Molecular docking and geographical information systems as tools to assess the potential impact of veterinary medicines on non-target organisms and the environment

Bowker, F.

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Faye Bowker

A thesis submitted in partial fulfilment of the requirements of the University of Westminster for the degree of doctor of philosophy

September 2015

Abstract

Veterinary medicines (VMs) from agricultural industry can enter the environment in a number of ways. This includes direct exposure through aquaculture, accidental spillage and disposal, and indirect entry by leaching from manure or runoff after treatment. Many compounds used in animal treatments have ecotoxic properties that may have chronic or sometimes lethal effects when they come into contact with non-target organisms. VMs enter the environment in mixtures, potentially having additive effects. Traditional ecotoxicology tests are used to determine the lethal and sometimes reproductive effects on freshwater and terrestrial organisms. However, organisms used in ecotoxicology tests can be unrepresentative of the populations that are likely to be exposed to the compound in the environment. Most often the tests are on single compound toxicity but mixture effects may be significant and should be included in ecotoxicology testing. This work investigates the use, measured environmental concentrations (MECs) and potential impact of sea lice treatments on salmon farms in Scotland. Alternative methods for ecotoxicology testing including mixture toxicity, and the use of *in silico* techniques to predict the chronic impact of VMs on different species of aquatic organisms were also investigated.

The Scottish Environmental Protection Agency (SEPA) provided information on the use of five sea lice treatments from 2008-2011 on Scottish salmon farms. This information was combined with the recently available data on sediment MECs for the years 2009-2012 provided by SEPA using ArcGIS 10.1. In depth analysis of this data showed that from a total of 55 sites, 30 sites had a MEC higher than the maximum allowable concentration (MAC) as set out by SEPA for emamectin benzoate and 7 sites had a higher MEC than MAC for teflubenzuron. A number of sites that were up to 16 km away from the nearest salmon farm reported as using either emamectin benzoate or teflubenzuron measured positive for the two treatments. There was no relationship between current direction and the distribution of the sea lice treatments, nor was there any evidence for alternative sources of the compounds e.g. land treatments. The sites that had MECs higher than the MAC could pose a risk to non-target organisms and disrupt the species dynamics of the area. There was evidence that some marine protected sites might be at risk of exposure to these compounds.

To complement this work, effects on acute mixture toxicity of the 5 sea lice treatments, plus one major metabolite 3-phenoxybenzoic acid (3PBA), were measured using an assay using the bioluminescent bacteria *Aliivibrio fischeri*. When exposed to the 5 sea lice treatments and 3PBA *A. fischeri* showed a response to 3PBA, emamectin benzoate and azamethiphos as well as combinations of the three. In order to establish any additive effect of the sea lice treatments, the efficacy of two mixture prediction equations, concentration addition (CA) and independent action

(IA) were tested using the results from single compound dose response curves. In this instance IA was the more effective prediction method with a linear regression confidence interval of 82.6% compared with 22.6% of CA.

In silico molecular docking was carried out to predict the chronic effects of 15 VMs (including the five used as sea lice control). Molecular docking has been proposed as an alternative screening method for the chronic effects of large animal treatments on non-target organisms. Oestrogen receptor alpha (ERα) of 7 non-target bony fish and the African clawed frog *Xenopus laevis* were modelled using SwissModel. These models were then 'docked' to oestradiol, the synthetic oestrogen ethinylestradiol, two known xenoestrogens dichlorodiphenyltrichloroethane (DDT) and bisphenol A (BPA), the antioestrogen breast cancer treatment tamoxifen and 15 VMs using Auto Dock 4. Based on the results of this work, four VMs were identified as being possible xenoestrogens or anti-oestrogens; these were cypermethrin, deltamethrin, fenbendazole and teflubenzuron. Further investigation, using *in vitro* assays, into these four VMs has been suggested as future work. A modified recombinant yeast oestrogen screen (YES) was attempted using the cDNA of the ERα of the zebrafish *Danio rerio* and the rainbow trout *Oncorhynchus mykiss*. Due to time and difficulties in cloning protocols this work was unable to be completed. Use of such *in vitro* assays would allow for further investigation of the highlighted VMs into their oestrogenic potential.

In conclusion, VMs used as sea lice treatments, such as teflubenzuron and emamectin benzoate may be more persistent and have a wider range in the environment than previously thought. Mixtures of sea lice treatments have been found to persist together in the environment, and effects of these mixtures on the bacteria *A. fischeri* can be predicted using the IA equation. Finally, molecular docking may be a suitable tool to predict chronic endocrine disrupting effects and identify varying degrees of impact on the ERα of nine species of aquatic organisms.

