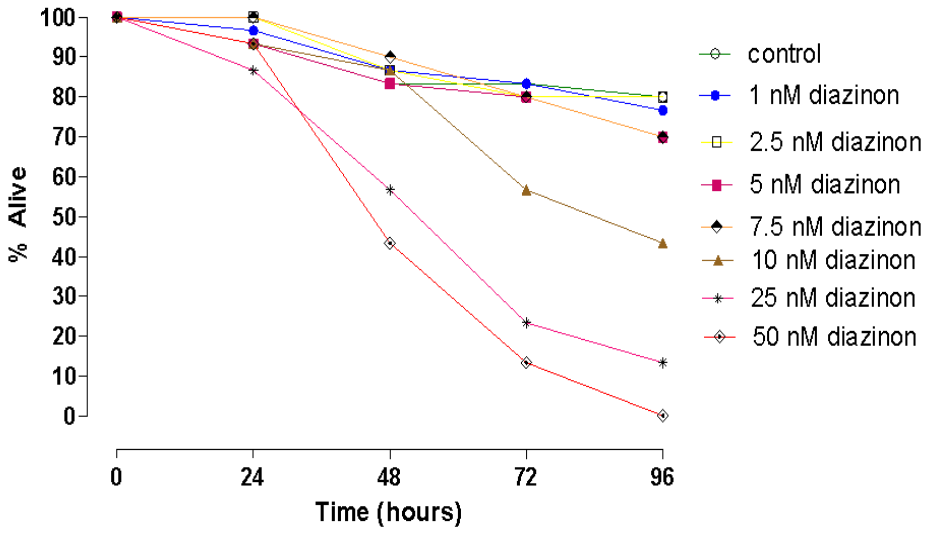


Figure 3.5 The time dependent survival of G. pulex exposed to different concentrations of diazinon in the commercial formulation (n=30, 10 organisms each replicate).

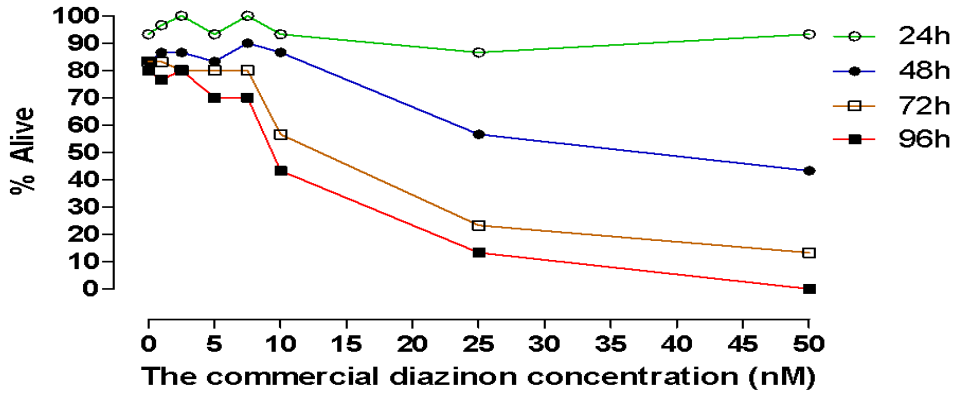


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Figure 3.6 Survival of G. pulex after exposure to various concentrations of diazinon in the commercial formulation for different time intervals (n=30, 10 organisms each replicate)

The relative toxicities of the chemicals can be represented by a composite graph (Figure 3.7) that directly compares the toxicity dose response curve. It took 96 h exposure to 50 nM of diazinon in the commercial diazinon formulation, 100 nM of diazinon oxon or more than 250 nM of diazinon to kill all test animals.

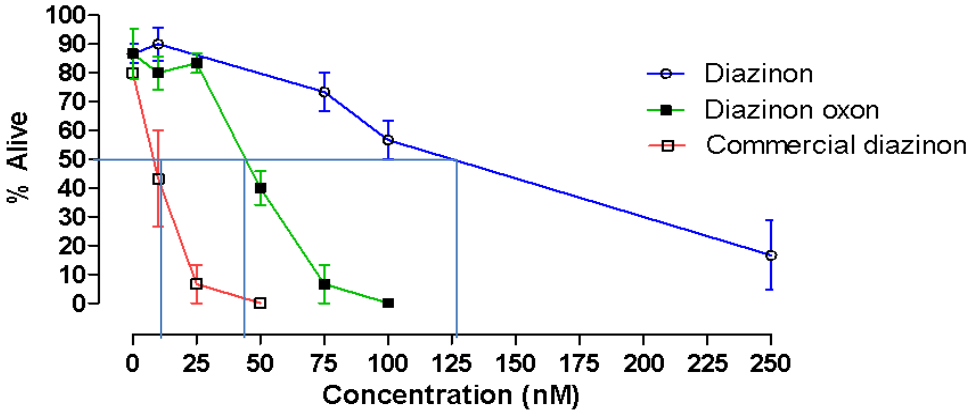

Figure 3.7 Survival of G. pulex after exposure to various concentrations of diazinon, diazinon oxon and diazinon in commercial formulation for 96 h (n = 3, 10 animals per replicate).

LC_{50} determination

Table 3.1 and Figures 3.1 to 3.6 demonstrate that the effect of exposure to diazinon, diazinon in the commercial formulation and the diazinon metabolite, diazinon oxon, is both concentration and time dependent.

Table 3.1 Trimmed Spearman-Karber analysis of toxicity data.

Exposure time / Statistics	Diazinon			Diazinon oxon			Diazinon in the commercial formulation		
	48h	72h	96h	48h	72h	96h	48h	72h	96h
LC_{50} nM	923. 1	222.6	140.1	87.5	65.3	45.7	-	16.1	10.4
Upper 95% Confidence Interval	1585.4	300.4	183.2	101.4	73.1	51.6	-	20.7	14.1
Lower 95% Confidence Interval	537.5	160.7	107.1	75.5	58.3	40.5	-	12.5	10.4

The data in Table 3.1 indicate that diazinon in the commercial formulation is significantly more toxic to *G. pulex* than diazinon oxon, which in turn is significantly more toxic than diazinon. This demonstrates the need to consider not just pure substances, but the formulation that is released into the environment.

The data were analysed with the use of the Trimmed Spearman-Karber method to estimate LC_{50} values and 95% confidence intervals (available from the US Environmental Protection Agency at http//:www.epa.gov/nerleerd/stat2.htm) and are given in nM. The 24 h LC_{50} value could not be calculated because mortality did not exceed 50 % in any treatment.

3.2.2 Behavioural experiments

Phototactic and Geotactic behaviour

The phototactic and geotactic behaviour of *G. pulex* after exposure to diazinon are shown in Figures 3.8 and 3.9. The ~~group~~ controls were more stable than the exposed groups butm nnnn there was no significant effect of diazinon exposure on the phototactic and geotactic behaviour of *G. pulex* for exposure to up to 100 nM diazinon for 96 h.

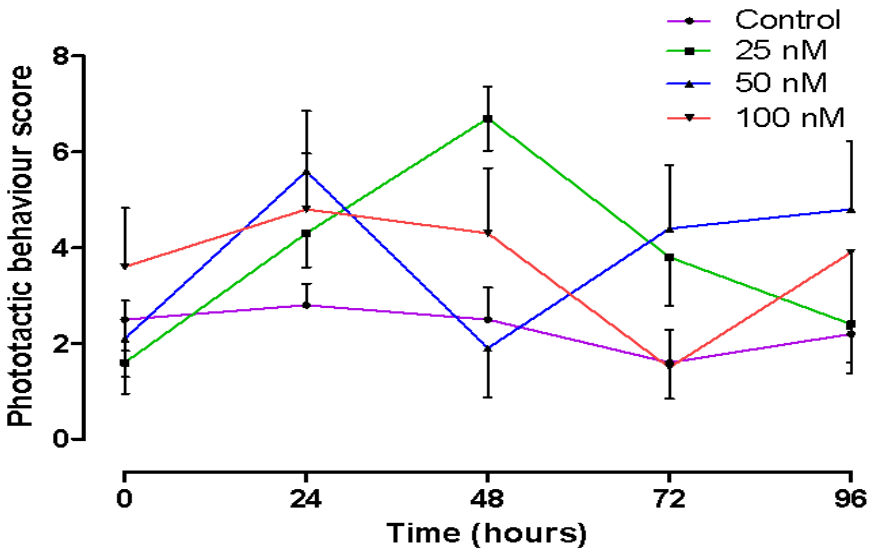

Figure 3.8 Effect of diazinon on phototactic behaviour of G. pulex

(means ± s.e, n = 10, one animal each tube).

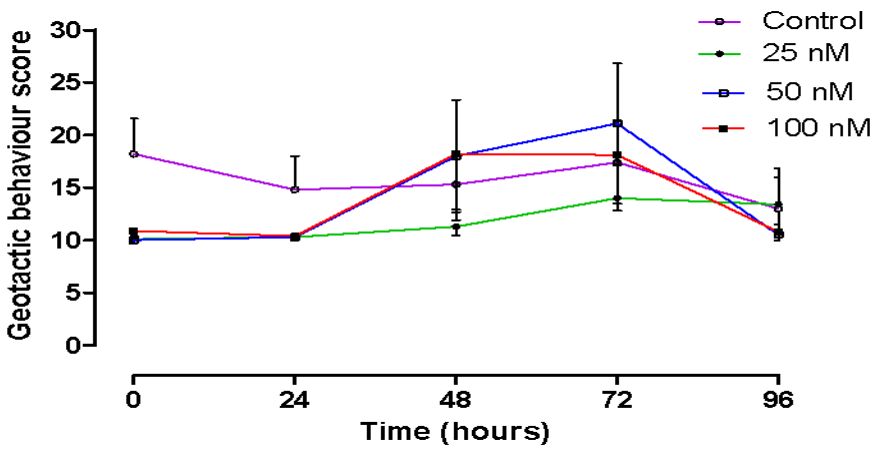

Figure 3.9 Effect of diazinon on geotactic behaviour of G. pulex

(means ± s.e, n = 10, one animal each tube).

Horizontal movement

The influence of diazinon on the horizontal movement behaviour of *G pulex* is shown in Figure 3.10. There was no significant effect of diazinon exposure on the horizontal movement behaviour of *G. pulex* for up to 100 nM diazinon for 24 h.

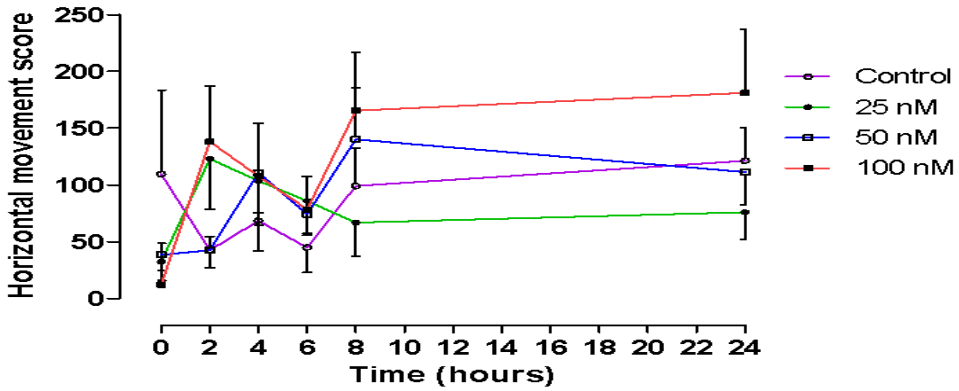

Figure 3.10 Effect of diazinon on horizontal movement in G. pulex

(means ± s.e, n = 10, one animal each petri dish).

Vertical movement activity

The effect of diazinon, diazinon oxon and diazinon in the commercial formulation on the vertical movement of *G. pulex* are shown in Figure 3.11. Diazinon at a concentration of 50 nM or greater significantly inhibited the number of times *G. pulex* came to the surface after 24 hours of exposure ($p<0.05$). Diazinon in the commercial formulation at a concentration of 25 nM or greater significantly reduced the number of surfacing of *G. pulex* came to the surface compared to both control and diazinon treated animals ($p<0.001$). However, there was no significant difference in the number of times of *G. pulex* surfaced between the control and the animal exposed up to 100 nM diazinon oxon.

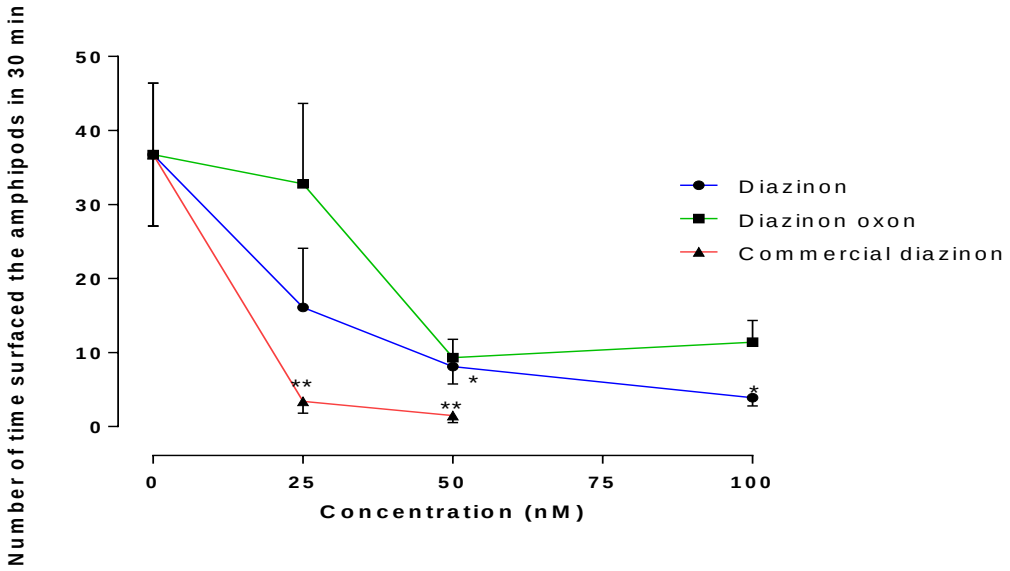

*Figure 3.11 Effect of diazinon, diazinon oxon and diazinon in the commercial formulation on the vertical swimming of G. pulex after 24 h exposure (means ± s.e, n = 10, * p<0.05 and ** p<0.001).*

Feeding behaviour

The effect of diazinon, diazinon oxon and diazinon in the commercial formulation on the feeding behaviour of *G. pulex* after 24 and 48 h exposure are shown in Figures 3.12 and 3.13, up to 25 nM diazinon oxon had no significant effect (one way ANOVA test). The feeding rate assay did not show any significant differences between animals exposed up to 25 nM of diazinon or diazinon oxon, and control for 24 h. However, the feeding rate after 48 h was significantly inhibited at 10 and 25 nM diazinon ($p<0.01$). In contrast, the feeding rate of *G. pulex* treated with 5 to 25 nM of diazinon in the commercial formulation was significantly lower than the control for both 24 and 48 h exposure.

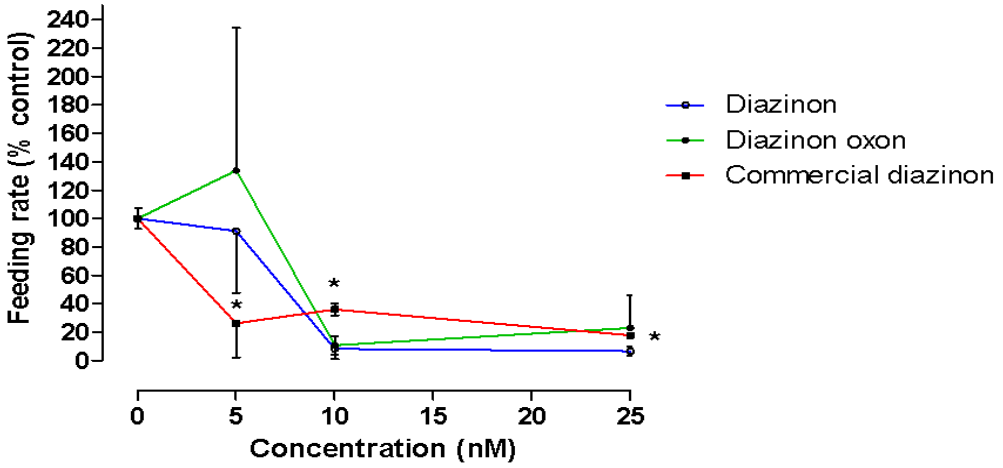

*Figure 3.12 Effect of diazinon, diazinon oxon and diazinon in the commercial formulation on the feeding rate of G. pulex after 24 h exposure (means ± s.e, n = 3, * p<0.01).*

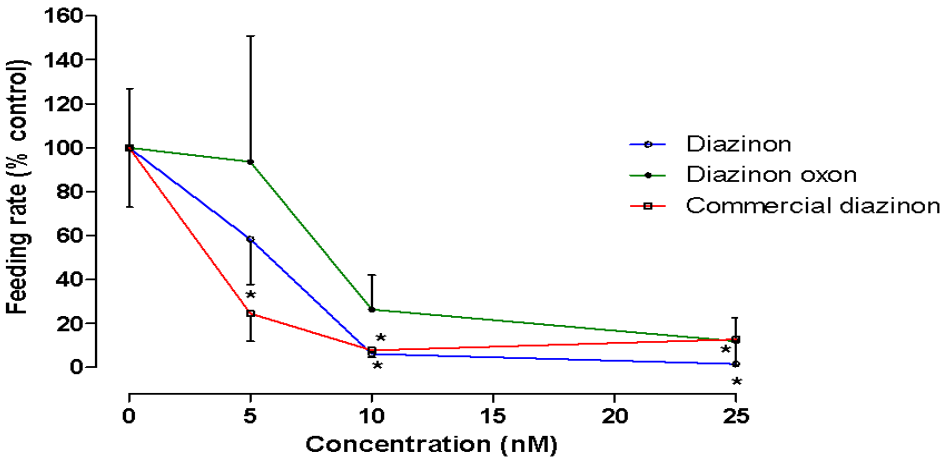

*Figure3.13 Effect of diazinon, diazinon oxon and diazinon in the commercial formulation on the feeding rate of G. pulex after 48 h exposure (means ± s.e, n = 3, * p<0.01).*

3.3 Discussion

Despite the environmental importance of *G. pulex*, there is very little information on the toxicity of organophosphate pesticides towards this organism. The data presented in this study show that the toxicity of diazinon in the commercial diazinon formulation, diazinon oxon and diazinon on *G. pulex* increased with increasing exposure period and concentration. In the present study, the 96 h LC_{50} diazinon in the commercial formulation was 13.5 fold and 4.4 fold more toxic than diazinon and diazinon oxon, respectively. This may be due to the presence of other compounds such as sulfotep in the commercial diazinon formulation, which may be more toxic than diazinon itself towards *G. pulex.* Ashauer *et al*. (2010) found that LC_{50} values for diazinon in *G. pulex* were 2 7.8, 18.7 and 13.6 nM for 48 h, 72 h and

96 h of exposure time, respectively. This difference may be due to differences between populations, the chemical composition of the experimental water, experimental conditions or may be due to dissolved organic matter (DOM). For example Kim *et al* (1999) found that the toxicity of copper to *Ceriodaphnia dubia* decreased with increasing copper–DOM reaction time. There are also differences between related species. For example, Johnson and Finley (1980) observed that *G. fasciatus* was very sensitive to diazinon with a 96 h LC_{50} of 7 nM, but a value of only 542 pM (165 ng/L) was recorded for *G. pseudolimnaeus* (Hall and Anderson, 2005). The 24 and 96 h LC_{50} values of diazinon for the tropical freshwater shrimp *Caridina laevis* were 2.5 µM (0.76 mg/l) and 1.9 µM (0.59 mg/L), respectively (Sucahyo *et al*., 2008). The 48 h LC_{50} of diazinon for other crustaceans was found 3 nM (0.92 µg/l) for *D. magna* and 7.9 nM (2.39 µg/l) for *Ceriodaphnia dubia* (Burkepile *et al*., 2000).

There are changes in the acute toxicity of diazinon for many fish species; the 96 h LC_{50} value of diazinon for fish ranged from tenths to several tens of milligrams per litre (Köprücü *et al*., 2006). Aydın and Köprücü (2005) found that the common carp *Cyprinus carpio* was sensitive to diazinon, with a 96 h LC_{50} value of 5 µM (1.5 mg/L). The Office of Pesticide Programs (2000) stated that the 96 h LC_{50} values of diazinon were between 459.9 nM to 1.5 µM (0.1–0.5 mg/L) for bluegill fry *Lepomis macrochirus*, 4.8 µM (1.5 mg/L) for sheepshead minnow *Cyprinodon variegates*, 5.4 µM (1.7 mg/L) for rainbow trout fry *Oncorhynchus mykis*, and 25.6 µM (7.8 mg/L) for fathead minnow *Pimephales minnow*. Oh *et al*. (1991) reported that three factors cause the selective toxicity of diazinon for various fish species. These include differences in the inhibition of AChE, as well as differences in the absorption and detoxification of pesticides. For example, Oh *et al*. (1991) indicated that Killifish appeared to absorb 4.5 times more diazinon than loach. Authors also observed that Killifish metabolize the diazinon much more actively than loach does.

The result in the present study is consistent with other studies that have compared parent and oxon forms diazinon. For example, the oxon derivatives of diazinon, malathion and chlorpyrifos were significantly more toxic than their parent. Diazinon oxon was approximately 10 times more toxic than diazinon for larval frog *Rana boylii* and malathion oxon was about 100 times more toxic than its parent. Similarly, 14.3 nM (0.005 mg/l) chlorpyrifos oxon killed all of larval *Rana boylii* and was at least 100 times more toxic than the concentration of chlorpyrifos that caused no mortality (Sparling and Fellers, 2007). Another study by Tsuda *et al*. (1997) also reported that the 48 h LC_{50} for diazinon oxon was 722.9 nM (0.22 mg/l) for the killifish *Oryzias latipes*, whilst it was 14.5 µM (4.4 mg/l) for diazinon.