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Acknowledgements

I would like to express my gratitude to my director of studies Dr Sharron McEldowney for her guidance, support and insightful comments. I would also like to thank my supervisors Dr Pamela Greenwell for all her help in the laboratory, and Dr Tamas Kiss for helping me get to grips with Linux. Thank you to Dr Miriam Dwek for the space in her laboratory and to Dr Anatoliy Markiv for the empty plasmids and to Dr Ian Bailey for the yeast transformation kit.

Thank you to Dr Farzad Pakdel for sending me plasmids containing rainbow trout and zebrafish oestrogen receptor cDNA.

To the technical staff, thank you for your support throughout especially Thakor, Harry and Paulette. Thank you to Dr Hans Heindl and Dr Samantha Walker for showing me the AutoDock ropes.

Thank you to the University of Westminster for providing me with a full scholarship, which without this project would not have been possible.

I would like to thank all of the friends I have made in the PhD room in C4.03 for making the PhD process that little bit less lonely.

Finally I would like to thank my friends and family for their love, support and putting up with me, especially my long suffering parents.

Declaration

I declare that the present work was carried out in accordance with the Guidelines and Regulations of the University of Westminster. The work is original except where indicated by special reference in the text.

The submission as a whole or part is not substantially the same as any that I previously or am currently making, whether in published or unpublished form, for a degree, diploma or similar qualification at any university or similar institution.

Until the outcome of the current application to the University of Westminster is known, the work will not be submitted for any such qualification at another university or similar institution.

Any views expressed in this work are those of the author and in no way represent those of the University of Westminster.

Signed:

Date:

Abbreviations

3D – 3 Dimensional
3PBA – 3-Phenoxybenzoic acid
AA – Amino acid
AChE – Acetylcholinesterase
ADME - Absorption, distribution, metabolism and elimination
AF - Assessment factor
AHL – Acetylated homoserine lactones
ANOVA – Analysis of variance
Arg - Arginine
Asp - Aspartic acid
AZE – Allowable Zone of Effects
BDL - Below level of detection
bp - Base pair
BPA – Bisphenol A
bw - bodyweight
CA - Concentration addition (Loewe additivity)
CHO – Chinese Hamster Ovary
CPRG - Chlorophenol red-ß-D-galactopyranoside
CPUE - Catch per Unit Effort
CVMP - Committee for Veterinary Medicinal Products
DDT – Dichlorodiphenyltrichloroethane
DES – Diethylstilboestrol
DMSO - Dimethyl sulfoxide
DNA - Deoxyribonucleic Acid
E1 – Oestrone
E2 – Oestradiol
E3 – Oestriol
EC - European Commission
EC_{50} – Effect Concentration 50%
ECHA - European chemicals agency
EDC - Endocrine disrupting chemicals
EE2 – Ethinylestradiol
EIA - Environmental impact assessment
EIC - Environmental introduction concentration
ELIZA - Enzyme Linked Immunosorbent Assay
EMBOSS - European Molecular Biology Open Software Suite
EMEA - European Medicines Agency
EQS – Environmental Quality Standards
EQSD - Environmental Quality Standards Directive
ER – Oestrogen Receptor
ERA – Environmental risk assessment
ERE – Oestrogen Response Element
ERL - Effects range low
EtOH - Ethanol
EU – European Union
GABA – γ-γ aminobutyric acid

GFP - Green fluorescent protein
gh – Growth hormone
GIS – Geographical information systems
Glu - Glutamic acid
His - Histidine
IA - Independent action (Bliss independent action)
IB - In bath
IF - In feed
igf1 - Insulin like growth factor
JNCC – Joint nature conservation committee
JRC - Joint research council
kb - Kilo base
Kcal/mol - Kilocalorie/mole
k_D – Dissociation constant
Ki – Inhibition constant
Kow - n-octal/water coefficient
LBD – Ligand binding domain
LD_{50} – Lethal dose 50%
LiAc - Lithium acetate
LOEC - Lowest observed effect concentration
MAC – Maximum allowable concentration
MEC – Measured environmental concentration
MEGA – Molecular evolutionary genetics analysis
MOA – Mode of action
MPA – Marine protected area
MSS - Marine Scotland science
n.d - No data
N/A – Not applicable
NaCl - Sodium chloride
NOEC – No observed effect concentration
NSAID – Non-steroidal anti-inflammatory drug
OD_{600} – Optical density at 600 nanometres (nm)
OECD - Organisation for economic cooperative and development
OHHL - N-3-oxohexanoyl-L-homoserine lactone
OL - Left operon
OP - Octylephenol
OR - Right operon
PCR – Polymerase chain reaction
PEC – Predicted environmental concentration
PEER - Public employees for environmental responsibility
PEP - Predicted oestrogenic potential
pKa - pH at which half of the side chain of an amino acid is charged
PMV – Python molecular viewer
PNEC – Predicted no effect concentration
POP – Persistent organic pollutant
QSAR – Quantitative structure activity relationships
RCSB - Research collaboratory for structural bioinformatics
REACH – Registration, evaluation, authorisation and restriction of chemicals