There are also differences in sensitivity of *G. pulex* to other organophosphate pesticides when compared with other gammarids. For example, the 96 h LC_{50} value for malathion was 1 nM (Ashauer *et al*., 2011), but 19.8 nM for fenitrothion and 12.2 nM for methyl parathion. However, for *G. fossarum* the 96 h LC_{50} value was 10.4 nM for fenitrothion and 9.6 nM for methyl parathion (Kuhn and Streit, 1994). Several organophosphate pesticides have been used in acute toxicity tests on different aquatic crustaceans. For example, Roast *et al*. (1999b) reported that chlorpyrifos was more toxic to *Neomysis integer* (Crustacean: Mysidacea) than dimethoate. The 96 h LC_{50}values were 0.5 nM and 2.3 µM for chlorpyrifos and dimethoate respectively. Furthermore, the 96 h LC_{50} values of chlorpyrifos and malathion for *G. palustris* were 856 pM (0.30 µg/L) and 14 nM (4.7µg/L) respectively (Leight and Van Dolah, 1999).

Other mechanisms may be affected by exposure of organisms to toxicants. For example, chlorpyrifos and endosulfan caused changes in the oxygen consumption rate of the freshwater crab *Trichodactylus borellianus* (Montagna and Collins, 2008), who also indicated that the oxygen consumption rate shifts occurred at 6 µM (2500 µg/l)

endosulfan and this may also be related to behaviour abnormalities. The effect on physiological performance had an influence on muscle activity affecting respiratory capacities and energy expenditure. In minnow, *Pimephales promelas* embryos a significant increase was observed in oxygen consumption rate upon exposure to 3.8 µM (1000 µg/L) pentachlorophenol, 10.9 pM (0.002 µg/L) cadmium chloride, and 695.5 nM (150 µg/L) atrazine for 2 h. (Sanchez *et al*., 2008). Chlorpyrifos also caused a significant increase oxygen consumption rate, but it reduced egestion rate and scope for growth of the mysid *Neomysis integer* (Roast *et al*., 1999b).

Reproductive development has a metabolic cost. A considerable amount of energy is needed to produce a batch of eggs and brood them (Sutcliffe, 1992). Thus, allocation of resources to reproduction may be reduced by pollutants. Any effect on reproduction will have effects on both the organismic and population levels (Meliyan, 1991).

It is suggested that any pollutant inducing effects on reproductive behaviour in *G. pulex* might potentially influence the population dynamics of this organism in the field. The reproductive traits of *G. pulex* were found to be very sensitive to esfenvalerate (Cold and Forbes, 2004). The number of precopula pairs, release of eggs or offspring from the brood pouch, and substantial delays in pair formation and subsequent reproduction were disrupted after exposure of *G. pulex* to 119 pM (0.05 µg/l) esfenvalerate for 1 h (Cold and Forbes, 2004). The toxicity of phenethyl isothiocyanate may be responsible for separation of reproductive pairs, and have implications for the sustainability or survival of populations of *G. pulex* (Dixon and Shaw, 2011). Heckmann *et al*., (2005) observed that no pre-copulatory behaviour of *G. pulex* was observed in the highest treatment 37 nM (16.7 µg/l) of lambda-cyhalothrin, because of nearly total mortality. However, the numbers of pre-copulatory pairs was significantly lower between individuals exposed to 30-min pulses of

778 pM (0.35 µg/l) and 4.4 nM (1.97 µg/l) at 72 and 120 h post exposure, respectively. The processes of pairing, copulation, fertilization and egg laying in the marsupium of *G. kischin-effensis* were affected by exposure to pesticides such as propiconazol and lindane (Meliyan, 1991). Blockwell *et al*., (1996) observed that 20.6 nM (6 µg/L) lindane decreased the growth of juvenile *G. pulex* during the 14 days test period. There were a decrease in viable offspring size and an increase in the number of aborted broods in *G. pulex* females after exposure to zinc for 4 weeks. Authors attributed these influences to decrease in the energy available for reproduction and growth, possibly *via* a decrease in feeding activity in metal exposed females (Maltby and Naylor, 1990).

A variety of studies have used *G. pulex* as a model to determine LC_{50} values of several pesticides (Table 1.1) which can be need to determine sublethal concentrations of these chemicals. Therefore a range of sublethal experiments have been developed for *G. pulex,* such as feeding rate (Alonso *et al*., 2009), percentage of the population (Taylor *et al*., 1994), growth (Blockwell *et al*., 1996), and oxygen consumption (Kedwards *et al*., 1996). All these experiments can be used to detect environmentally relevant concentrations. On the other hand, they are all effectual by one or more practical problems, such as requiring relatively expensive equipment, requiring substantial operator input whilst the experiment is running or longer exposure times (Lloyd Mills *et al*., 2006).

Movement behaviour has been widely used to assess the effect of environmental contamination on aquatic organisms. For example, *D. magna* exposed to 1.6 µM (37 µg/L) dimethoate for 15 min exhibited significantly increased phototactic behaviour (Martins *et al.*, 2007). Kwak *et al*. (2002) chose a variety of movement patterns of medaka *Oryzias latipes* and used artificial neural networks to model diazinon induced alternations in movement. They found that 328.6 nM (0.1

mg/L) diazinon over 2 days significantly increased the shaking movement of medaka, but reduced their smooth behaviour. Abnormal behaviours of fish after exposure to OP may be linked to the inability to release energy, this could cause severe stress, leading to the death of animals (Dembélé *et al*., 2000).

In gammarids, as elsewhere movement is essential to find food, avoid predation and obtain mates. Any poison that interferes with these behaviours could possibly reduction the fitness of affected animals (Lloyd Mills *et al*., 2006). It is clear that vertical swimming activity was a more sensitive indicator of diazinon exposure than horizontal movement behaviour in *G. pulex*. Twenty four hours exposure to 25 nM or above of the diazinon in the commercial formulation, or 50 nM or above of diazinon significantly inhibited vertical swimming activity, but up 100 nM diazinon had no significant effect on horizontal movement. This may be due to vertical swimming requiring more energy than horizontal swimming. An alternative explanation that the pesticide induces a disturbance in normal geotactic behaviour is not supported. The experiments that attempted to demonstrate this failed to find any significant effect (Figure 3.9). Baillieul and Blust (1999) have reported that the swimming activity of freshwater organisms such as the cladoceran *D. magna* (Straus) is closely linked to energy metabolism and external environmental conditions.

Several studies carried out to determine the effects of pollutants on the physiology of aquatic animals have led to the development of behavioural markers that can be used for environmental measurements. One of the important behavioural endpoints is feeding, as decrease in feeding rate may lead to significant reductions in growth rate and reproductive fitness (McLoughlin *et al*., 2000). Some studies have shown that feeding activity is a sensitive indicator of toxic stress in both marine and freshwater species (Maltby *et al*., 1990; Satapornvanit, 2006). McLoughlin *et al*. (2000) indicated that, in *G.*

pulex exposed to the OP pirimiphos-methyl, feeding activity was 13-fold more sensitive to the toxicity of this pesticide than AChE activity. Similarly, in the present study, feeding rate was a sensitive endpoint, being able to discriminate between control, diazinon and the commercial diazinon formulation treatments. However, diazinon oxon did not induce significant changes in feeding rate in *G. pulex* exposed to up to 25 nM for 24 and 48 h. Diazinon only caused significant inhibition after 48 h exposure at 10 and 25 nM.

In contrast, the commercial diazinon formulation significantly inhibited the feeding behaviour of *G. pulex* after 24 and 48 h exposure at 5 nM or above. A possible explanation of the observed decrease in the feeding rate of *G. pulex* may be due to poor energy utilisation and expenditure of more energy by metabolism when the organism was under stress, or the ability to capture food decreased due to inhibition of AChE. Sandahl *et al*. (2005) found that brain AChE inhibition of juvenile coho salmon (*Oncorhynchus kisutch)* correlated with reductions in feeding activity and spontaneous swimming activity after exposure to 7 µM (2.5 mg/L) chlorpyrifos for 96 h.

Diazinon exposure also decreased the feeding rate of *D. magna* at 1.5 µM (0.5 µg/L) for 24 h (Fernandez-Casalderrey *et al*., 1994). Yuan *et al*. (2004) found that the feeding rate of freshwater swamp shrimps *Macrobrachium nipponense* was reduced to 82.5% of the untreated group when exposed to 30.3 nM (0.01 mg/L) malathion for 24 h, but it was 49.15% of the control for 7 day exposure. Xuereb *et al.* (2009) found that 2.7 nM chlorpyrifos reduced the feeding rate (41.5%) of *G. fossarum* for 96 h. However, the effect of pesticides on the feeding activity of shrimps will depend on the organisms, pesticides and the experimental conditions, such as exposure method, time and temperature (Tu *et al*., 2010). Studies on effects of sublethal concentrations of toxins on the behaviour, feeding and reproduction of organisms have been increasing awareness in recent years. Since the

level of environmental contamination might be low, it may be possible to use behavioural assays rather than acute toxicity tests to focus on survival (Fernandez-Casalderrey *et al*., 1994). A variety of ecotoxicity tests with non-lethal toxicities have been carried out on *G. pulex* (Table 3.2).

Table 3.2 Comparison of non-lethal ecotoxicity tests with G. pulex

Toxic	96 h LC_{50}	Marker	Reference
Cadmium	730.4 µM (82.1 µg/l)	Feeding rate significantly decreased at 133.4 µM (15 µg/l) for 168 h	Felten *et al*. (2008)
		Movement activity significant decreased at 66.7 µM (7.5 µg/l) for 120 h	
Carbofuran	40.7 µM (9 µg/l)	Feeding rate significant decreased at 13.6 µM (3 µg/l) for 168 h	Matthiessen *et al*. 1995
Esfenvalerate	314.4 nM (0.132 µg/l)	Reproductive traits disrupted at 119 nM (0.05 µg/l) for 1 h	Cold and Forbes (2004)
pirimiphos-methyl	9 µM (2.78 µg/l)	Feeding rate significantly decreased at 19.7 nM (0.006 µg/l) for 144 h	McLoughlin *et al*. 2000
Diazinon	140.1 nM (42.6 µg/l)	Feeding rate significantly decreased at 10 nM (3 µg/l) for 48 h	In the present study
		Vertical swimming significantly decreased at 50 nM (15.2 µg/l) for 24 h	

CHAPTER FOUR: Acetylcholinesterase

4.1 Introduction

The main mechanism of toxicity of many OP pesticides has been identified as inhibition of acetylcholinesterase (AChE). This enzyme breaks down the neurotransmitter acetylcholine, which restricts cholinergic stimulation. OP pesticides disrupt the function of AChE by making it unable to breakdown acetylcholine, which is important in cholinergic nerves. Inhibition of AChE cause an accumulation of acetylcholine in the synaptic cleft, which leads to convulsions, neuromuscular block and respiratory failure.

OPs are highly neurotoxic. These pesticides have been designed to be effective inhibitors of AChE located at neuromuscular junctions in the central and peripheral nervous system (Ansari and Kumar, 1984; Fowler *et al*., 2001; Barata *et al*., 2004; Rickwood and Galloway, 2004; Sandahl *et al*., 2005). Since the 1970s, the inhibition of AChE activity has been widely used to assess the environmental impact of selected pollutants in many parts of the world (Xuereb *et al*., 2007; Falfushynska and Stolyar, 2009; Mdegela *et al*., 2010). A range of studies have successfully used AChE inhibition in invertebrates as a bioindicator of pesticide exposure. These studies have measured AChE activity in different extracts such as hemolymph, nerve ganglia, muscle tissue, and whole body tissue (Dembélé *et al*., 2000; Fulton and Key, 2001; García-de La Parra *et al*., 2006 ; Xuereb *et al*., 2009; Tu *et al*., 2010). Edwards and Fisher (1991) have indicated that AChE activity in many aquatic species can be significantly inhibited by sub-lethal exposure to pesticides. These authors suggested that this enzyme could be a sensitive bioindicator with which to assess the effect of pesticides in the environment.

Monitoring of AChE in environmental fauna might offer many advantages over the use of chemical analysis alone. The inhibition of AChE is the principal mechanism by which OP pesticides produce toxicity. Rompas *et al*. (1989) observed that AChE inhibition was more sensitive to the oxon-forms of OP compared to the thiono-forms in tiger shrimp larvae. The utility of biomarkers is thus directly related to the chemical's toxic way of action. When significant inhibition of AChE activity is found, it not only means that the species has been exposed, but also that enough of the toxin has reached the target site to cause a physiological influence. Furthermore, most OP pesticides are rapidly degraded in the environment. Their concentrations might decrease below measurable levels in hours to days. In contrast, AChE inhibition in many organisms persists for up to several weeks (Post and Leasure, 1974; Fulton and Key, 2001). *Gammarus* is one of the most sensitive genera of organisms to anti-cholinesterase compounds (Kuhn and Streit, 1994; Xuereb *et al.*, 2007). Alterations in the ACh neuronal of pathways leads to disturbance of higher integrated processes. For example, the feeding rate and locomotory impairment of *G. fossarum* were directly related to levels of AChE inhibition for both the OP pesticide chlorpyrifos and the carbamate pesticide methomyl (Xuereb *et al*., 2009).

In almost every case of diazinon toxicity a decrease in cholinesterase activity levels is observed. Diazinon exerts its toxic influence *via* the binding of its oxygen analogue to AChE for a significant time post exposure (Pan and Dutta, 1998). However, diazinon also affects other metabolic activities in exposed animals such as antioxidant defence systems and lipid peroxidation, and the combined effect leads to death (Ansari *et al*., 1987; Oruc, 2010).

Diazinon has a variety of different trade names including Knox-out, Dianon, Diazintol and Basudin (Adedeji, 2011). Various studies have evaluated the effect of diazinon in commercial formulation on AChE

activity in aquatic organisms. For example, following exposure to Basudin 60EM, brain AChE activity of the freshwater guppy fish *Poecilia reticulate* was significantly inhibited by the equivalent of 492.8 nM (150 µg/L) diazinon. In contrast, the same exposure level did not inhibit the muscle and gill AChE activity (Sharbidre *et al*., 2011). Banaee *et al*. (2011) observed that 328.6 nM (0.1 mg/L) of diazinon in Basudin 60EM significantly reduced AChE activity in the plasma of the rainbow trout *Oncorhynchus mykiss* after 7 days exposure. Pan and Dutta (1998) found that 1/10 of the 24-h LC_{50} 1.18 µM (90 µg/L) of diazinon from the formulation produced by Celex Corp significantly inhibited juvenile largemouth bass *Micropterus salmoides* brain AChE activity. The inhibition of AChE can produce physiological and behavioural modifications that decrease survival of the animals (Pan and Dutta, 1998).

In vitro assays are relatively quick to perform compared to *in vivo* experiments. These include measurement of key enzymes such as AChE and the effect of sublethal exposure on their activity (Ibrahim *et al*., 1998). *In vitro* studies have found that organisms from various classes exhibit large differences in sensitivity to organophosphates and carbamates (Wang and Murphy, 1982). *In vitro* assays have also shown that AChE is involved in the resistance to different insecticides in many species of aquatic organisms including, the shrimp *Palaemon serratus* (Galgani and Bocquene, 1990), channel catfish fingerling (Straus and Chambers, 1990), the freshwater snail *Potamopyrgus antipodarum* (Gagnaire *et al*., 2008) and *G. pulex* (Xuereb *et al*., 2009).

In vivo experiments are more time consuming than *in vitro* assays. However, these take additional factors into account. It is vital to evaluate these before the use of AChE activity as a response indicator in aquatic bioassays. Especially, the reaction to inhibitory pesticides such as OPs has to be demonstrated if AChE inhibition is to have a

diagnostic value (Ibrahim *et al*., 1998). Several studies have used *in vivo* methods to determine the effect of OP pesticides on the AChE activity of invertebrate organisms such as *G. pulex* (Kuhn and Streit, 1994), the shrimp *Litopenaeus vannamei* (García-de La Parra *et al*., 2006) and *G. fossarum* (Xuereb *et al*., 2009). Xuereb *et al*., (2007) indicated that *G. pulex* have only one ChE which displays the typical properties of an AChE.

The aim of this study was to evaluate the effect of the OP pesticide diazinon on AChE activity of *G. pulex*. This was compared to the diazinon metabolite diazinon oxon and diazinon in the commercial formulation Osmond's Gold Fleece. The time course of development of AChE inhibition was also investigated.

4.2 Results

In vitro exposure

In vitro exposure up to 10 µM diazinon or diazinon in the commercial formulation did not lead to inhibition of AChE activity in *G. pulex* extracts (Figure 4.1). In contrast, diazinon oxon at 5 µM or greater induced significant inhibition of AChE activity compared to the control extract ($p<0.001$), with an IC_{50} value of 3 µM.

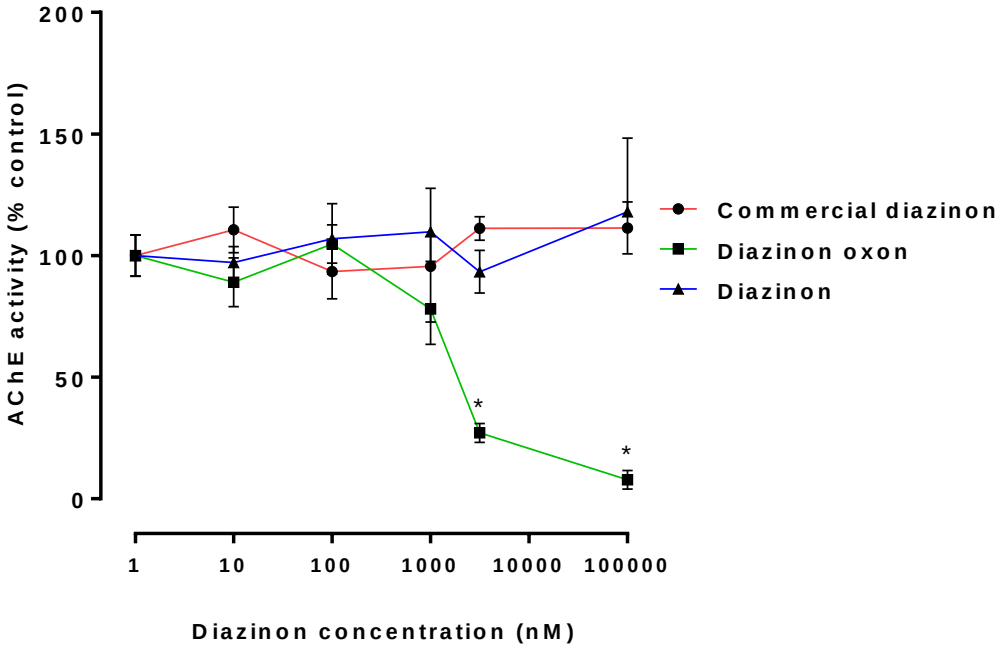

Figure 4.1 Effect of diazinon, diazinon oxon and diazinon in the commercial formulation on the in vitro acetylcholinesterase activity of G. pulex (means ± s.e., n = 3, significantly different from control p<0.001).*

In vivo exposure

Twenty four hour exposure

Following *in vivo* exposure for 24 h, diazinon significantly inhibited the AChE activity of *G. pulex* at 10 nM or greater ($p<0.001$), whilst diazinon in the commercial formulation caused significant inhibition at 5 nM and above ($p<0.001$) (Figure 4.2). The IC_{50} values were 10 nM for diazinon in the commercial formulation and 17.5 nM for diazinon. However, diazinon oxon did not cause a significant inhibition of enzyme activity up to 50 nM.

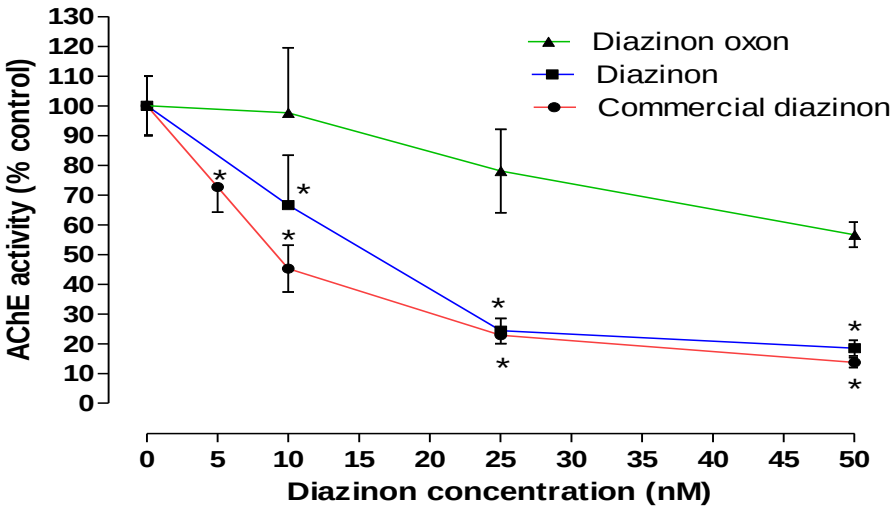

Figure 4.2. Effect of diazinon, diazinon oxon and diazinon in the commercial formulation on the acetylcholinesterase activity of G. pulex after 24 h exposure (means ± s.e., n = 3; significantly different from control <0.001).*

Time course experiments

There was no significant effect of 10 nM diazinon at any time point up to 24 h (Figure 4.3). However, *G. pulex* exposed to 25 nM diazinon exhibited a significant level of AChE inhibition from 12 h to 24 h (P< 0.001).