RIC_{20} – Relative inhibitory concentration 20%
RLU - Relative light units
RNA - Ribonucleic Acid
RQ – Risk quotient
SAC – Special area of conservation
SEPA – Scottish environmental protection agency
SMILES - Simplified molecular-Input line-entry system
SPA – Special protected area
STP – Sewage treatment plant
thra - Thyroid hormone receptor alpha
TRP - Tryptophan
UK – United Kingdom
URA – Uracil
VICH - International cooperation on harmonisation of technical requirements for registration of veterinary medicinal products
VM - Veterinary medicines
VMD - Veterinary medicines directorate
VRC – Variance ratio criterion
VSDB – Veterinary substances database
VTG - Vitellogenin
w/v - mass/volume
WFD - Water framework directive
YES – Yeast oestrogen Screen
ZFL – Zebrafish Liver

Table of Species

Latin name	Common name
Acartia tonsa	Marine copepod*
Aliivibrio fishceri	Bioluminescent marine bacteria*
Anabaena flos-aquae	Cyanobacteria*
Aphodius constans	Dung beetle
Apis cerana	Asiatic honey bee
Caligus elongatus	Sea louse
Chironomus riparius	Harlequin fly/bloodworm
Chironomus tentans	Non-biting midge/bloodworm
Chironomus yoshimatsui	Non-biting midge/bloodworm
Corophium volutator	Amphipod crustacean*
Crangon septemspinosa	Sand shrimp
Crassostrea gigas	Pacific oyster
Danio rerio	Zebra fish
Echinogammarus marinus	Amphipod crustacean*
Eisenia fetida/E. Andrei	Brandling worm
Escherichia coli	Gram-negative bacteria*
Euprymna scolopes	Hawaiian bobtail squid
Gasterosteus aculeatus	Three spined stickleback
Gavia stellata	Red-throated loon
Gyps bengalensis	White-rumped vulture
Hediste diversicolor	Ragworm
Homarus americanus	American lobster
Homarus gammarus	European lobster
Homo sapiens	Human
Lemna minor	Duckweed
Lepeophtheirus salmonis	Sea louse
Leptometra celtica	Filter feeding echinoderm*
Lutra lutra	European otter
Marisa cornuarietis	Giant ramshorn snail
Musca autumnalis	Face fly
Mysid sp.	Opossum shrimp
Mytilus galloprovincialis	Mediterranean mussel
Navicula pelliculosa	Diatom*
Nitocra spinipies	Epibenthic copepod*
Nucella lapillus	Dog whelk
Onchrynchus mykiss	Rainbow trout
Ophiopsila annulosa	Gravel brittle star
Oreochromis niloticus	Nile tilapia
Palaemon elegans	Rock shrimp
Phaeodactylum tricornutum	Diatom*
Phoca vitulina	Harbour seal
Pimephales promelas	Fathead minnow
Placostegus tridentatus	Rare tubeworm*

* = species description due to common name being unavailable

Table of Species continued

Latin name	Common name
Pseudokirchneriella subcapitata	Microalga*
Rutilus rutilus	Common roach
Saccharomyces cerevisiae	Brewer's yeast
Salmo salar	Atlantic salmon
Scathophaga stercoraria	Yellow dung fly
Skeletonema costatum	Diatom*
Sparus aurata	Gilt head bream
Spodoptera exigua	Beet armyworm
Tisbe holothuriae	Harpacticoid copepod*
Trialeurodes vaporariorum	Whitefly
Vibrio harveyi	Bioluminescent marine bacteria*
Xenopus laevis	African clawed frog