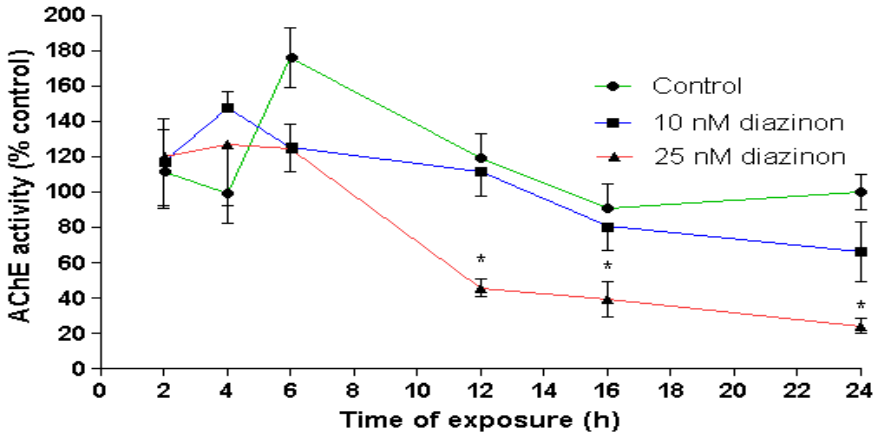

Figure 4.3 Time course for the effect of diazinon on the acetylcholinesterase activity of G. pulex (means ± s.e., n = 3; significantly different from control p<0.001).*

In contrast, neither 10 nor 25 nM diazinon oxon had any significant effect on the AChE activity of *G. pulex* for up to 24 h exposure (Figure 4.4).

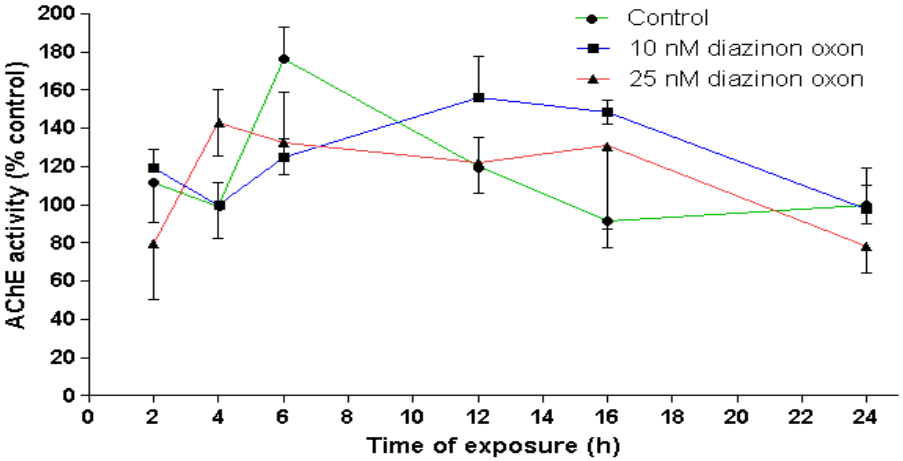

Figure 4.4 Time course for the effect of diazinon oxon on the acetylcholinesterase activity of G. pulex (means ± s.e., n = 3).

At 10 nM diazinon in the commercial formulation, AChE activity was significantly inhibited only after 24 h ($p<0.005$). However, *G. pulex* exposed to 25 nM of diazinon in the commercial formulation exhibited significant inhibition after only 16 h ($p<0.001$).

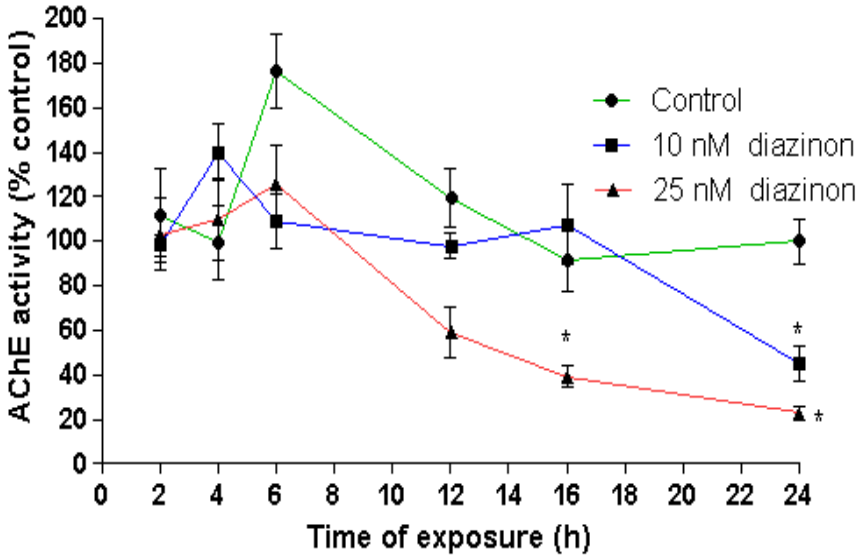

Figure 4.5 Time course for the effect of diazinon in the commercial formulation on the acetylcholinesterase activity of G. pulex (means ± s.e., n = 3, significantly different from control p<0.001).*

The effect of AChE on feeding rate and vertical movement activity

Figures 3.11- 3.13 demonstrate that diazinon exposure impairs both vertical swimming activity and feeding rate. The major mechanism of diazinon toxicity is AChE inhibition. By directly comparing inhibition of vertical swimming and feeding rate with the reduction in AChE activity, the importance of AChE inhibition of these processes can be estimated. Figures 4.6 and 4.7 show that there is no direct relationship between

AChE activity and feeding rate, or AChE activity and vertical movement. Unfortunatley due to lack of data (5 nM AChE activity lacking for diazinon and diazinon oxon, 50 nM for feeding and 5 and 10 nM for vertical movement) there are only 3 data points for each pesticide for each graph. Even so, it does demonstrate that behaviour is not simply governed by AChE activity.

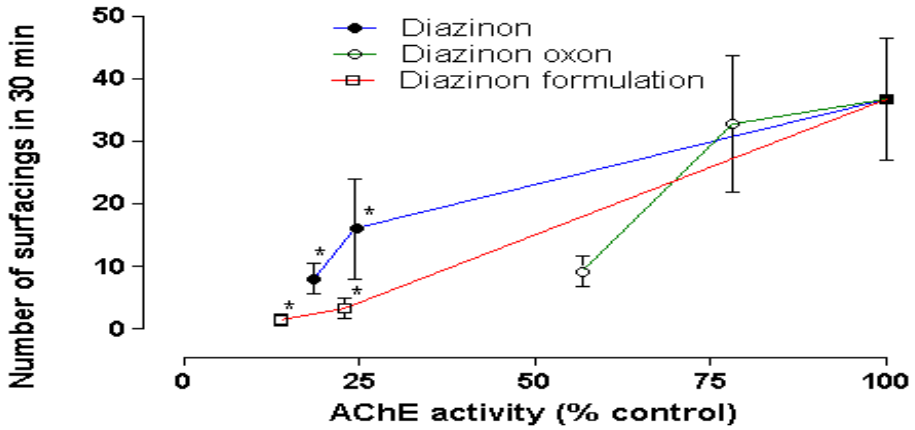

Figure 4.6 Vertical movement and AChE activity (means ± s.e., n = 3; significantly different from control p<0.001)*

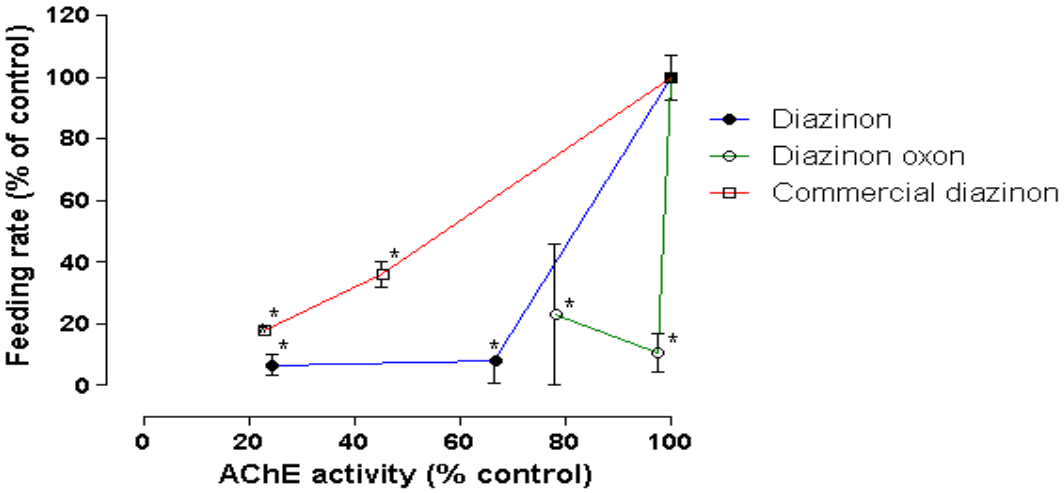

Figure 4.7 Feeding rate and AChE activity (means ± s.e., n = 3; significantly different from control, p<0.001 for diazinon and diazinon in the commercial formulation and p = 0.009 for diazinon oxon).*

4.3 Discussion

The inhibition of AChE activity has been extensively used as a biomarker to evaluate the effect of pesticides or other water contaminants on aquatic organisms such as invertebrates, and vertebrates such as fish. The activity of AChE is vital for many physiological functions of aquatic organisms, such as prey location, predator evasion, and orientation toward food (Roex *et al*., 2003; Adedeji, 2011). Many studies have used various tissues of freshwater organisms, such as brain, gills and muscle to assess the activity of AChE. In contrast, other studies have used the whole body. In this study, preliminary experiments determined that the 9000g supernatant of whole body extracts of *G. pulex* was a good model to assess the effect of diazinon on AChE activity.

The present study shows that AChE activity of *G. pulex* was inhibited by *in vivo* exposure to diazinon and diazinon in the commercial

formulation. By contrast, up to 25 nM diazinon oxon did not inhibit the enzyme activity under *in vivo* conditions for all time points up to 24 h. However, 25 nM diazinon and diazinon in the commercial formulation caused significant inhibition of AChE activity at 12 h and 16 h exposure, respectively. This suggests that the AChE of *G. pulex* is much more sensitive to diazinon and diazinon in the commercial formulation than to diazinon oxon by *in vivo* exposure, with IC_{50} values for diazinon and diazinon in the commercial formulation of 17.5 and 10 nM, respectively. When enzyme assays were carried out following *in vitro* incubation of gammarid extracts, neither diazinon nor diazinon in the commercial formulation inhibited AChE until 10 µM, almost full inhibition (92%) was observed at a concentration of 10 µM diazinon oxon.

Possible explanations for these results include more rapid degradation and/or less uptake of diazinon oxon *in vivo*. Kretschmann *et al*. (2011a) found that the toxic metabolite diazinon oxon could hardly be detected in *D. magna* during exposure to diazinon for 21 h. They suggested that this could be due to lower accumulation than diazinon. 50% inhibition of AChE activity led to significant mortality of *G. fossarum* after exposures to chlorpyrifos. However, no mortality was observed despite 66% inhibition of AChE after exposure of *G. fossarum* to the carbonate pesticide methomyl (Xuereb *et al.,* 2009). They suggested that the death observed with chlorpyrifos may have been caused by this compound's additional modes of toxic action. However, in this study there was a relationship between the inhibition of AChE activity and parameters such as feeding and locomotory behaviours (Xuereb *et al.,* 2009). Other studies indicated different links between the change in AChE activity and immobility. For example, Printes and Callaghan (2003) found that AChE activity of *D. magna* was inhibited by 70% after exposure to 100 µM (18.3 µg/l) of the OP acephate, yet no immobility was observed. This means that

significant inhibition of AChE activity does not always lead to immobility (Jemec *et al*., 2007a).

In agreement with the current study, 591.4 nM (180 µg/L) diazinon caused significant inhibition of AChE activity (63%) of largemouth bass *Micropterus salmoides* after 24 h (Pan and Dutta, 1998), whilst 3.3 µM (1 mg/L) induced significant inhibition of AChE activity (93%) in brain of the Nile tilapia *Oreochromis niloticus* after 7 days (Üner *et al*., 2006). The activity of AChE in whole body extracts from *G. fossarum* significantly declined (53.2 %) at 1.4 nM chlorpyrifos (Xuereb *et al*., 2009). In addition, after *in vivo* exposure, chlorpyrifos induced a significant AChE inhibition with a 96-h IC_{50} of 0.99 nM, corresponding to 0.35 µg/L. However, *in vitro* exposure to chlorpyrifos caused significant inhibition (95%) of AChE activity of *G. pulex* at 100 µM. This emphasises the importance of considering *in vivo* exposure routes, whereby the kinetics of uptake, distribution, metabolism and excretion have profound influences (Xuereb *et al*,. 2007).

The accumulated chlorpyrifos in *G. pulex* is quickly eliminated, which may be due to biotransformation capacities in this organism (Ashauer *et al*, 2006). For instance, Ashauer *et al*. (2006) observed that 50% of total accumulated chlorpyrifos in *G. pulex* was eliminated through the first 72 h of the depuration phase. Berger and Sultatos *et al*. (1997) also suggested that mice exposed to fenitrothion may change estradiol metabolism by inhibition of certain P450 isozymes.

The effects of sublethal concentration of fenitrothion and parathion-methyl OPs on *G. pulex, G. fossarum* and *G. tigrinus*, indicated that *G. pulex* was the most sensitive species; fenitrothion and parathion–methyl concentrations as low as 3.6 nM fenitrothion and 2.8 nM (1 µg/L) parathion–methyl significantly inhibited AChE activity (61-65 % activity) in *G. pulex* after 24 h; however the least sensitive species was *G. tigrinus*. Significant AChE inhibition might not be observed at lower

than 10.8 nM (3 µg/L) fenitrothion (80% activity) and lower than 13.8 nM (5 µg/L) parathion–methyl (83% activity) in *G. tigrinus* (Kuhn and Streit, 1994). A clear inhibition of AChE activity of *G. pulex* was observed after exposure to 458.5 µM (140 mg/L) pirimiphos methyl OP insecticide for 8 days compared with unexposed organisms, whilst 16.4 µM (5 mg/L) caused additional lower activities onday 11 when compared with 193.2 nM (0.059 mg/L) and control treatments (Crane *et al*., 1999).

Normally, organisms exposed to diazinon exhibit a resultant reduction in AChE activity (Pan and Dutta, 1998; Üner *et al*., 2006). However, Jemec *et al*. (2007b) found that no changes in AChE activity occurred in *D. magna* after exposure to 23 nM (7µg/L) diazinon for 48 h. They suggested that this may be due to metabolism of diazinon in *D. magna*. Furthermore, the toxicity of diazinon is species-dependent. It depends on the level of bioactivation of this pesticide by conversion to the potent diazinon oxon, detoxification in the species, and the affinity of AChE for diazinon within *D. magna* (Keizer *et al*., 1995).

The greater sensitivity of fish to diazinon may be related to rates of absorption in different organisms. For example, the toxicity of diazinon to loach *Misgurnus anguillicaudatus* was 14 fold stronger than killifish and inhibition of AChE in loach by diazinon oxon 21 fold stronger than killifish. This is due, in part, to reduced uptake whereby loach appeared to absorb 4.5 times less diazinon than killifish (Oh *et al*., 1991). The excretion rate constants (k) and biological half-lives of the pesticides and their oxidation products are dependent on pesticide. For example, that the excretion rates of malaoxon and diazinon oxon could not be measured in killifish may be due decreased torpidity. However, the excretion half-life ethyl p-nitrophenyl thionobenzenephosphonate oxon (EPN oxon) (1.2 h) and fenitrothion oxon (2. 3 h) were faster than those of ethyl p-nitrophenyl thionobenzenephosphonate (EPN) (34.7 h) and fenitrothion (6.3 h) (Tsuda *et al*., 1997).

It is well known that the reduction of AChE activity disturbs numerous physiological and behavioural processes. These may be manifested as disturbances to the species' feeding activity, avoidance of predators, and spatial orientation (Pan and Dutta, 1998; García de La Parra *et al*., 2006). As observed in the current study, exposure of *G. pulex* to diazinon and diazinon in the commercial formulation for 24 h significantly inhibited AChE activity. Behaviour is not simply governed by AChE activity since there is no direct relationship between AChE and feeding rate, or AChE and vertical movement in the current study.

Death of organisms could be due to reduced activity of other enzymes, such as metabolic enzymes and digestive enzymes (Ansari and Kumar, 1984). Diazinon not only reduces AChE activity but also affects other metabolic activities such as protein metabolism in the species and the combined effect leads to mortality (Ansari *et al*., 1987; Adedeji, 2011).

In this study, the disparity between the relative toxicities of diazinon and diazinon in the commercial formulation, as indicated by LC_{50} values and inhibition of AChE activity, suggests that there are non-cholinergic toxicity targets and/ or that formulation components other than diazinon may influence toxicity.

CHAPTER FIVE: Heat shock proteins and phosphoproteins as potential molecular markers of toxicity

5.1 Introduction

Several studies aiming to develop sensitive approaches for evaluating the environmental risk of low concentrations of xenobiotic compounds have used cellular heat shock protein (HSP) responses as potential biomarkers of environmental stress (Sanders, 1993; Sanders and Martin, 1993; Werner and Nagel, 1997; Walker *et al*., 2005; Bedulina *et al*., 2010). Among different animals, HSPs are a group of intracellular proteins that have an uncommonly high degree of identity at the amino acid level. This group of proteins are normally referred to as stress proteins (Iwama *et al*., 1998; Ling *et al*., 2009), since they are induced by environmental stress such as temperature, hypoxia, or exposure to ethanol, heavy metals and pesticides (Eder *et al*., 2009; Dorts *et al*., 2009; Ceyhun *et al*., 2010).

Available data show that HSPs can reduce the harmful impact from environmentally induced disturbances on cellular function (Werner and Nagel, 1997). The expression of this group of proteins is also up-regulated by several physiological perturbations such as oxidative stress, nutritional deficiencies and ultraviolet radiation (Pockley, 2003). HSPs are expressed in cells to conserve critical cellular processes linked to protein folding, translocation and fidelity, and they enhance survival by protecting important cellular functions (Iwama *et al*., 1998). These proteins are also important in immunology, as they have been revealed to act as main antigens in the immune response to pathogens, and mechanisms involving HSPs have been implicated in

the pathogenesis of a diversity of autoimmune illnesses, including systemic lupus erythematosus and rheumatoid arthritis (Chant, 1999).

HSPs are classified by their molecular weight and they are also recognized as molecular chaperones, which help to recover the native states of proteins after their damage by external stress (Clayton *et al*., 2000; Tomala and Korona, 2008). A range of aquatic species has been extensively used to evaluate environmental contamination due to their morphological, ecological and economic significance, as well as their physiological and ecological diversity in aquatic environments. Applying toxicity tests in aquatic species is also necessary to assess the aquatic ecosystems (Guven, 1994). In the laboratory, life-history traits such as development, stress resistance, life span and fecundity can be affected by very low levels of induced HSPs (Sørensen and Loeschcke, 2001; Sørensen *et al*., 2003).

Freshwater amphipods (Crustacea) have been used as model organisms to investigate the effect of stress on HSP response (Sures and Radszuweit, 2007; Timofeyev *et al*., 2008; Shatilina *et al*., 2010). Pesticides stimulate HSP expression in aquatic species. However, this differs from one organism to another even though similar stressors are employed. For example, diazinon significantly increased the expression of HSPs at a concentration of 1.97 µM (0.6 mg /L) in the freshwater amphipod *Hyalella azteca*at, at 9.8 µM (3 mg/L) and 98.6 µM (30 mg/L) and in the marine and estuarine amphipods *Rhepoxynius abronius* and *Ampelisca abdita,* respectively (Werner and Nagel, 1997). The fact that HSPs can be induced by pesticide exposure suggests that they may represent useful markers of diazinon exposure in *G. pulex*.

Another potential biomarker could be the phosphorylation status of cellular proteins. Protein phosphorylation is a common type of post translational modification used in signal transduction. It is involved in

most cellular processes such as metabolism, growth, cell division, muscle contraction, differentiation, motility, learning and memory, membrane transport, organelle trafficking and immunity, the deregulation of which is typical of cancer and other diseases (Manning *et al*., 2002; Ubersax and Ferrell, 2007; Gamez-Pozo *et al*., 2011). Protein phosphorylation is of considerable interest, since it is involved in signal transduction, cell division and cancer (Sjaastad and Nelson, 1997; Yan *el al*., 1998). To control cellular processes such as signal transduction, gene expression, cytoskeletal regulation and the cell cycle, animals use reversible phosphorylation of proteins

Since the various sites of phosphorylation on a protein may exert different influences on activity, there is a great need for techniques that correctly determine these sites (McLachlin and Chait, 2001). Antibody detection is a commonly used approach to detect the specific phosphoamino acids in phosphoproteins. This method can be used to observe phosphorylation of proteins in sectioned material of organs or whole animals. To recognize which proteins have been phosphorylated, SDS-PAGE, followed by western blotting with the phospho-specific antibodies can be used to detect the phosphoproteins in a protein extract. Immunoblotting is a sensitive technique, which is very useful for the detection of specific residues in individual phosphoproteins (Abu-Lawi and Sultzer, 1995; Yan *el al*., 1998). Due to the availability of well characterised antibodies that recognise protein phosphorylation sites it was of interest to apply the above mentioned approaches to this study of diazinon toxicity in *G. pulex.*

Proteins are the primary effector molecules in all living systems, since any adaptive reactions to physiological, environmental or pathological conditions will be regulated by changes in protein content or activity, (Bradley *et al*., 2002; Huang and Huang, 2011). Recently, proteomic techniques have become important tools of developmental biology; two-dimensional PAGE (2D-PAGE) has been used to detect protein

changes after specific signalling events (Janini and Veenstra, 2002; Wolfgang and Craig, 2008). The separation of proteins via 2D PAGE occurs according to size as well as the charge of each respective protein (Marko-Varga, 2004). This method is suitable to detect the alterations in protein amounts as well as shifts in isoform distribution directly on the gel. The amount of protein loading in 2D-PAGE should be suitable because highly abundant proteins may mask the proteins by overlapping weaker protein spots. On the other hand, low abundance proteins may not be detectable because the amount of sample on the 2D-PAGE gel may not be sufficient, as the cellular concentration of proteins differs widely (Wilkins *et al*., 1998; Gygi *et al*., 2000; Wolfgang and Craig, 2008). With these potential drawbacks in mind, it was considered to be of interest to perform preliminary 2D-PAGE analysis for initial biomarker identification in the current work.

5.2 Results

In order to determine whether HSP levels could be indicative of toxic exposure to OPs, gammarid tissue fractions were analysed by western blotting using antibodies to HSP 24, HSP 60, HSP 70 and HSP 90. To avoid any loading errors, all 9000g supernatant and 9000g pellet results for HSPs and phosphoproteins were normalized to blots probed with anti ß-actin antibody for 9000g supernatant samples and anti HSP 60 antibody for 9000g pellet samples. Typical probed blots for these housekeeping proteins are shown in Figure 5.1. In figures 5.2-5.12 significant differences from the control (*) are the result of ANOVA followed by a Tukey test.

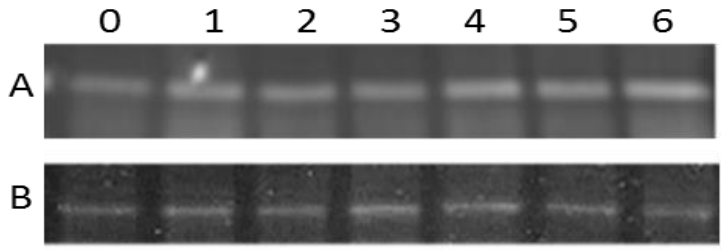

Figure 5.1 Western blots probed with, A: anti ß-actin antibody and B: anti HSP 60 antibody. Shown in panel A and B are probed blots of extracts from gammarids prepared after exposure to 10 and 50 nM of diazinon (1 & 2), diazinon oxon (3 & 4) and diazinon in the commercial formulation (5 & 6) for 24 h

5.2.1 HSPs as potential molecular markers of toxicity

Small HSP

The results presented in Figure 5.2 show that exposure to 50 nM diazinon and diazinon in the commercial formulation, but not diazinon oxon significantly increased the relative level of small HSP in supernatant samples of *G. pulex* compared to untreated control extracts ($p < 0.001$). However, small HSPs were not detected in pellet samples (not shown).

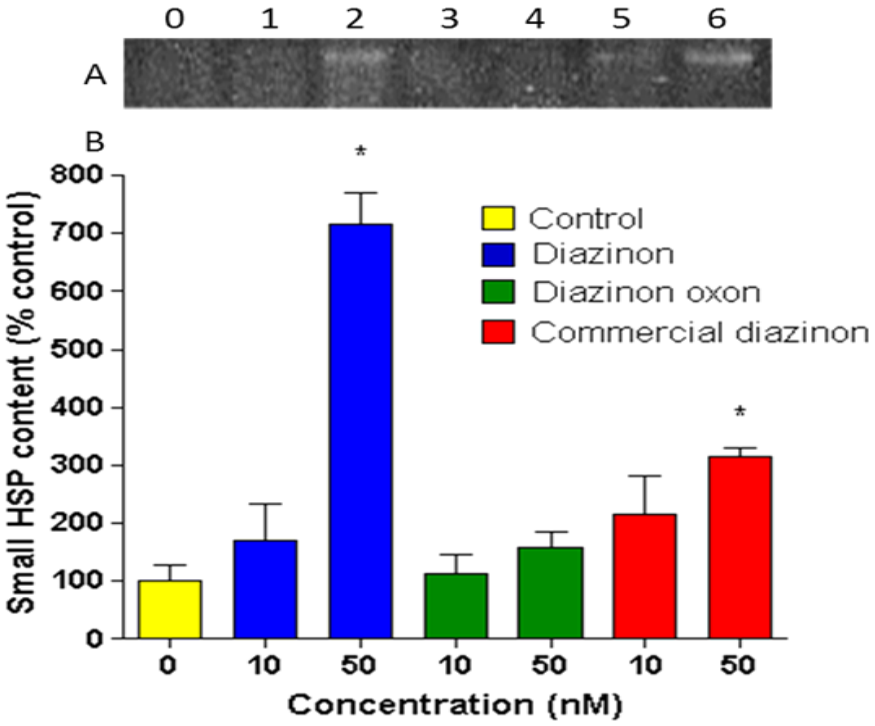

*Figure 5.2 Small HSP content in the 9000g supernatant sample of G. pulex. Shown in panel A is a probed blot of extracts from gammarids prepared after exposure to diazinon (1 & 2), diazinon oxon (3 & 4) and diazinon in the commercial formulation (5 & 6) for 24h. Panel B shows densitometric analysis, (means ± s.e., n= 3). * significantly different from control. In panel A, blots were probed with antibody to HSP 24.*

Hsp60

Up to 50 nM diazinon, diazinon oxon and diazinon in the commercial formulation did not significantly change the relative level of HSP 60 in pellet samples of *G. pulex,* compared to untreated control extracts (Figure 5.3). HSP 60 was not detected in supernatant samples (not shown).

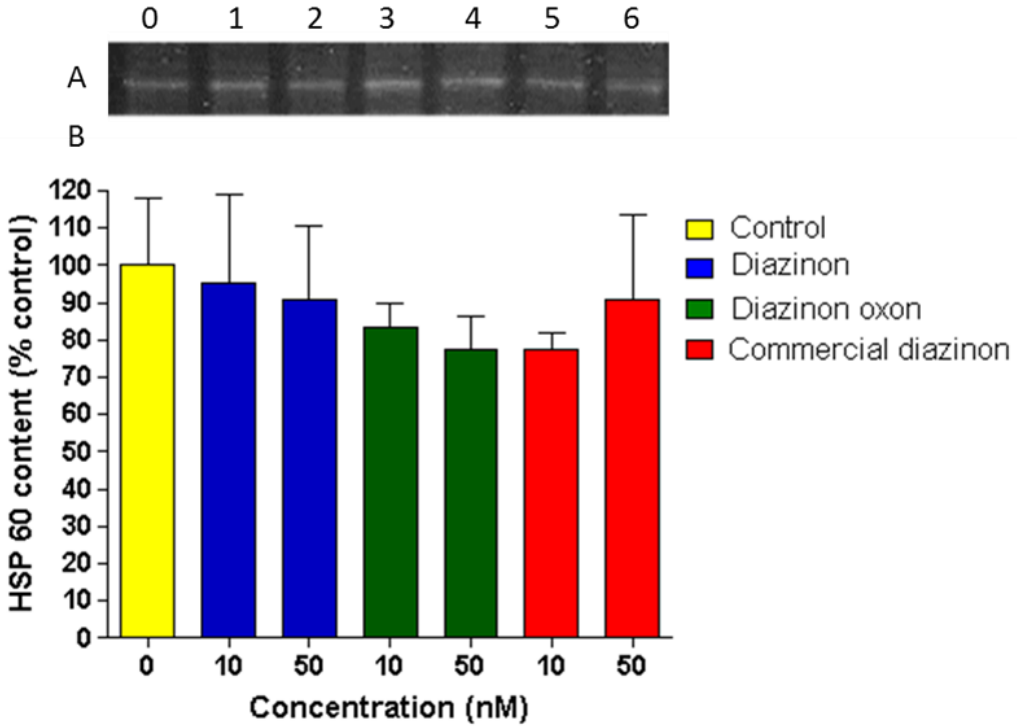

Figure 5.3 HSP 60 content of the pellet sample of G. pulex. Shown in panel A is a probed blot of extracts from gammarids prepared after exposure to diazinon (1 & 2), diazinon oxon (3 & 4) diazinon in the commercial formulation (5 & 6) for 24h. Panel B shows densitometric analysis, (means ± s.e., n = 4). In panel A, blots were probed with antibody to HSP 60.

HSP 70

In contrast to the lack of effect on HSP 60, 50 nM diazinon significantly increased the relative level of HSP 70 in supernatant samples ($p<0.001$). However, exposure of *G. pulex* to concentrations up to 50 nM diazinon oxon or diazinon in the commercial formulation had no effect (Figure 5.4).

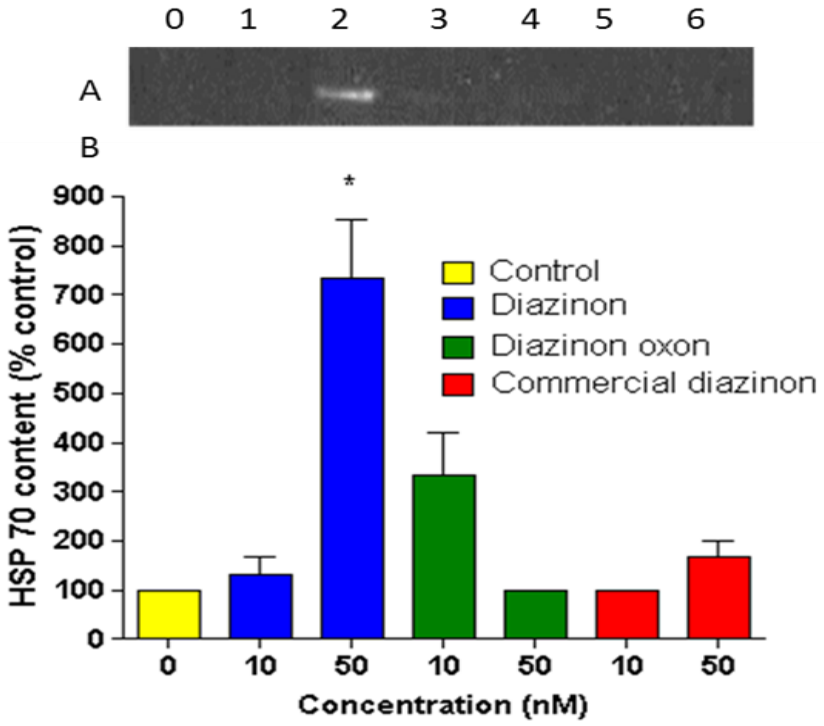

*Figure 5.4 HSP 70 content of the 9000g supernatant of G. pulex. Shown in panel A is a probed blot of extracts from gammarids prepared after exposure to diazinon (1 & 2), diazinon oxon (3 & 4)diazinon in the commercial formulation(5 & 6) for 24h. Panel B shows densitometric analysis, (means ± s.e., n = 4). * Significantly different from control. In panel A, blots were probed with antibody to HSP 70.*

Exposure to 50 nM diazinon or diazinon in the commercial formulation, but not diazinon oxon, significantly increased the relative level of Hsp70 in pellet samples (Figure 5.5) of *G. pulex* compared to untreated control extracts ($p<0.001$).

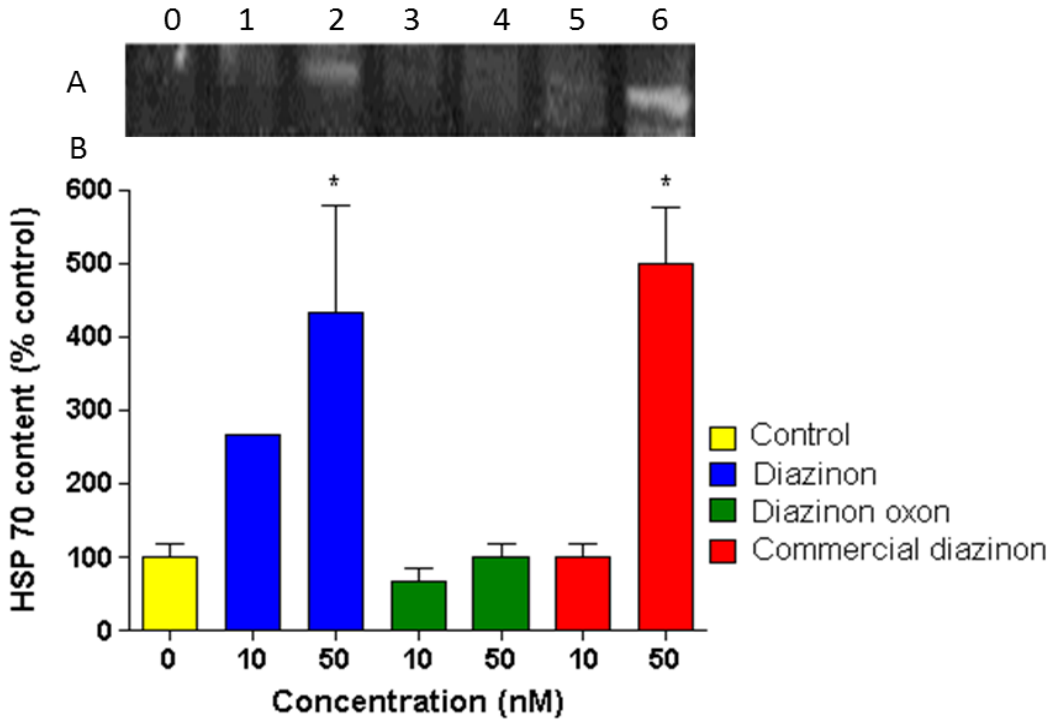

*Figure 5.5 HSP 70 content of the 9000g pellet fraction of G. pulex. Shown in panel A is a probed blot of extracts from gammarids prepared after exposure to diazinon (1 & 2), diazinon oxon (3 & 4) diazinon in the commercial formulation (5 & 6) for 24 h. Panel B shows densitometric analysis, (means ± s.e., n = 4). * significantly different from control. In panel A, blots were probed with antibody to HSP 70.*

HSP 90

The relative level of HSP 90 in supernatant samples of *G. pulex* were significantly raised after exposure of organisms to 50 nM diazinon or diazinon in the commercial formulation for 24 h compared to untreated samples ($p<0.005$). However, up to 50 nM diazinon oxon did not significantly increase the relative level of HSP 90 in these samples (Figure 5.6).

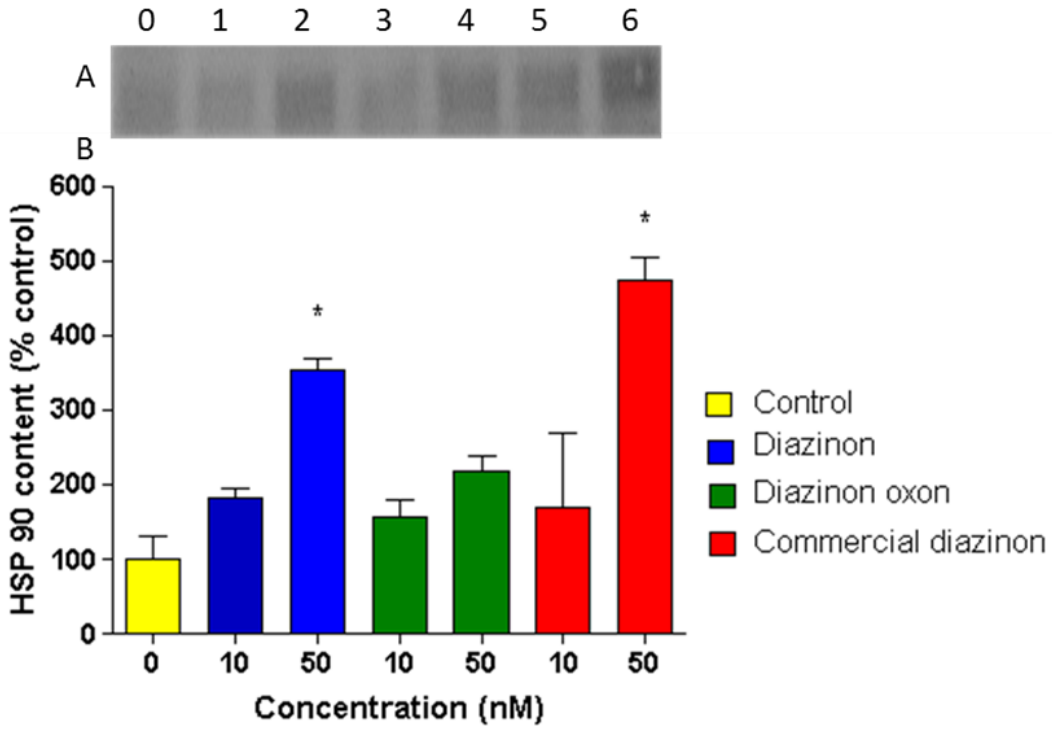

*Figure 5.6 HSP 90 content of the supernatant sample of G. pulex. Shown in panel A is a probed blots of extracts from gammarids prepared after exposure to diazinon (1& 2), diazinon oxon (3 & 4) and diazinon in the commercial formulation (5 & 6) for 24 h. Panel B shows densitometric analysis, (means ± s.e., n = 3). * significantly different from control. In panel A blots were probed with antibody to HSP 90.*

Figure 5.6 shows that exposure to 50 nM diazinon in the commercial formulation for 24 h caused a significant increase in HSP 90 levels compared with the control extract ($p<0.001$). On the other hand, up to 50 nM diazinon and diazinon oxon did not significantly alter the expression of this protein.

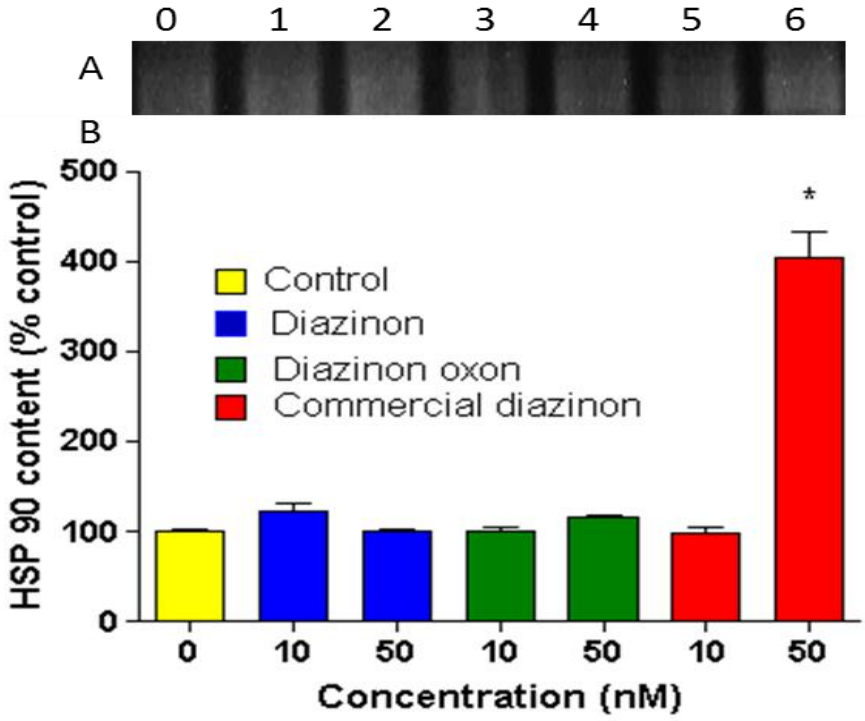

*Figure 5.7 Expression of HSP 90 in pellet sample of G. pulex. Shown in panel A is probed blot of extracts from gammarids prepared after exposure to diazinon (1 & 2), diazinon oxon (3 & 4) and diazinon in the commercial formulation (5 & 6) for 24 h. Panel B shows densitometric, (means ± s.e., n = 4). * significantly different from control. In panel A, blots were probed with antibody to HSP 90*

5.2.2 Phosphoproteins as potential molecular markers of toxicity.

Previous studies on phosphoproteins have focused mainly on mammalian systems. In contrast, the effect of pesticides on the induction of phosphoproteins in aquatic organisms, especially those in freshwater, have seldom been mentioned. This study considers aspects of proteomic alteration of *G. pulex* due to exposure to diazinon, diazinon oxon and diazinon in the commercial formulation. As a result,

some differential changes in proteins might be established that sheds new light on the mechanisms underlying diazinon toxicity. The work could also assist in the development of bioindicators for integrating OP pesticide pollution levels in aquatic environments. This may aid in the assessment of the environmental hazard of pesticides to aquatic animals as well as to human health.

Dot blot analysis

Phosphotyrosine

Exposure to 50 nM diazinon, and to 10 and 50 nM diazinon in the commercial formulation, but not diazinon oxon, significantly increased the total relative level of phosphotyrosine in the 9000g supernatant sample of *G. pulex* (Figure 5.8 A) compared to unexposed control gammarid extracts ($p<0.001$). However, exposure to 10 and 50 nM diazinon, diazinon oxon and diazinon in the commercial formulation did not significantly change the total relative level of phosphotyrosine content in the pellet sample of *G. pulex* compared to control organism extracts (Figure 5.8 B).

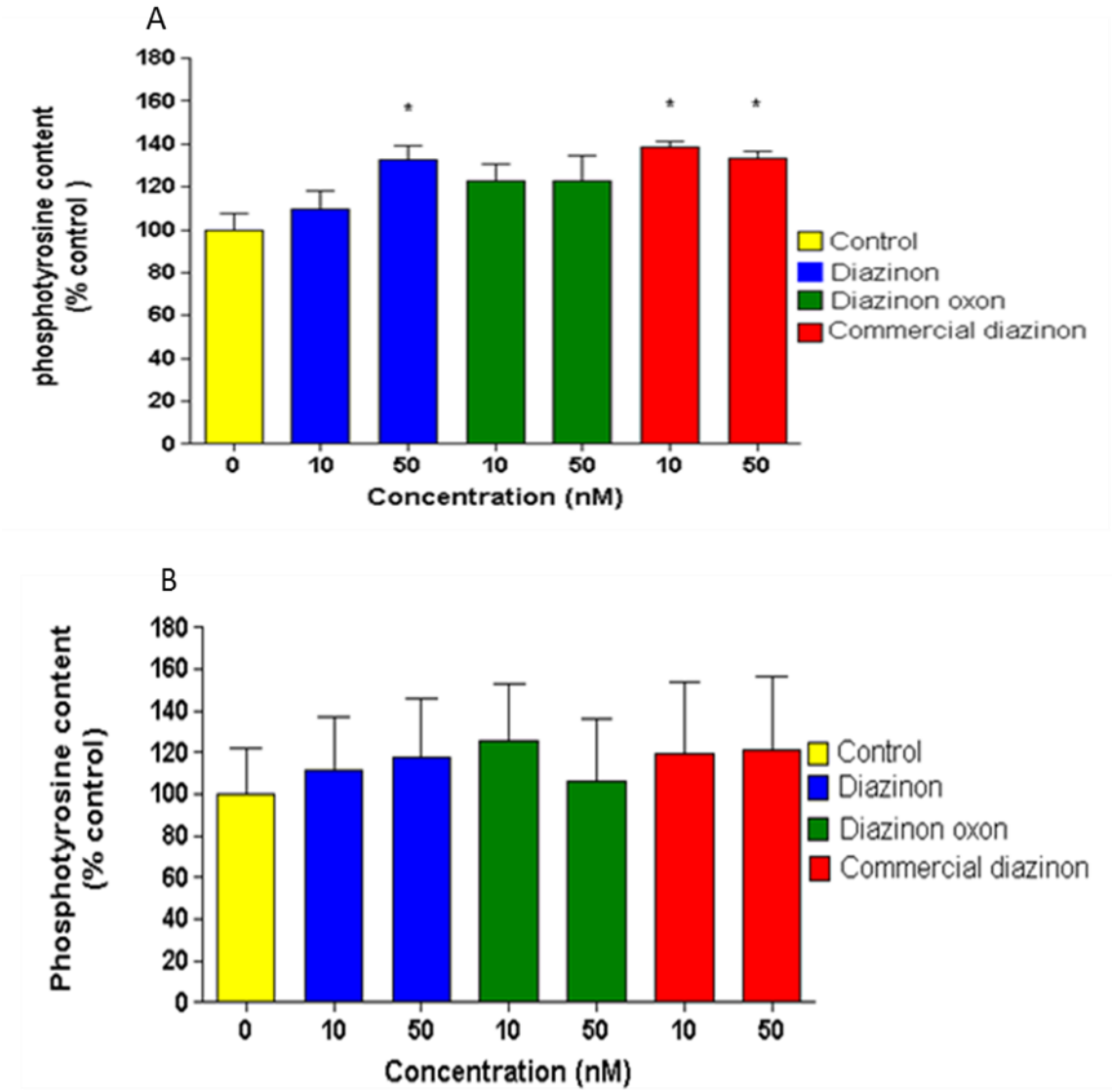

*Figure 5.8 Phosphotyrosine content of the supernatant sample (A) and the pellet sample (B) of G. pulex after exposure to diazinon, diazinon oxon and diazinon in the commercial formulation for 24 h (means ± s.e., n = 4). * significantly different from control.*

Phosphoserine

Up to 50 nM diazinon, diazinon oxon and diazinon in the commercial formulation did not significantly affect the total relative level of phosphoserine content in the supernatant or pellet sample of *G. pulex* compared to control organism extracts (Figures 5.9 A and B).

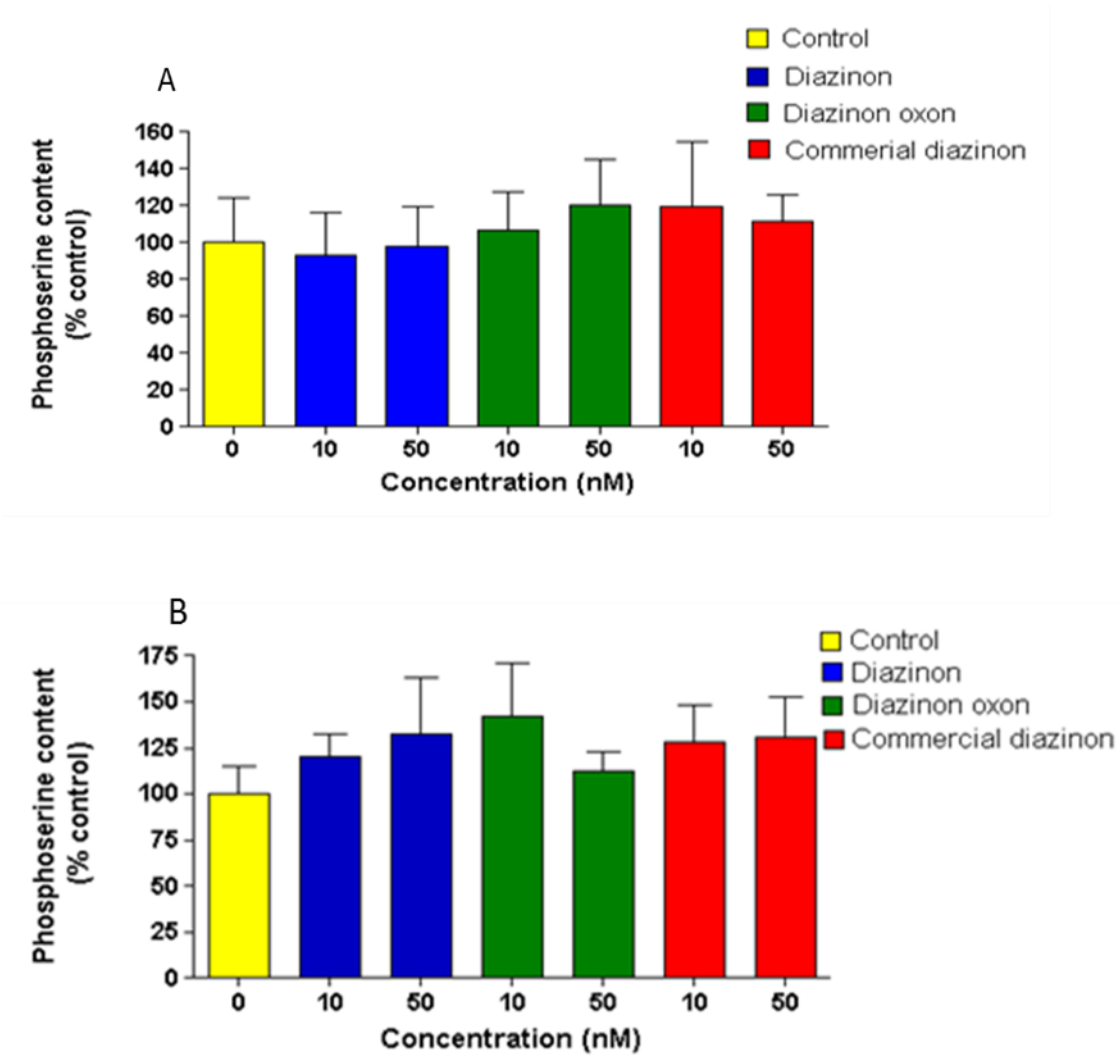

Figure 5.9 Phosphoserine content of the supernatant sample (A) and the pellet sample (B) of G. pulex after exposure to diazinon, diazinon oxon and diazinon in the commercial formulation for 24 h (means ± s.e., n = 4).

Phosphothreonine

Up to 50 nM diazinon, diazinon oxon and diazinon in the commercial formulation did not significantly alter the total relative level of phosphothreonine content in the supernatant and pellet sample of *G. pulex* compared to unexposed control extracts (Figures 5.10 A and B).

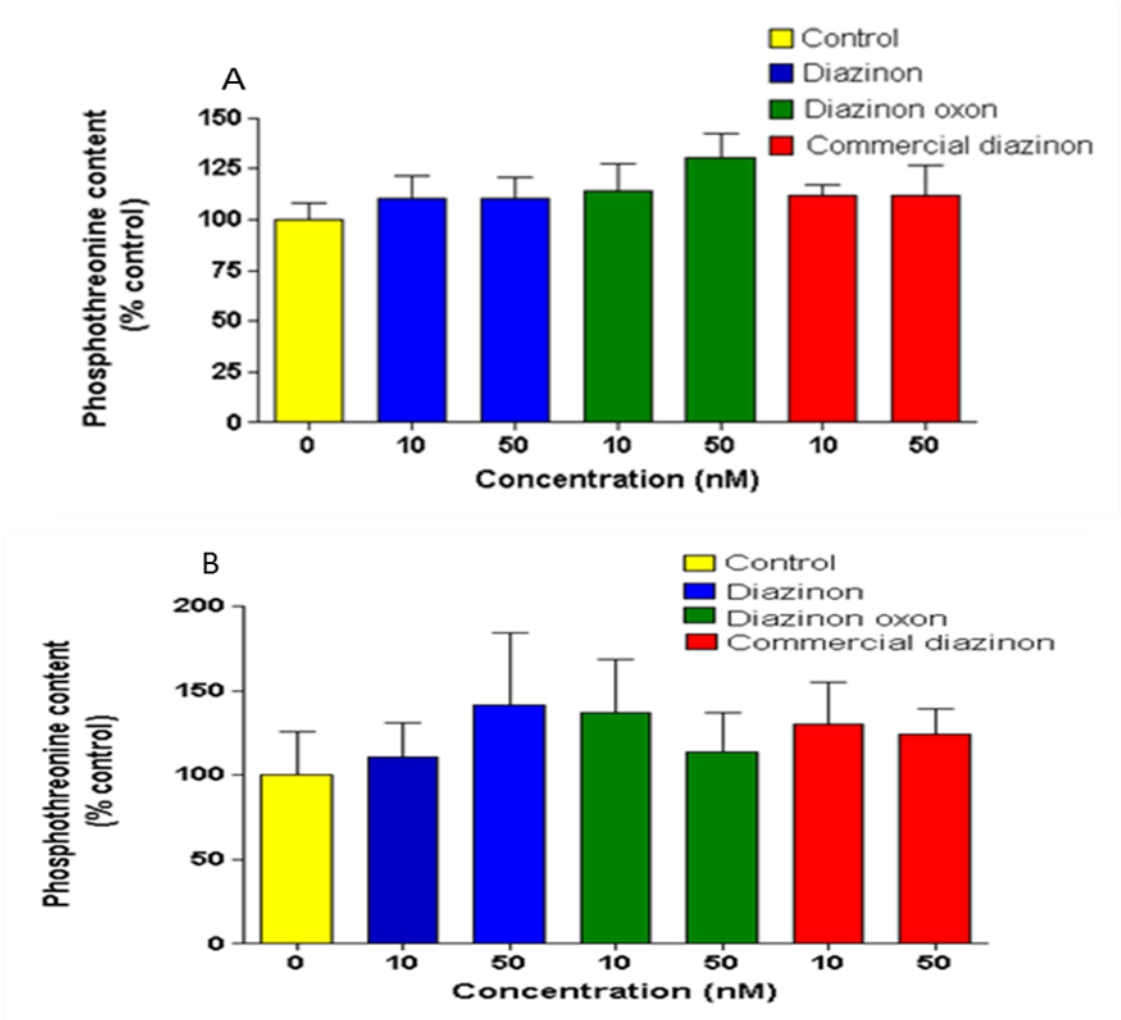

Figure 5.9 Phosphothreonine content of the supernatant sample (A) and the pellet sample (B) of *G. pulex* after exposure to diazinon, diazinon oxon and diazinon in the commercial formulation for 24 h (means ± s.e., n = 4).

SDS-PAGE and Western blotting analysis

As dot blot analysis only gives a measure of total (net) phosphorylation, it was of interest to determine whether any individual proteins were affected differently. This was achieved by Western blot analysis. Only the 9000g supernatant samples of *G. pulex* were used to study the effect of diazinon, diazinon oxon and diazinon in the commercial formulation on phosphoproteins, because the result of dot blot analysis indicated that there was only a significant increase in the total relative level of phosphotyrosine in the 9000g supernatant sample of *G. pulex.* Typical Western blots of whole gels used for the analysis of phosphotyrosine, phosphoserine and phosphothreonine content are shown in Appendix 1.

Phosphotyrosine

The effect of diazinon, diazinon oxon and diazinon in the commercial formulation on the level of phosphotyrosine in *G. pulex* after 24 h exposure is shown in Figure 5.10. Reactivity of antibodies with bands corresponding to 75 and 51 kDa was significantly increased by exposure to 50 nM diazinon and diazinon in the commercial formulation compared with unexposed control ($P<0.001$). However, treatment with 10 nM diazinon and diazinon in the commercial formulation and up to 50 nM diazinon oxon had no significant effect on the level of reactivity with these bands (75 and 51 kDa) compared with control.

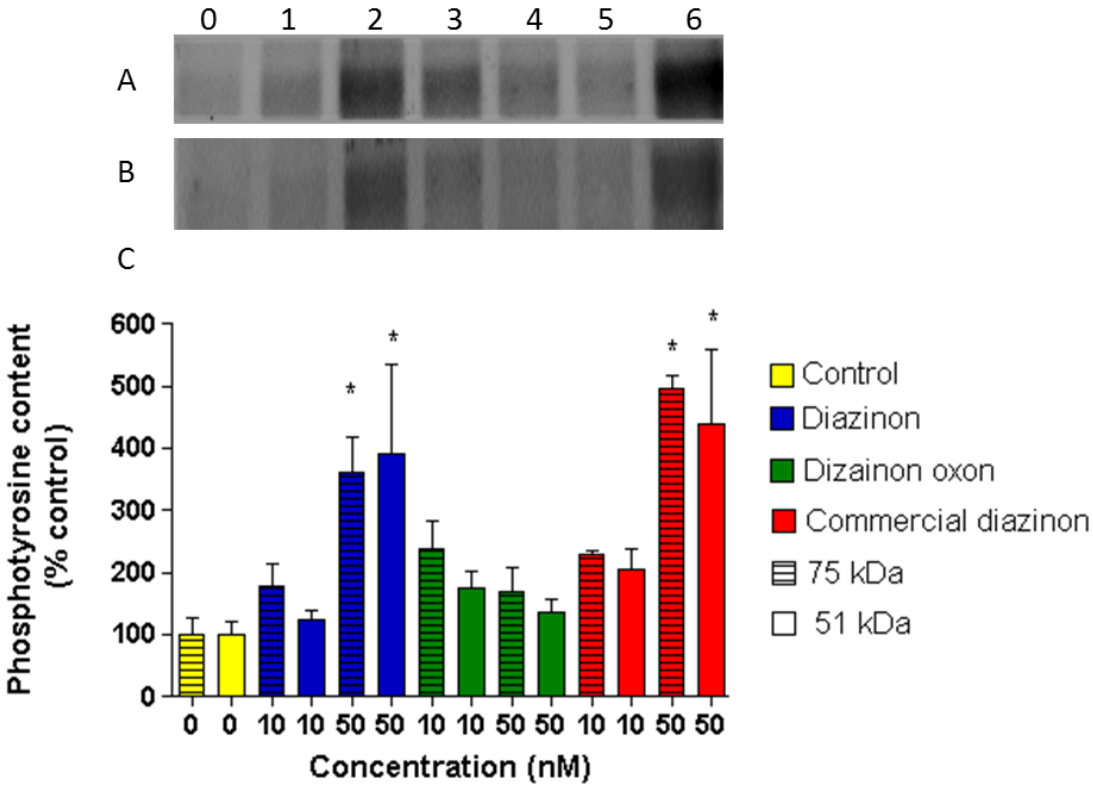

*Figure5.10 Western blot analysis of phosphotyrosine content of the supernatant sample of G. pulex. Shown in panel A and B are probed blots of extracts from gammarids prepared after exposure to diazinon (1 & 2), diazinon oxon (3 & 4) and diazinon in the commercial formulation (5 & 6) for 24 h (means ± s.e., n = 3), A: band at 75 kDa; B: band at 51 kDa. C : densitometric analysis. * significantly different from control.*

Phosphoserine

Western blotting analyses of the level of phosphoserine after exposure of *G. pulex* to diazinon, diazinon oxon and diazinon in the commercial formulation are presented in Figure 5.11. Reactivity of antibodies with bands corresponding to 75 kDa significantly increased after *G. pulex* exposure to 10 and 50 nM diazinon and diazinon in the commercial formulation compared with the unexposed control ($p<0.001$). On the other hand, exposure to 10 and 50 nM diazinon oxon did not

significantly increase the level of phosphoserine antibody reactivity with the same band. For the 51 kDa band the level of phosphoserine significantly increased with 50 nM diazinon, diazinon oxon and diazinon in the commercial formulation compared with untreated control ($p<0.01$). However, at 10 nM these pesticides did not significantly affect the level of phosphoserine in this protein band.

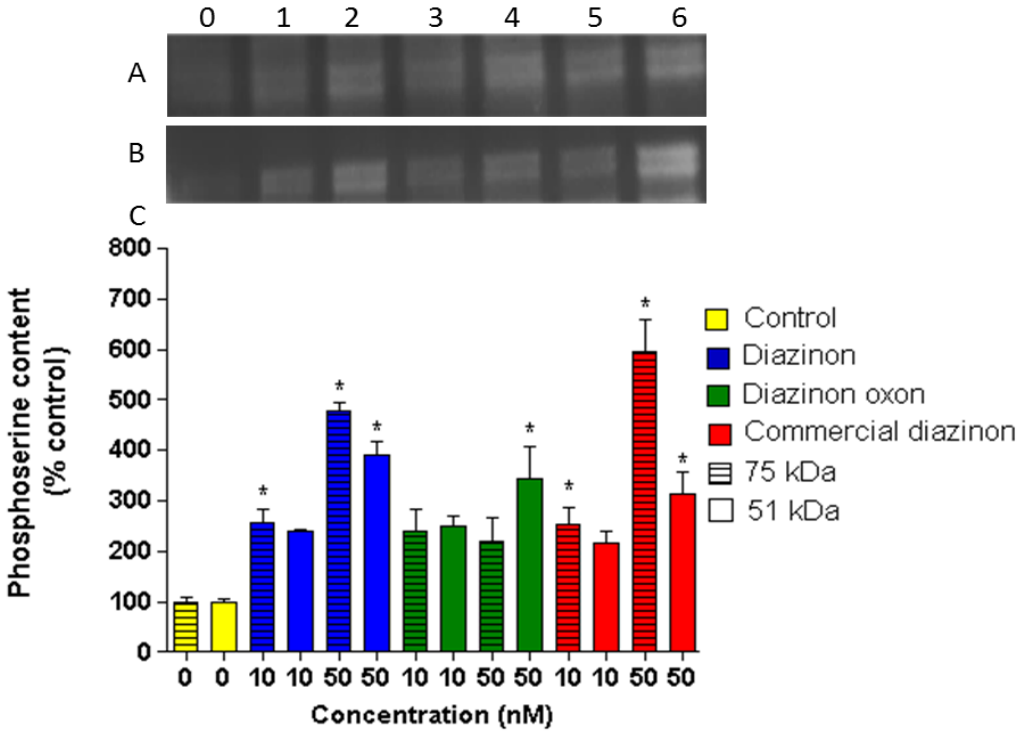

*Figure 5.11 Western blot analysis of phosphoserine content of the supernatant sample of G. pulex. Shown in panel A and B are probed blots of extracts from gammarids prepared after exposure to diazinon (1 & 2), diazinon oxon (3 & 4) and diazinon in the commercial formulation (5 & 6) for 24 h (means ± s.e., n = 3), A: bands at 75 kDa; B: bands at 51 kDa and C: densitometric analysis. * significantly different from control.*

Phosphothreonine

The level of phosphothreonine antibody reactivity with extracts of a 75 kDa band in *G. pulex* significantly increased in extracts of gammarids after exposure for 24 h to 50 nM diazinon and diazinon in the commercial formulation. However, diazinon oxon exposure did not have a significant effect on the level of phosphothreonine in the 75 kDa bands (Figure 5.12).

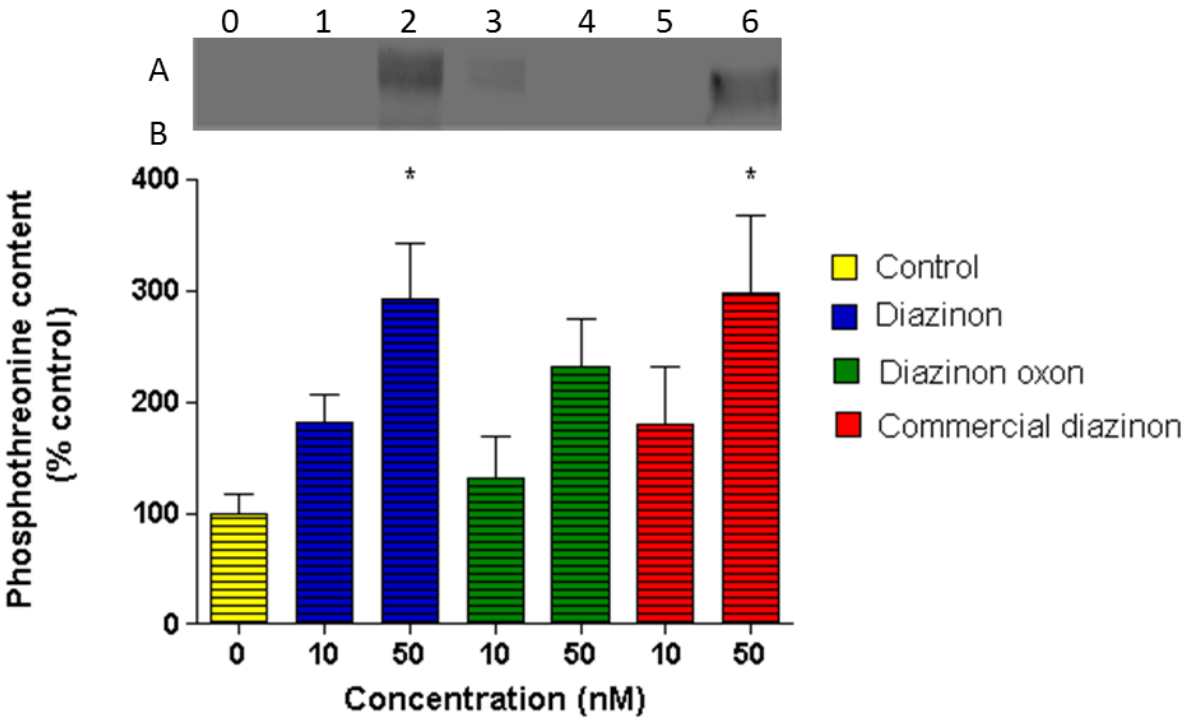

*Figure 5.12 Western blot analysis of phosphothreonine content of the supernatant sample of G. pulex. Shown in panel A is a probed blot of extracts from gammarids prepared after exposure to diazinon (1 & 2), diazinon oxon (3 & 4) and diazinon in the commercial formulation (5 & 6) for 24 h (means ± s.e., n = 3), A: bands at 75 kDa and B: densitometric analysis. * significantly different from control.*

5.2.3 2D-PAGE

In the current study, the 2D-PAGE technique was used to investigate the response of the proteome from 9000g supernatant samples of *G. pulex* to 50 nM diazinon after 24 h exposure. Approximately 150 protein spots were detected from the 9000g supernatant sample when sufficient protein was applied on 2D-PAGE. Changes in certain protein levels were quantified by image analysis. Four proteins represented in Table 5.1 and Figure 5.13 are shown decreased by ≥ 1.8 fold (green circles). However, three proteins were increased by ≥ 2.2 fold (red circles).

None of the detected changes were in high abundance polypeptides, making identification impractical. Though not statistically significant at $P<0.05$, these spots showed changes that were approaching significance, highlighting potential polypeptides for future study.

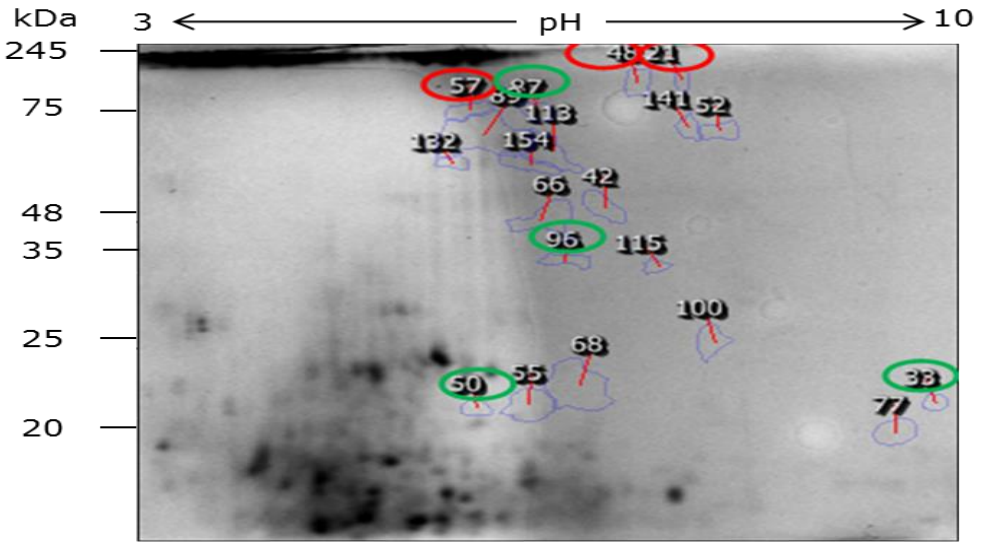

Figure 5.13 Representative silver-stained 2D gel of 9000g supernatant sample of G. pulex after exposure to 50 nM diazinon for 24 h.

Table 5.1 Changes in certain protein levels quantified by image analyses from triplicate samples. (No Anovas were significant)

	Protein spot	Anova (p)	Fold change
Decreasing proteins	33	0.101	2.7
	50	0.056	2.3
	87	0.104	1.9
	96	0.101	1.8
Increasing proteins	21	0.077	3.1
	48	0.171	2.4
	57	0.143	2.2

5.3 Discussion

HSPs play important roles in protein folding and they are up-regulated in a variety of general stress responses. HSPs are divided by molecular weight into several families: HSP90 (90 kDa), HSP70 (70 kDa), HSP60 (60 kDa) and small HSPs from 43 to 12 kDa (Sanders, 1993; Shatilina *et al*., 2010). The induction of HSPs in fish has been described in extracts from different samples. For example, in the tissues of whole animals, cell lines and primary cultures of different cells (Iwama *et al*., 1998) and they have been used as bioindicators in a variety of species to indicate sublethal stress due to pesticide exposure (Köhler *et al*., 2001; Eder *et al*., 2009).

Small heat shock proteins (sHSP) are a large and heterogenic family, which contains proteins with molecular weights from 12 to 43 kDa. They have a highly conserved region known as the the α- crystallin domain (Plesofsky and Brambl, 2002). Anti-αA/αB-Crystallin, pAb was

used in the current study to determine the effect of diazinon, diazinon oxon and diazinon in the commercial formulation on sHSP of *G. pulex.*

Protopopova *et al.* (2011) observed that the molecular weight of sHSP in amphipods (*G. lacustris and G. pulex*) was in the range 35-37 kDa. In the present study, the molecular weight of sHSP was between 30-37 kDa, which is similar to the findings of Protopopova *et al.* (2011). The result of expression shows that only exposure to 50 nM diazinon and diazinon in the commercial formulation significantly increased the relative level of sHSP expression in the 9000 g supernatant samples of *G. pulex* compared to untreated control extracts. Diazinon oxon exposure did not significantly increase sHSP levels in this sample, whilst sHSP was not detected in pellet samples. The detection of sHSP in supernatant samples of *G. pulex* may be due to the abundance of this protein in the cytosol as opposed to the membrane. The ability of only diazinon and diazinon formulation to induce sHSP synthesis suggests that only the parent compound is responsible for this stress response.

The increase of HSP by weak stress effects allows species to obtain resistance to stronger stresses (Sanders, 1993; Shatilina *et al*., 2010). Timofeyev *et al*. (2008) have observed well-expressed induction of sHSP, immunochemically related to the a-crystallin group, in *Eulimno gammarus verrucosus, Eulimno gammarus cyaneus* and *G. lacustris* after exposure to cadmium chloride for 24 h. The relative stress needed to induce HSP expression is species-specific and depends on environmental factors of animals (Shatilina *et al*., 2010). The impact of natural organic matter such as humic substances was to increase the abundance of HSP 70 and sHSPs of *G. pulex* after 30 min exposure (Bedulina *et al*., 2010).

In the current study, the molecular weight of anti-HSP 60 reactive band was between 55- 60 kDa, consistent with its known molecular

weight. The present result shows that sublethal exposure of diazinon, diazinon oxon and diazinon in the commercial formulation (10 and 50 nM for 24 h) did not induce HSP 60 in 9000 g pellet sample, whilst HSP 60 was not detected in 9000 g supernatant sample of *G. pulex*. The lack of response in HSP 60 observed in the present study suggests that these toxicants do not induce HSP 60 in *G. pulex* or that the tested concentrations and/or exposure time used did not reach the threshold required to the induction of HSP 60. In addition, the lack of effect may indicate that the mitochondrial matrix proteins are not major targets. As this protein is a mitochondrial matrix protein (Paranko *et al*., 1996).

Stressors similar to toxicants could induce HSP 60, and this induction has been used as a suitable bioindicator in ecotoxicological research (Clayton *et al*., 2000; Eder *et al*., 2009). Animals respond to protein-toxicity with the induction of HSP, which are able to repair partly denatured proteins (Hallare *et al*., 2004). Up to 20.8 nM (7.3 µg/L) chlorpyrifos did not affect HSP 60 levels in muscle and gills of Chinook salmon (*Oncorhynchus tshawytscha*). There was no difference in HSP 60 level in the American lobster, *Homarus americanus* between control and methoprene-exposed (Walker *et al*., 2005). However, esfenvalerate significantly increased HSP 60 levels in muscle but not in gill samples in the same organism (Eder *et al.,* 2009).

HSP 60 has been detected in *Hyalella azteca* (freshwater amphipod) in both supernatant and pellet samples after exposure to diazinon for 24 h. Exposure of *H. azteca* to diazinon significantly increased HSP 58 and HSP 64 levels in the supernatant and pellet samples. However, HSP 54 and 64 were detected predominantly in the pellet fraction of the estuarine amphipod *Ampelisca abdita* (Werner and Nagel, 1997). The same authors also found that HSP 54 and HSP 64 levels in supernatant samples of the marine amphipod *Rhepoxynius abronius* were significantly increased after exposure to diazinon. Whereas, HSP 64 in pellet samples was only elevated in pellets after increase the in

concentration of diazinon. Eder *et al*. (2004) found that the HSP 60 in muscle tissue of juvenile Chinook salmon (*Oncorhynchus tshawytscha*) did not significantly change after exposure to chlorpyrifos for 96 h. By contrast, esfenvalerate caused a significant increase of HSP 60 compared with the control. Therefore, although not significantly affected by exposure to diazinon, diazinon and diazinon in the commercial diazinon formulation in the current work, HSP 60 may be a useful analytical indicator to investigate the effect of diazinon or other pesticides and environmental toxins in other marine organisms.

In the current study, the expression of HSP 70 level was studied in *G. pulex* following exposure to various concentrations of diazinon, diazinon oxon and diazinon in the commercial formulation for 24 h. The fact that HSP 70 was detected in both the 9000g supernatant and pellet fractions, may reflect its abundance in both the mitochondrial and post mitochondrial extracts. This group of proteins is abundance in cytosol, mitochondria and endoplasmic reticulum (Gupta *et al*., 2010). The results in the current study indicated that only 50 nM diazinon induced significant increases in HSP 70 expression in the 9000g supernatant sample of *G. pulex*. The observation that expression of HSP 70 was significantly increased in the 9000g pellet of *G. pulex* after exposure to 50 nM diazinon and diazinon in the commercial formulation but not by the oxon metabolite, suggests that only the parent compound is responsible for this stress response.

Elevated expression of HSP 70 due to exposure to diazinon has also been observed in other amphipods. Werner and Nagel (1997) observed that HSP 71 was significantly increased in both samples of *Hyalella azteca* and *Rhepoxynius abronius* after exposure to diazinon for 24 h. However, in the pellet fraction of *Ampelisca abdita*, HSP 71 showed no response. Diazinon also increased the level of HSP 70 the *zebrafish* (Scheil *et al*., 2009). The induction of HSP 70 in aquatic organisms due to other organophosphate pesticides has also been investigated. For

example, Eder *et al*., (2004) found that juvenile Chinook salmon *Oncorhynchus tshawytscha* exposed to chlorpyrifos for 96 h exhibited significantly increased HSP 70 levels in muscle tissue. However, gilthead sea bream (*Sparus aurata*) exposed to dichlorvos did not show significant changes in the expression of HSP 70 compared with the control. Therefore, quantification of HSP 70 content of *G. pulex* may serve as useful indicators of the deterioration of environmental health.

In eukaryotic cells, HSP 90 is enriched in the cytosol and under physiological conditions it has been found to be linked with different intracellular proteins such as calmodulin, actin, tubulin, kinases, and some receptor proteins (Gupta *et al*., 2010). The molecular weight of HSP 90 in eukaryotes ranges from 82-90 kDa (Gupta *et al*., 2010).

In this study, the molecular weight of HSP 90 was between 80-90 kDa and the current study shows that HSP 90 was detected in both 9000g supernatant and in 9000 g pellet fractions of *G. pulex*. In addition to its cytosolic expression, however, HSP 90 has been detected in mitochondria (Kang *et al*., 2007). Alterations in HSP 90 expression in *G. pulex* in response to diazinon, diazinon oxon and diazinon in the commercial formulation indicated that, in supernatant samples, 50 nM diazinon and the diazinon in commercial diazinon formulation for 24 h significantly increased the relative expression of the HSP 90, but diazinon oxon (50 nM) did not affect the expression of this protein. This suggests that only the parent compound of diazinon may have the ability to induce increased HSP 90 synthesis in *G. pulex*. However, only 50 nM diazinon in the commercial formulation significantly increased the relative level of this HSP in pellet samples, and up to 50 nM diazinon and diazinon oxon had no significant effect on the level of HSP 90 compared with unexposed samples.

HSP 90 expression has been studied with different organisms and pesticides. Eder *et al*. (2004) found that 3.4 nM (1.2 µg/L) chlorpyrifos and 238.2 pM (0.1 µg /L) esfenvalerate significantly increased the relative level of HSP 90 in the muscle of juvenile Chinook salmon after 96 h, whilst no rise in stress protein content was observed in gills of this organism. Thus, HSP 90 induction represents a useful biomarker of diazinon exposure in *G. pulex* and could be particularly useful for studying the physiological disturbances in aquatic organisms by naturally or accidentally modified environmental factors.

The results from dot blot analysis indicated that the total protein phosphotyrosine content of *G. pulex* in the 9000g supernatant fractions (but not pellets) was significantly increased by 50 nM diazinon and 10 and 50 nM diazinon in the commercial formulation whereas total phosphoserine and phosphothreonine levels were not significantly increased in either fraction following the same treatments. Coupled with the fact that diazinon oxion had no effect under any conditions tested, these findings suggest that diazinon but not diazinon oxon is capable of activating tyrosine kinases and/or inactivating tyrosine phosphatases.

The use of anti-phosphotyrosine, phosphoserine and phosphothreonine antibodies in Western blot analysis allowed detection of changes in the phosphorylation of individual phosphoproteins in 9000g supernatant fractions. Data indicated that only exposure to 50 nM diazinon and diazinon in the commercial formulation significantly increased the level of tyrosine phosphorylation of polypeptide bands corresponding to 75 and 51 kDa compared with the unexposed organism. The level of phosphoserine content of *G. pulex* was significantly increased in polypeptide bands corresponding to 75 kDa after exposure to 10 and 50 nM diazinon and diazinon in the commercial formulation, but not following treatment with 10 or 50 nM diazinon oxon. However, exposure to 50 nM diazinon, diazinon oxon and diazinon in the

commercial formulation induced significant increases in the level of phosphoserine content in bands corresponding to 51 kDa compared with untreated samples. The quantitative data of the level of phosphothreonine content of *G. pulex* indicated that only 50 nM diazinon and diazinon in the commercial formulation caused significant increases in the intensity of bands corresponding to 75 kDa compared with control fractions. Identification of these specific phosphoproteins would be of value. Indeed, given the similarity in size between the higher molecular weight phosphoprotein showing changes on Western blots and HSP 70, it would be interesting to determine whether the latter is also a phosphorylation target following exposure to diazinon.

Thus, in contrast to the findings from dot blot assays, which measure total phosphoamino acid content, the quantitative Western blotting data have shown a clear effect of diazinon, diazinon oxon and diazinon in the commercial formulation on the levels of phosphotyrosine, phosphoserine, and phosphothreonine content in specific proteins in gammarid. The explanation of these differences is that, while there may be no change in total levels of phosphorylation on dot blots, the phosphorylation of individual proteins may rise or fall, as highlighted on probed Western blots, indicating that analysis of individual bands is more informative than total phosphoprotein.

Walker *et al*. (2005) indicated that there were different patterns of protein phosphorylation in comparable fractions after exposure of *Homarus americanus* to methoprene compared with the control animals. Miyazawa and Matsumura (1990) also observed that protein phosphorylation and dephosphorylation activities in the nervous system were of *Homarus americanus* affected by exposure to the pyrethroid ester insecticide deltamethrin. One of the proteins of which the rate of phosphorylation was affected by this pesticide had an apparent molecular weight of 260 kDa. Burlando *et al*. (2003) found that copper, mercury and hydrogen peroxide induce a relevant

increase in tyrosine phosphorylation in cultured RTH 149 trout hepatoma cells. Matsumura *et al*. (1989) observed that deltamethrin exposure caused a significant alteration in protein phosphorylation activities, which follow depolarization of squid, *Loligo pealei*. In contrast, Rani and Sivaraj (2010) found that the level of phosphothreonine in the control gills of freshwater fish *Clarias batrachus* (Linn.) was higher than chromium-treated gills after 28 days, whereas, protein bound phosphotyrosine was not detected in gills of treated or control *C. batrachus*. Thus, ranges of environmental toxins are capable of disrupting different types of protein phosphorylation in a range of aquatic organisms.

In the current study preliminary analysis by 2D-PAGE has shown that 3 protein spots from the 9000g supernatant sample were up-regulated by ≥ 2.2 fold, whilst four proteins were shown to be decreased by ≥ 1.8 fold after exposure of *G. pulex* to 50 nM diazinon. Identification of such proteins was hampered by their low abundance. Leroy *et al*. (2010) found that, of more than 560 spots detected in the 15000 g supernatant of *G. pulex*, 21 proteins showed significant expression differences following exposure to polychlorinated biphenyls (PCB). Ling *et al.* (2009) also observed that 18 protein spots in gill tissue of *Paralichthys olivaceus* exposed to cadmium were significantly changed compared to a control sample. Huang and Huang (2011) indicated that there was significant alteration of 13 protein spots of membrane protein in zebrafish liver after exposure organism to methyl parathion. Thus, 2D-PAGE represents a potentially useful technique for the identification of novel protein biomarkers following exposure of aquatic organisms to environmental toxin such as Ops.

CHAPTER SIX: General discussion

6.1 Test performance for *G. pulex*

Aquatic organisms such as crustaceans have been commonly used in aquatic toxicity studies. Amongst these species, *Daphnia pulex*, *Daphnia magna* and *Ceriodaphnia dubia* are often used in aquatic toxicity testing for a variety of reasons (US-EPA, 1996; Sucahyo *et al*., 2008). The availability, abundance and familiarity with the species may have contributed to their popularity (Hickey, 1989 and Sucahyo *et al*., 2008). In the present study, *G. pulex* was shown to be suitable for toxicity testing following standardized test guidelines that are used for *Daphnia* and associated species. *G. pulex was chosen because it is* relatively easy to collect from streams using a hand net and is widespread in the UK (Lloyd Mills *et al*., 2006). The *G. pulex* population in Creswell Crags Nature Reserve, Derbyshire was available throughout the year and is in a relatively unpolluted site. Gammarids can easily be cultured in the laboratory and can be fed on a variety of food such as wheat germ. There was little mortality during acclimation and control survival during testing was also high, more than 90%.

6.2 Acute Toxicity

Comparative toxicity of different types of diazinon on *G. pulex*

It is important to study the effect of diazinon on aquatic organisms due to the extensive use of this compound in aquaculture and in sheep dips. This has led to increased concern about their release into watercourses flowing through agricultural land and its potential subsequent toxicity towards organisms and ecosystems. This compound is of particular interest since diazinon is a component of sheep dip pesticides used in the UK. However, the market

authorisation for these pesticides has recently been suspended pending further assessment of their environmental impact. Several environmental metabolites such as 2-isopropyl-6-methyl-4-pyrimidinol (IMP), diethylphosphate (DEP), and diethylthiophosphate (DETP) can also be found in the environment due to the break down of diazinon (Morgan *et al*., 2010). Therefore, a range of non-target species are likely to be exposed. This may even include humans where water bodies are contaminated. Bioconcentration of pesticides and/ or metabolites is also possible. Fish in particular may be at significant risk in affected water. But they tend to be less sensitive to diazinon. Diazinon has sublethal effects, the fish need not exhibit obvious outward signs of diazinon poisoning to be a risk for human consumption.

According to the United States Geological Survey under the National Water Quality Assessment Program (NAWQA) stream quality network programs, and other sources, diazinon was the most commonly detected pesticide in surface water (US EPA, 2006). Higher diazinon concentrations were detected in urban sites than in agricultural locations. Surface waters sampled for both agricultural and urban pesticide use include rivers, streams, and creeks. For example, 35% of NAWQA samples were found to contain ~~containing~~ diazinon and its concentrations ranged from below detectible levels up to 12.5 nM (3.8 µg/L) (US EPA, 2006). The acceptable maximum concentration of diazinon in drinking water is 0.02 mg/L (Technical Support Document for Ontario Water Standards, 2006). US EPA (2006) reported that diazinon is moderately to very highly toxic to invertebrates. The LC_{50} values were from 13.8 nM to 3.3 µM (4.2 to < 1000.0 µg/l). The acute No Observable Adverse Effect Concentration (NOAEC) in shrimp was < 8.9 nM (< 2.7 µg/l). For freshwater aquatic invertebrates, diazinon has very high acute toxicity with EC_{50} values ranging from 690 pM to 115 nM (0.21 to 35.0 µg/l). NOAEC was found in *D. magna* at 558.6 pM (0.17 µg/l), when organisms were exposed chronically to diazinon. No

observable effect concentrations (NOEC) at which which there is no biological or statistically significant effect of diazinon were 0.23 and < 0.55 mg/l for mysid shrimp and brook trout respectively, whilst, lowest observed effect concentrations were 1.05, 1.4 and 1.8 µM (0.32, 0.42 and < 0.55 mg/l) for *D. magna*, *mysid shrimp* and *brook trout* respectively (US EPA, 2006).

The main source of water contamination arises as run off from the soil, and one of these contaminations is diazinon (Fenlon *et al*., 2011 and Kanda and Glendinning, 2011). For example, in 1998, diazinon was found at 52% of 107 Welsh sites that had their water quality tested. Also in 1990, diazinon was found in all 20 catchments sampled by the Scottish Tweed River Purification Board, with concentrations of diazinon ranging from 14-124 ng/L (46-407.4 pM) (Boucard *et al*., 2004). During July through September 2000 in baseflow in southeastern New York, 37 pesticides and/or pesticide degradates were found. Nine compounds were found at concentrations greater than 0.10 µg/L. Three of them were diazinon (328.6 pM), carbaryl (497 pM) and imidacloprid (391.1 pM) (Phillips and Bode, 2002). Gerecke *et al*. (2002) found that the level of atrazine was significantly higher in wastewater treatment plants and the river Aabach in Switzerland between mid-May and end of June 1999. The concentration of atrazine in the Aabach always exceeded the Swiss quality goal of 0.1 µg/l for surface waters. Evans (2009) indicated that some pesticides were present above the threshold level of 0.1 µg/l. in the late 1990s in the river? Cherwell UK. For example, Isoproturon from October to March, Chlorotoluron from October to December, Propyzamide from September to February and Simazine from April to May.

Results on the toxicity of diazinon were determined using three types of diazinon which were diazinon, diazinon oxon and diazinon in the commercial formulation. There were notable differences in LC_{50}

between them (Table 3.1). LC_{50} values in the current study indicated that diazinon in the commercial formulation (which is only 60% diazinon with the remaining 40% comprising excipient and trace impurities) was 4.4 fold and 13.5 fold more toxic than diazinon oxon and diazinon, respectively. The 96 h LC_{50} values for these pesticides were 140.1 nM for diazinon, 45.7 nM for diazinon oxon and 10.4 nM for diazinon in the commercial formulation.

A possible explanation of these differences in LC_{50} may be due to the commercial diazinon formulation including other compounds, which may be more toxic towards *G. pulex*. Indeed the toxicity of the formulation to insects might primarily be due to the impurities. One of the toxic impurities of diazinon formulations is (O,O,O',O'-tetraethyl dithiopyrophosphate) sulfotepp, which also may concentrate in the environment and cause ecological problems and unanticipated health issues (Meier *et al*., 1979). For example, sulfotepp has a higher toxicity than diazinon for fathead minnow, bluegill (*Lepomis macrochirus*) and rainbow trout. The 96 LC_{50} of sulfotepp and diazinon for these organisms were 552.3 nM and 33.8 µM (0.178 and 10.3 mg/L) for fathead minnows 5 and 394.3 nM (0.0016 and 0.12 mg/L) for bluegill and 55.8 nM and 4.4 µM (0.02 and 1.4 mg/L) for rainbow trout respectively (Meier *et al*., 1979).

Aquatic organisms are normally exposed to a large range of possible toxic compounds at the same time in the natural environment. However, the most available toxicity data deals with the effects of single pure compounds (Adam *et al*., 2009). In the current study, the commercial diazinon formulation was 13.5 fold and 4.4 fold more toxic than diazinon and diazinon oxon (96 h LC_{50}) for *G. pulex*. A possible explanation is that 40% concentration comprises excipient and trace which impurities maybe increase the toxicity of commercial diazinon formulation. Another study by Adam *et al*. (2009) found that cypermethrin (pyrethroid insecticide) was very toxic to *G. pulex*, whilst

3-iodo-2-propinyl butyl carbamate was moderately toxic and propiconazole and tebuconazole (triazoles fungicides) were not toxic for *G. pulex*. However, when *G. pulex* was exposed to a mixture (45.8% propiconazole, 18.8% 3-iodo-2-propinyl butyl carbamate, 18.2% of cypermethrin and 17.2% tebuconazole) the toxicity was equal to that of the most toxic competent of cypermethrin. The toxic effects were markedly higher after exposure of *G. pulex* to the real commercial mixture including pesticides, solvents and additives. Additives might also act since they are themselves are toxic, or they reduce the activity of detoxification mechanisms, or they facilitate pesticide intake (Adam *et al*., 2009). Jemec *et al*. (2007b) compared the toxicity of imidacloprid (IMI) and its commercial liquid formulation (Confidor SL 200) on *D. magna* and authors observed that these compounds have similar effects on the reproduction of *D. magna*. However, survival was affected at lower concentrations of Confidor SL 200 than IMI, this may due to the synergism between the solvents and IMI.

The toxicity of diazinon oxon was higher than diazinon in the current study and this agreed with the study by Sparling and Fellers (2007), who found that diazinon oxon also was more toxic than diazinon to larval frogs *Rana boylii*. Numerous studies have been performed using other species of gammarids as a model to investigate the effect of diazinon on these species (Johnson and Finley, 1980 and Ashauer *et al*., 2010). Ashauer *et al*. (2010) indicted that the 96 h LC_{50} (13.6 nM) of diazinon on *G. pulex* was less than the 96 h LC_{50} in this study. Johnson and Finley, (1980) also observed that the closely related *G. fasciatus* was more sensitive to diazinon compared with *G. pulex* in the present study. These differences of the toxicity of diazinon may be due to different organisms or different experimental conditions. It is important to know where and at which stages the toxic reactions of diazinon occur. An essential part in understanding the toxicity of diazinon is its requirement for bioactivation. Cantrell *et al*. (1998)

indicated that a cytochrome P450 inhibitor, ipiperonyl butoxid, blocked both embryo toxicity and DNA degradation by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Moore and Waring (1996) observed significantly decreased rates of the reproductive steroids in Atlantic salmon *Salmo salar* after exposure to 985.7 pM (0.3 µg/l) diazinon for 120 h.

In the current study, it was clear that the toxicity of all types of diazinon was significantly increased with increasing concentrations of pesticides and exposure time (Figures 3.1 to 3.6). Other authors also observed these results (Köprücü *et al*., 2006; Adedeji *et al*., 2010) though the actual LC_{50} values varied between organisms. Differences in the toxicity of diazinon may be due to variation in the sensitivity of AChE, as well as different detoxification and absorption kinetics (Oh *et al*., 1991). Altinok *et al*. (2006) indicted that the difference of methiocarb toxicity may be due to the different size of fish or water quality parameters.

Toxicological data is required for many levels of biological organization to evaluate the possible hazards of pollutants on aquatic resources. Initial acute toxicity testing is necessary, cost effective and the clear end points allow easy calculation of mortality. However, studies on the effect of pollutants on survival, growth, reproduction, spawning hatching success and foraging ability, etc., are more environmentally relevant. Classical toxicity testing also is expensive, and labour intensive and takes 96 h to conduct. It is also subject to operator error, because each test chamber needs to be processed at suitable times since acute and chronic tests need to be manually processed (Girling *et al*., 2000 and Lloyd Mills *et al*., 2006). The development of more environmentally relevant (semi) automated toxicity tests would be of great benefit to industry and environmental regulators.

6.3 Potential behavioural markers of toxicity

Behavioural studies of an organsim following contaminant exposure offer a useful understanding of the probable environmental effects of toxic pollution rather than lethal consequences, particularly when behaviour is affected at environmentally realistic toxicant levels (Faimali *et al*., 2006). Mortality does not always occur in organism exposed to stress under natural conditions. The normal activities such as movement, survival, feeding and reproduction may interfere with behaviour, and are likely to have an effect at the population level. Behavioural measurements are mainly useful as these endpoints are easy to measure and they are relevant to ecology (Roast *et al*., 1999b; Wallace and Estephan, 2004).

The results of movement behaviour in the current study indicate that the effect of diazinon, diazinon oxon and diazinon in the commercial formulation on movement behaviour of *G. pulex* was dependant on the type of movement, type of pesticides and concentrations used. Therefore, up to 100 nM diazinon did not significantly change the phototactic behaviour, geotactic behaviour and horizontal movement of *G. pulex* after 24 h exposure. Also up to 32.9 µM (10.0 mg/L) diazinon had no effect on swimming activity of chinook salmon (*Oncorhynchus tshawytscha)* after 2 h exposure (Scholz *et al*., 2000). However, the vertical swimming experiment in the current study indicated that 50 and 100 nM of diazinon and 25 and 50 nM of diazinon in the commercial formulation significantly decreased the number of surfacings of *G. pulex* after 24 h of exposure. But up to 100 nM of diazinon oxon produced no significant difference in the number of surfacings of *G. pulex* compared with control organisms. However, phototactic behaviour in *D. magna* (Straus) was suitable for examining the effects of low concentrations of a range of toxic compounds such as pesticides (dimethoate and lindane) and heavy metals such as mercury (Martins *et al*., 2007). Alteration in phototactic behaviour of

juvenile *Gammarus* corresponded to the body size (Hunte and Myers, 1984). Some behaviours of an organism may be more or less sensitive to the contaminant exposure than others. For instance, changes in prey capture by grass shrimps *Palaemonetes pugio* from contaminated environments have been observed. However, predator avoidance of this organism was not affected (Perez and Wallace, 2004).

It was clear that only the vertical swimming behaviour of *G. pulex* was affected by diazinon compared with horizontal movement, phototactic behaviour and geotactic behaviour. The variances of sensitivity of *G. pulex* movements toward diazinon, diazinon oxon and diazinon in the commercial formulation may be due to the energy required for these behaviours. Therefore, *G. pulex* may consume more energy in vertical swimming than other movements. Similarity, vertical swimming of *G. lawrencianus* was more sensitive to cadmium than horizontal movement activity, due to the supposed greater energetic rates linked with producing sufficient thrust to attain the lift essential to make a vertical rise in the water (Wallace and Estephan, 2004) than the forward movement created by the beating of the pleopods (Boudrias, 1991; Wallace and Estephan, 2004). Lawrence and Poulter (1998) studied the effect of sublethal toxicity on swimming behaviour of *G. duebeni* and their results indicated that swimming endurance (i.e., length of time swimming into a current) is a suitable indicator of exposure to organic (e.g., benzo[a]pyrene) and copper pollutants . In contrast, with this study, horizontal swimming activity of *G. lawrencianus* was decreased with increased cadmium concentration and it could be possible that the different sensitivity between vertical swimming and horizontal swimming in *G. lawrencianus* may be related to differences in the energetic costs of these two behaviours (Wallace and Estephan, 2004).

The effect of diazinon on the vertical movement and the surface movements of Medaka *Oryzias* could be related to the decrease in the

AChE activity and behavioural responses of Medaka after exposure to low concentrations of diazinon had a neuro-physiological background (Chon *et al*., 2005). The effect of diazinon on swimming speeds of adult zebrafish *Brachydanio rerio* was dependent on the concentrations of diazinon Low concentrations increased swimming speeds, but higher doses decreased speeds (Wall, 2000). Thus, the assessment of vertical swimming activity in *G. pulex* and related species is ecologicaly relevant and is easy to measure, making it a suitable tool to assess toxic effects on aquatic organisms.

Feeding activity and growth level are sensitive indicators to sublethal exposure since they can have an effect on higher levels of biological organisation (Rinderhagen *et al*., 2000). Feeding rate could be decreased in the presence of pesticide or other contaminants due to its effect on the sensing system (Satapornvanit, 2006). Feeding activity of *G. pulex* in the current study depended on the type of diazinon pesticide, its concentration and the exposure period. The feeding rate did not significantly change after exposure to up to 25 nM diazinon oxon for 24 and 48 h (Figure 3.12 and 3.13) but diazinon significantly inhibited the feeding rate of *G. pulex* after exposure to 10 and 25 nM for 48 h. Also, feeding behaviour of *G*. *pulex* was more sensitive to diazinon in the commercial formulation than diazinon and diazinon oxon since feeding activity was significantly reduced after exposure to 5 and 25 nM diazinon in the commercial formulation for both periods of exposure of 24 h and 48 h.

G. pulex has also been used as a model species by Blockwell *et al*. (1998) to study the effect of sublethal toxicities. Decreases in feeding activity of gammarids were observed after exposure to 3,4-dichloroaniline for 240 h, lindane and copper for 96 h. However, decreasing in the concentration of lindane with increased exposure time (240 h) significantly increased the feeding activity of *G. pulex* compared with control organisms. The authors explained that the

increase in feeding activity may be due to the stimulatory effect related to the toxicant action of lindane.

In the current study, a 24 h exposure time of sublethal diazinon and diazinon in the commercial formulation and 48 h exposure period to sublethal level diazinon and not diazinon oxon seems to be enough to inhibit the feeding activity of *G. pulex*. Similarity the feeding rate of *D. magna* was decreased after exposure to sublethal concentrations of diazinon for 24 h (Fernandez-Casalderrey *et al*., 1994). However, the feeding activity of *G. fossarum* and *Macrobrachium rosenbergii* required more than 24 h exposure time for inhibition by sublethal concentrations of chlorpyrifos (Xuereb *et al*., 2009) and dimethoate (García de La Parra *et al*., 2006).

The inhibition of feeding behaviour by exposure to diazinon may be due to it affecting motivation to feed, its search effectiveness, or ability to capture prey (Gaworecki and Klaine 2008). Usually the first response to environmental stress is the feeding activity (McLoughlin *et al*., 2000). If reduced, feeding activity has possible effects on other life history traits, for example on reproduction, growth and eventually the survival of organisms (Maltby, 1999 and Pestana *et al*., 2007). Influences on the feeding activity of individuals may be linked to effects on populations and, more vital than that, to impacts on important ecosystem functions (Pestana *et al*., 2007). The decrease of *G. pulex* feeding behaviour may be related to a decrease in its abundance and also with a reduced rate of detritus processing and macroinvertebrate diversity in environmental contaminations (Maltby *et al*., 2002). Feeding assays are relatively cheap and simple to run compared with other physiological endpoints (Pestana *et al*., 2007). The results presented here show that *G. pulex* appear to be promising test organisms and that physiological endpoints such as their feeding behaviour can be used to detect deleterious effects of pesticides such as OPs.

6.4 Acetylcholinesterase

There is a body of information available on using AChE as a particular bioindicator of insecticide exposure in aquatic organisms, but, these studies are difficult to compare as the authors use a variety of different units such as nmol min^{-1} $(mg\ P)^{-1}$ and % of control activity without stating basal activity (Xuereb *et al*., 2007). These authors observed that *G. pulex* is one of the most sensitive organisms for using AChE as a biomonitoring tool to evaluate the effect of OP exposure.

AChE monitoring in the environment offers clear advantages over the use of analytical chemistry alone (Fulton and Key, 2001). It is able to offer reliable data in real-time without or with a minimum sample preparation, and is cheap and simple to handle. AChE activity is essential for various physiological functions such as predator evasion and orientation toward food and prey location (Roex *et al*., 2003; Adedeji, 2011). Therefore, terrestrial and freshwater aquatic ecosystems have been widely used to monitor the inhibition of AChE *via* exposure to OPs and physiological effects in exposed organisms. Its utility as a bioindicator is directly related to the substance's toxic mode of action of the toxicants. A significant reduction of AChE activity not only means that the animal has been exposed, but also that a high enough amount of the pollutant has reached the target site to induce a physiological effect (Edwards and Fisher, 1991; Fulton and Key, 2001). AChE activity of aquatic invertebrates has been measured in different extracts including whole *G. pulex* (Xuereb *et al*., 2007), the head capsule of *G. pulex* (Crane *et al*., 1999), muscle and eyes of *Litopenaeus vannamei* (García de La Parra *et al*., 2006) and the gills of *Mytilus galloprovincialis* (Mora *et al*., 1999). A number of studies have indicated that the activity of AChE using whole bodies was lower compared with this activity in the head of *G. pulex* (Crane *et al*., 1999; McLoughlin *et al*., 2000). This may be because the head has a higher amount of AChE than other parts of the body and also this enzyme is

one of the most efficient enzymes of the nervous system. The activity level of AChE in *G. pulex* is observed to be of the same order of magnitude as those estimated in most aquatic invertebrates (Xuereb *et al*., 2007).

The current study found a significant inhibitory effect of exposure to sublethal concentrations of diazinon, diazinon oxon and diazinon in the commercial formulation on AChE activity in whole homogenates (9000g supernatant) of *G. pulex*. Hamm *et al*. (2001) observed that diazinon was biotransformed to a more potent oxon metabolite that inhibited AChE activity in whole homogenates of stage 34 embryos and 24 h larvae of *Oryzias latipes*.

It is well known that OPs produce toxicity by inhibiting AChE in both vertebrate and invertebrate organisms. The inhibition of AChE led it to its being unable to break down acetylcholine, which is important in cholinergic nerves, since the muscle involved cannot relax when the neurotransmitter is not broken down after it has served its function. This might produce spasms and paralysis which lead to muscular convulsions (Fulton and Key, 2001; Üner *et al*., 2006). The data shown in this thesis indicate that 10 µM diazinon oxon caused almost full inhibition (92%) of AChE in gammarid extracts *in vitro* with an IC_{50} value of 3 µM. However, up to 10 µM of diazinon and diazinon in the commercial formulation did not inhibit the enzyme activity in the same experiment. In marked contrast, *in vivo* exposure inhibited AChE activity following exposure to diazinon and diazinon in the commercial formulation for 24 h, and the IC_{50} values were 17.5 and 10 nM, respectively. However, diazinon oxon did not significantly inhibit the AChE activity via the *in vivo* exposure route.

Kretschmann *et al*. (2011a) observed that AChE in *D. magna*is was very sensitive towards diazinon oxon. One possible explanation for these results may be that diazinon oxon is less toxic *in vivo* on AChE

activity of *G. pulex* than diazinon and diazinon in commercial formulation due to a more rapid degradation rate and/or reduced uptake. Kretschmann *et al*. (2011a) also observed that the maximum inhibition speed of AChE on *D. magna* is reached when diazinon oxon is present at the target. According to the study obtained by Ashauer *et al*. (2006) accumulated chlorpyrifos is quickly eliminated in *G. pulex*. *In vitro* and *in vivo* inhibitions were used also by Xuereb *et al*. (2007) to investigate the effect of chlorpyrifos on the AChE activity of *G. pulex*. Their results indicated that chlorpyrifos exposure led to inhibition of *G. pulex* AChE activity in both *in vitro* and *in vivo* conditions. The AChE activity was only 5% at 100 µM compared with 100% control activity *in vitro*, whilst chlorpyrifos induced a significant AChE inhibition (the 96 h IC_{50} was 0.99 nM) *in vivo* experiment. Also the *in vivo* study by Barata *et al*. (2004) supported the current study: AChE activity of *D. magna* declined with increasing exposure time to malathion and chlorpyrifos.

The results obtained in this thesis indicated that diazinon oxon has a higher toxicity than diazinon on *G. pulex*. However, diazinon caused strong inhibition of AChE activity in *G. pulex*, but diazinon oxon did not significantly inhibit this enzyme at up to 50 nM *in vivo* study. Hence, the mortality of *G. pulex* exposed to diazinon oxon may not be directly related to AChE inhibition but to an additional mechanism. Similarity Xuereb *et al*. (2009) suggested that the observed mortality of *G. fossarum* after exposure to chlorpyriphos was not directly related to AChE inhibition, but that an additional toxic mode of action occurred.

Generally, exposure of organisms to diazinon leads to inhibition of AChE activity (Üner *et al*., 2006), but AChE activity of *D. magna* did not change after exposure to sublethal concentrations of diazinon (16.4 nM, 5 µg/l) for 21 days (Jemec *et al*., 2007b). They suggested that this might be due to metabolism of diazinon in this organism. Daizinon with low water solubility should be readily be absorbed

across permeable surfaces such as the gills. US EPA (2007) indicated that daizinon oxon is very toxic to aquatic animals compared with daizinon. However, diazinon oxon is short-lived in the environment and its concentrations are likely to be lower when compared to diazinon.

The increased uptake and bioactivation of diazinon in medaka *Oryzias latipes* was allied with the increased sensitivity of AChE activity (Hamm *et al*., 2001). The bioactivation to oxon metabolites is a key component of the toxicity of OPs. Therefore, toxicity of these diazinon compounds may be related to the ability of their metabolites to be bioactivated (Hamm *et al*., 2001).

The guppy *Poecilia reticulate* is the most sensitive fish species to diazinon, and has the highest capacity to bioactivate this pesticide, whilst the carp *Cyprinus carpio* had a low ability to bioactivate diazinon and was insensitive (Keizer et al., 1995). Hamm *et al*. (2001) used an incubation system in which inhibition of an exogenous AChE source is correlated to metabolic conversion of diazinon to the more potent AChE-inhibiting oxon metabolite. This experiment demonstrated the role of metabolic activation in the sensitivity of pesticides. The metabolism may play a role in the altered toxicity of diazinon in the zebrafish and the guppy by triggering two different mechanisms of action. This may be due to the effective oxidation of diazinon to diazinon oxon and may be responsible for the higher toxicity of this OP in the guppy than in the zebrafish during in *vivo* studies (Keizer *et al*., 1995).

Other studies have found that POs have a lack of AChE inhibition after exposure to levels based on their respective LC_{50} values (Fulton and Key, 2001). To achieve a 50% reduction in AChE activity in tiger shrimp *P. japonicas* larvae, fenithrothion concentrations up to 50 times higher than the 24 h LC_{50} were required. Also in the same organism, diazinon concentrations 300 times higher than the 24 h LC_{50} were

needed to inhibit AChE activity by 50% (Rompas *et al*., 1989; Fulton and Key, 2001). Key (1995) did not find IC_{50} for 24 h of AChE activity after exposure of adult grass shrimp *Palaemonetes pugio*un to malathion. In addition, to avoid complete mortality of grass shrimp, organisms cannot be exposed to more than six times the 48 h LC_{50} for malathion. Even at this level grass shrimps were either moribund or dead. Therefore, a 50 % inhibition of AChE activities could not be achieved without killing the exposed organism. The inhibition of AChE activity in adult shrimps surviving malathion exposure was no more than a 30 % reduction (Fulton and Key, 2001).

6.5 Potential molecular markers of toxicity

HSPs

HSPs are groups of proteins which function as molecular chaperones and they are classified into HSP groups by their molecular weight. The common families of HSPs are HSP 100, HSP 90, HSP 70, HSP 60 and the HSP 12-43 kDa. HSP concentration can be induced by cellular stress and the presence of denatured proteins. HSPs stabilize proteins and are involved in the folding of denatured proteins (Malinovska *et al*., 2012). These families can be induced or increased after exposure to several environmental stressors such as tributyltin, pesticides, heavy metals and as well as other pollutants, which make it more difficult for proteins to form their right structures and cause some already structured proteins to unfold. Left uncorrected, miss-folded proteins form aggregates that may finally kill the cell (Sanders and Martin, 1993; Paranko *et al*., 1996; Iwama *et al*., 1998).

In the current study, the 9000g supernatant and 9000g pellet fractions were used to evaluate the effect of sublethal concentrations of

diazinon, diazinon oxon and diazinon in the commercial formulation on HSPs of *G. pulex* after 24 h exposure. HSP 60 was only detected in pellet fractions. This may be due to HSP 60 in enriched mitochondria. However, HSP90 and HSP 70 could be detected in the 9000g supernatant and 9000g pellet fractions, suggesting that these proteins were abundant in both extracts. sHSP levels were only detected in the 9000g supernatant and not in the pellet samples. One possible explanation is that protein is more sensitive to pesticides in supernatant than in the pellet sample. HSP 60 and sHSP were not detected in the 9000g supernatant and 9000g pellet respectively. It is possible that these proteins may require increased concentrations or exposure time to be detected.

Werner and Nagel. (1997) have used different cell extracts (supernatant or soluble fraction and pellet, insoluble, or membrane fraction) of two infaunal (estuarine *Ampelisca abdita*; marine *Rhepoxynius abronius*) and one epifaunal freshwater amphipod (*Hyalella azteca*) to investigate the effect of chemical compounds such as cadmium, diazinon and dieldrin on HSPs expression. Their results indicated that xenobiotic compounds influenced intracellular distribution of HSPs in *A. abdita*, R. *abronius* and *H. azteca.* Bensaude *et al*. (1990) cited in Werner and Nagel (1997) indicated that HSPs are soluble proteins under normal conditions. The alterations may represent structural modification of HSPs or they could be a consequence of the overall structural variations of proteins in stressed cells. HSPS were detected primarily in insoluble extracts of *Ampelisca abdita* after exposure to diazinon, whilst HSP 60 was observed in the soluble fraction only after strong exposure. Therefore, HSP 60 was detected in both sample extracts of *R. abronius*. In agreement with the current study HSP 70 was detected in both pellet and supernatant fractions of *H. azteca* (Werner and Nagel. 1997). Another study by Iwama *et al*. (1998) used fish cell lines to study the alterations in HSP expression in response to environmental contaminants. Monitoring HSP

expression is important in multiple tissues or organs, because, the intensity of HSP response is organ-specific (Eder *et al*., 2009).

The data obtained in this thesis indicated that sHSP levels in the 9000g supernatant of *G. pulex* significantly increased following exposure of the animals to 50 nM diazinon and diazinon in the commercial formulation but not to diazinon oxon for 24 h. However, this protein was not detected in 9000g pellet samples. The ability of only diazinon and diazinon formulation to induce sHSP level suggests either that only the parent compound is responsible for this stress response, or this could be due to different sensitivities of proteins in both samples. Another reason is that it may be richer in soluble protein than membrane protein. Bedulina *et al*. (2010) observed that HSP 70 and sHSP of *G. pulex* increased following exposure to natural organic matter (humic substance contents) for 30 min. Similarity Timofeyev *et al*. (2008) found that 24 h exposure to cadmium caused induction of sHSP in *Eulimnogammarus verrucosus*, *Eulimnogammarus cyaneus* and *G. lacustris.*

As HSP 60 was detected in pellet samples but not in supernatant samples, it is possible that HSP 60 is in enriched mitochondrial protein. Meinhardt *et al*. (1995) indicated that the presence of HSP 60 corresponds with mitotic activity suggesting the presence of active protein assembly machinery and mitochondrial protein importing more mitochondria for the dividing cells. In addition, all concentrations of all three pesticides did not significantly increase the content of this protein in the insoluble extract. It is possible that HSP 60 was less sensitive. A similar study indicated that HSP 58 of two crustaceans *H. azteca* and *A. abdita* significantly increased in supernatant samples after increasing the concentration of diazinon (Werner and Nagel, 1997). Since there is no effect of diazinon, diazinon oxon and diazinon in the commercial formulation on HSP 60 of *G. pulex*, this protein may not be a useful indicator to investigate the effect of diazinon.

Diazinon oxon did not significantly change the content of HSP 70 in either sample, whilst 50 nM diazinon significantly increased HSP 70 levels in supernatant samples of *G. pulex*. On the other hand HSP 70 levels significantly increased in pellet samples of *G. pulex* after exposure to 50 nM diazinon and diazinon in the commercial formulation for 24 h.

HSP 70 levels in muscle tissue of juvenile Chinook salmon significantly increased after exposure to 3.4 nM (1.2 µg/L) chlorpyrifos for 96 (Eder *et al*., 2004), whilst, HSP 71 was significantly increased in *Hyalella azteca* and *Rhepoxynius abronius* at 98.6 nM (30 µg/L) diazinon for 24 h. However, in the pellet fraction of *Ampelisca abdita*, HSP 71 showed no response (Werner and Nagel, 1997).

HSP 90 in the supernatant sample was significantly increased after exposure to 50 nM diazinon and diazinon in the commercial diazinon, whilst only 50 nM diazinon in the commercial formulation significantly increased the relative level in pellet samples. However, diazinon oxon did not significantly increase the content of this protein in both samples compared with the unexposed samples. This may suggest that only diazinon and diazinon in the commercial formulation have the ability to induce HSP 70 and HSP 90 levels.

3.4 nM (1.2 µg/L) chlorpyrifos and 238.2 pM (0.1 µg/L) esfenvalerate significantly increased the relative level of HSP 90 in muscle tissue of juvenile Chinook salmon for 96 h However, this protein was not detected in the gills of this organism (Eder *et al*., 2004).

The content of HSPs in the 9000g supernatant and pellet samples after diazinon treatment is best described as offering a yes or no response, demonstrating whether or not the extracts have been exposed to contaminants with the potential to induce a stress response (Clayton *et al*., 2000).

There are several advantages of using active biomonitoring such as: limited impact of field exposure; experiments can be performed for a known exposure period; it is easy to compare different sites if the animals are normally not present at exposure location (Smolders *et al*., 2003). As HSP levels are believed to rise in response to the accumulation of harmed proteins in the cell, HSP content patterns can serve as a biomarker of pollutant effects (Ananthan *et al*., 1986). Therefore, using HSP 70 and HSP 90 in *G. pulex* as biomarker are appropriate for the analysis of chemical toxicity (such as diazinon) of water, and would be particularly useful for studying physiological disturbances in aquatic organisms by naturally or accidentally modified environmental factors.

Phosphoproteins

Protein phosphorylation is the most common kind of post-translational modification used in signal transduction. This protein affects each process, such as metabolism, organelle trafficking, muscle contraction, growth, division, differentiation, motility, immunity, learning and memory. Protein phosphatase and kinases are enzymes catalysing the transfer of phosphate between their substrates. A protein phosphatase catalyses the transfer of the phosphate from a phosphoprotein to a water molecule, whilst a protein kinase catalyses the transfer of γ-phosphate from ATP to its protein substrates (Ubersax and Ferrell, 2007; Cheng *et al*., 2011).

Despite the environmental importance of *G. pulex*, there is very little information on the toxicity of OP pesticides on phosphoproteins in this organism. Two techniques were used in phosphorylation studies to detect phosphoproteins in the current study. Firstly dot blot system (total proteins) and secondly SDS-PAGE and Western blotting analysis.

It is clear that the results obtained by the second techniques were more useful than the total protein method because, it showed clear effects of diazinon, diazinon oxon and commercial diazinon formulation in soluble protein. In the dot blot analysis, only the phosphotyrosine content of *G. pulex* of the total proteins in supernatant extracts was significantly increased by 50 nM diazinon and 10 and 50 nM of diazinon in the commercial formulation, but not the pellet extract. However, the content of phosphoserine, and phosphothreonine was not affected by exposure of *G. pulex* to required concentrations of diazinon, diazinon oxon and diazinon in the commercial formulation for 24 h.

In contrast, by using SDS-PAGE and Western blotting analysis, these pesticides increased the levels of phosphoproteins in supernatant extracts of *G. pulex* after 24 h. Therefore, the phosphorylation of phosphotyrosine content significantly increased the bands correspond to 75 and 51 kDa after exposure to 50 nM diazinon and diazinon in the commercial formulation compared with the unexposed organism. Interestingly, diazinon oxon, diazinon and diazinon in the commercial formulation at 50 nM significantly increased phosphorylation of the band corresponding to 51 of phosphoserine content. However, bands corresponding to 75 kDa of this protein were significantly increased phosphorylation after exposure of the organism to only 10 and 50 nM diazinon and diazinon in the commercial formulation, but not at up to 50 nM diazinon oxon. Similarly, only 50 nM diazinon and diazinon in the commercial formulation significantly increased phosphorylation of the bands corresponding to 75 kDa of phosphothreonine content of *G. pulex* compared with control fractions. The alteration of protein phosphorylation may be one of the mechanisms related to the toxicity of diazinon in *G. pulex*.

The measurable data from SDS-PAGE followed by Western blotting showed a clear effect of diazinon, diazinon oxon and diazinon in the commercial formulation on the levels of phosphotyrosine,

phosphoserine, and phosphothreonine content in whole gammarid protein extracts, compared with the total protein extracts using the Biorad manifold dot blot 69 well system. The explanation of these differences is that, while there may be no change in total levels of phosphorylation on dot blots, the phosphorylation of individual proteins may rise or fall, as highlighted on probed Western blots, indicating that analysis of individual bands is more useful than total phosphoprotein

Protein phosphorylation has been used as a biomarker to investigate the effect of pesticides on intracellular protein of organisms. For example, Miyazawa and Matsumura (1990) studied the effect that deltamethrin has on protein phosphorylation in *Homarus americanus*. Their results indicated that exposing this organism to deltamethrin affected protein phosphorylation and dephosphorylation activities in its nervous system. Bykova *et al*. (2003) indicated that phosphoprotein is an essential posttranslational modification, the role of which is essentially unexplored in mitochondria. Walker *et al*. (2005) observed that exposure of *H. americanus* to methoprene caused differing patterns of protein phosphorylation in the comparable fractions of this species. Liu *et al*. (2008) claimed that the effects of deltamethrin and methamidophos on protein phosphorylation could be one of the toxicity actions of these substances to Japanese pine sawyer and other insects. Liu *et al*. (2008) found that most protein phosphorylation from the fat body and head of *Monochamus alternatus* was significantly inhibited after *in vitro* exposure to deltamethrin, with proteins at 113, 98 and 51 KDa completely inhibited. However, deltamethrin significantly increased the phosphorylation of a 138 kDa protein from the fat body.

The important signal to clarify the mechanisms of various actions at the molecular level has been offered by these studies on protein phosphorylation (Miyazawa and Matsumura, 1990; Liu *et al*., 2008). Phosphorylation is important for many processes in living cells. It

regulates protein functions by inducing conformational alterations or by disturbance and creation of protein-protein interaction surfaces.

Phosphorylation is essential to transfer a phosphate group to a molecule, which holds a hydroxyl group, or a terminating–OH. For example, before a glucose molecule is broken down it is phosphorylated twice to yield energy needed by the cell. Kinases use the chemical energy from adenosine triphosphate (ATP) to add phosphate groups to the sugar (Mullis, 2008). Matsumura *et al*. (1989) found that deltamethrin effects a significant alteration in protein phosphorylation activity in the optic lobe of squid, *Loligo pealei*. These authors suggested that calcium-calmodulin dependent protein kinase and synapsin gene I (synapsin I) were the best indicator proteins reacting to deltamethrin. Liu *et al*. (2008) suggested that methamidophos may inhibit a Ca^{2+}/CaM activated kinase of *Monochamus alternatus*, which is responsible for phosphorylation of the 51 kDa protein. However, it is unclear how pesticides affect protein phosphorylation in *G. pulex*.

2D PAGE can separate proteins on the basis of their the size as well as the charge of each respective protein (Marko-Varga, 2004). Whilst it is a sensitive and mature technique, questions remain concerning its ability to characterize all of the elements of a proteome. Proteome analysis combines 2D PAGE to separate and visualize proteins and mass spectrometry (MS) for protein identification (Gygi *et al*., 2000).

2-D PAGE is able to resolve thousands of proteins, however only the most abundant ones are visualized by staining and can be identified. This technique is still the most commonly used multidimensional separation method for the analysis of proteome mixtures (Janini and Veenstra, 2002).

In the current study only 9000g supernatant samples of whole *G. pulex* were used to investigate the effect of 50 nM diazinon after exposure for 24 h. Seven protein spots were quantified by image analyses from around 150 protein spots detected. Four proteins were decreased by ≥ 1.8 fold and three proteins were increased by ≥ 2.2 fold. 21 proteins of 15000g supernatant of *G. pulex* showed significant differences after exposure to PCB (Leroy *et al*., 2010) whilst, 18 protein spots of gill tissue of *Paralichthys olivaceus* significantly changed after exposure to cadmium (Ling *et al*., 2009). Membrane proteins of zebrafish liver were significantly altered after exposure to methyl parathion (Huang and Huang, 2011). Therefore, 2D-PAGE represents a possible suitable technique for the identification of novel protein bioindicators following exposure of aquatic organisms to environmental toxins such as OPs.

6.6 Conclusions

In conclusion, this thesis provides information on the effects of diazinon, diazinon oxon and diazinon in the commercial formulation on the freshwater shrimp *G. pulex*. It determined the 96 h LC_{50} values of these pesticides in *G. pulex*, then investigated the effect of sub-lethal concentrations of the chemicals on behavioural and molecular markers in *G. pulex*.

The findings can be summarised as follows:

• Diazinon in the commercial formulation was 13.5 fold and 4.4 fold more toxic than diazinon and diazinon oxon (96 h LC_{50}).

• Exposure to 50 nM diazinon and 25 nM diazinon in the commercial formulation for 24 h significantly reduced vertical swimming behaviour.

• The 24 h IC_{50} values for *in vivo* inhibition of AChE activity were 17.5 nM for diazinon and 10 nM for the diazinon in the commercial formulation, whilst diazinon oxon had no significant effect. In contrast, with *in vitro* diazinon and diazinon in the commercial formulation there was no significant effect up to 10 µM, whilst the IC_{50}inhibition of AChE by diazinon oxon was 3 µM.

• The feeding rate of *G. pulex* significantly decreased only after 48 h of exposure to 10 and 25 nM diazinon, whereas the feeding rate of *G. pulex* exposed to diazinon in the commercial formulation was significantly inhibited after only 24 h at 5, 10 and 25 nM.

• Small HSP levels significantly increased in 9000g supernatant sample only after exposure of *G. pulex* to 50 nM diazinon and diazinon in the commercial formulation. However, small HSPs were not detected in the 9000g pellet sample.

• Up to 50 nM diazinon, diazinon oxon and diazinon in the commercial formulation did not significantly affect the level of HSP 60 in 9000g pellet extract. However, HSP60 was not detected in 9000g supernatant samples.

• HSP 70 levels significantly increased in 9000g pellet samples only after exposure of *G. pulex* to 50 nM diazinon and diazinon in the commercial formulation. However, only 50 nM diazinon caused significant increase the level of HSP 70 in 9000g supernatant sample.

• The relative content of HSP 90 in 9000g supernatant sample of *G. pulex* significantly increased after exposure of organisms to 50 nM diazinon and diazinon in the commercial formulation. Only 50 nM diazinon in the commercial formulation significantly increased the relative level of HSP 90 in pellet sample.

• Dot blot assay with phosphorylation antibodies found that *G. pulex* exposed to 50 nM diazinon and 10 & 50 nM diazinon in the commercial formulation had significantly increased levels of phosphotyrosine.

• SDS-PAGE and Western blotting was carried out on the 9000g supernatant fraction. 51 and 75 kDa phosphotyrosine bands were significantly increased by 50 nM diazinon and diazinon in the commercial diazinon formulation. The 75 kDa phosphoserine band was significantly increased by exposure of organisms to 10 and 50 nM diazinon and diazinon in the commercial formulation. In contrast the 51 kDa phosphoserine band only significantly increased after exposure to 50 nM diazinon, diazinon oxon and diazinon in the commercial formulation. With the phosphothreonine antibody, only the 75 kDa band was significantly increased by 50 nM diazinon and diazinon in the commercial formulation.

In order to set a maximum recommended environmental level of diazinon in the environment it is necessary to take account of the most sensitive ecotoxicity assays and the relative toxicities of diazinon and its metabolites. Diazinon in the commercial formulation was shown to be more toxic, presumably due to components of the formulation enhancing its toxicity over that of pure diazinon. The formulation is the most likely type of diazinon to be encountered in the environment, so this is the form that needs to be considered. The most sensitive ecotoxicity assays were feeding and AChE, both of which detected a significant inhibition relative to unexposed control G. pulex at 5 nM. This equates to a diazinon concentration of 1.52 ug/l. Bearing in mind that this is the lowest tested observable adverse effect concentration achieved in a short term toxicity test, a safety margin of 100 suggests that 0.015 ug/l would be a realistic level for an environmental quality standard. This is very close to the suggested short term (0.02 ug/L) and long term (0.01 ug/L) predicted no effect concentration for diazinon proposed by the Environment Agency (2007). However, the

UK technical advisory group on the water framework directive recommended that the short term EQS for diazinon remain at 0.1 ug/L in its 2012 report. Based on the results in this thesis it is likely that the EQS has been set too high to protect against adverse effects in gammarids and other sensitive species.

6.7 Future work

It would be of interest to further characterise the molecular biomarkers in a number of ways, as indicated below.

- According to the result of 2D-PAGE, it will be necessary to design strategies to produce extracts enriched in these proteins. Sufficiently abundant spots of interest showing statistically significant changes from 2D- PAGE will be processed by tryptic digestion and mass spectrometry (MS) in order to identify them.

- Identification of 75 kDa phosphoprotein bands upregulated in diazinon organisms The protein band will be digested with trypsin and the resulting peptides will be analysed by MS. The peptides are fragmented in MS to yield diagnostic patterns that can be matched to protein sequence databases.

CHAPTER SEVEN: References

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Appendix 1

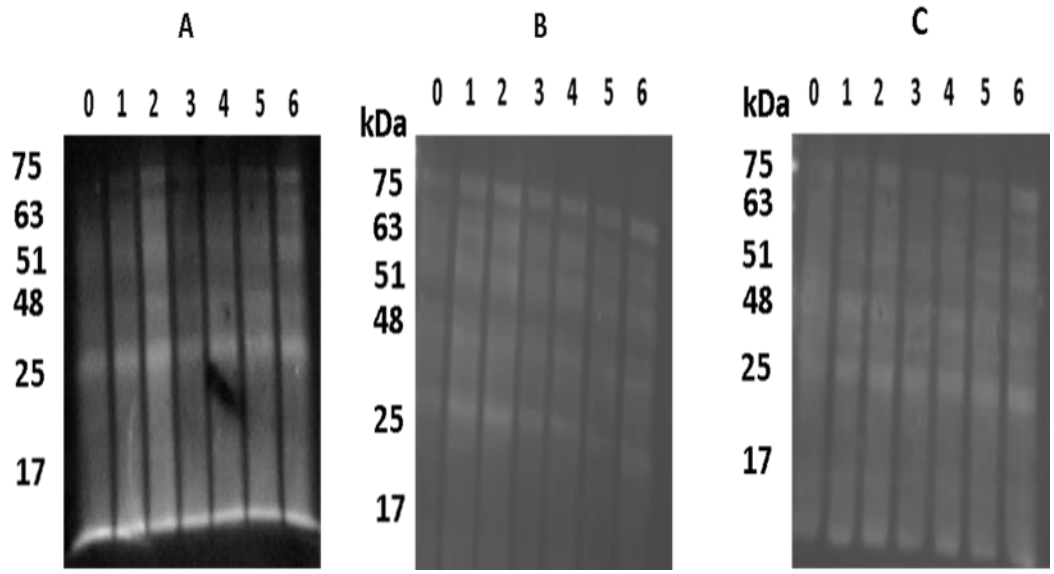

Figure 1. The whole of western blots probed with,A: phosphotyrosine antibody, B: anti phosphoserine antibody and C: phosphothreonine antibody. Shown in panel A, B and C are probed blots of extracts from gammarids prepared after exposure to diazinon (1 & 2), diazinon oxon (3 & 4) and diazinon in the commercial formulation (5 & 6) for 24 h