* = species description due to common name being unavailable

Chapter 1

General Introduction

Large animal treatments, including veterinary medicines (VMs), endo and ectoparasiticides, aquaculture treatments and antibiotics are widely used in agriculture throughout the world. They are essential for good animal welfare, producing healthy animal products and preventing financial loss (Capleton *et al.*, 2006). These compounds have the potential to pollute both the terrestrial and aquatic environment, through point source (direct) and diffuse (indirect) entry; potentially damaging non-target organisms (table 1.1). While human pharmaceuticals and VMs consist of broadly the same active ingredients, with the exception of some parasiticides, entry into the environment differs. The main route of entry to the environment from human pharmaceuticals is through the sewage system and so potential threats are to aquatic ecosystems, commonly at continuous, low concentrations (Crane *et al.*, 2006). However, with the exception of aquaculture treatments, VMs are applied in much larger doses and are often administered to a whole flocks and herds. In contrast, VMs used to treat companion animals are considered to be of lower risk than those used in food production animals in terms of environmental pollution due to their relatively low administration levels (Boxall *et al.,* 2003). The main concern with terrestrial VMs is that manure containing the parent and/or metabolised active ingredient reaches the terrestrial environment, creating spots of contamination which are a particular concern for non-target terrestrial organisms (Svendsen *et al.*, 2005). There is also the potential that VMs may leech into the aquatic environment, although the most likely route of entry to aquatic environments is though aquaculture, topical administration, spillage or disposal (Crane *et al.*, 2006; De Knecht *et al.*, 2009).

Table 1.1 Most common routes of entry for VMs.

Route of Entry	**Type of Administration**	**Example**
Runoff	Topical	Following application of topical treatment contamination can occur through precipitation or through treated animals directly entering a water source.
Waste material	Oral, parental, topical	Manure spreading, wastewater from aquaculture, wastewater from sheep dip, wastewater from clean up, residues on administrators' clothes.
Excretion	Oral	Via faeces and urine.
Spillage	Oral, parental, topical	Accidental or deliberate spillage directly into watercourse or onto land.
Disposal	Oral, parental, topical	Containers of VMs hold residues, when disposed into regular waste VMs can enter the environment.

Adapted from (De Knecht *et al.*, 2009)

Sheep dips are of particular concern, not only are they considered highly toxic, but the method of disposal is either via soakaways or direct application to 'sacrificial' land (Boucard *et al.*, 2008). Aquaculture is also beginning to attract attention and concern. It is a rapidly growing industry and the need for VMs such as ectoparasiticides, disinfectants and antibacterials is increasing. Salmon farms are situated directly in the ocean with no physical barrier between the farm and the marine environment except a metal cage. This makes it inevitable that VMs used in aquaculture are quickly dispersed into the surrounding water column and sediment, with a resultant risk to non-target organisms. Aquaculture VMs are also likely to enter the environment as mixtures, as treatments may be used in conjunction. At present, ecotoxicology tests are generally single species, single compound tests and do not take into account the additive effects of mixtures. A report published by Kortenkamp *et al.* (2009) for the European Union (EU) commission (EC) highlighted the importance of testing mixtures of compounds that are likely to enter the environment together. This has not yet been embedded in ecotoxicology regulation.

Perhaps the most documented case of a VM causing environmental damage is that of diclofenac in India and Pakistan. Populations of *Gyps* spp. vultures considerably declined in the early 1990's. It was found that they were feeding on the carcasses of livestock that had been previously treated with diclofenac (Taggart *et al.*, 2007). Diclofenac has been used in human pharmaceuticals since the 1970s, but as it is readily metabolised and has a low potency, environmental impacts were never thought to be a problem (EMEA, 2004). The 50% lethal dose (LD_{50}) data for rats, mice and rabbits is 53 mg/kg, 95 mg/kg and 157 mg/kg respectively making it a low risk drug (CVMP, 2004). Aquatic ecotoxicology data for diclofenac is limited, effect concentration 50% (EC_{50}) data is available for the water flea *Daphnia magna* which Cleuvers (2004) reported to be at 68 mg/L. Schwaiger *et al.* (2004) found that a 28 day EC_{50} chronic effect of diclofenac on the rainbow trout *Oncorhynchus mykiss* occurred at 5 µg/L and concluded that a no observed effect concentration (NOEC) for the species was 1 µg/L. It has also been reported that some rivers have been known to contain up to 2 µg/L (Schwaiger *et al.*, 2004). Toxicity tests on the white-rumped vulture *Gyps bengalensis* have shown that even the recommended low dose for treatment of mammals, 0.25 mg/kg bodyweight (bw), can cause death by renal failure and visceral gout (Oaks *et al.*, 2004). This serves as an excellent illustration of why using LD_{50} data from model species is flawed. Not only does the LD_{50} concentrate on acute toxicity, disregarding chronic effects, data is only available for a few select test organisms. Thus finding sensitivity in species that have not previously been considered becomes difficult. As part of the Water Framework Directive (WFD), 'mechanism to identify emerging pollutants', a recently compiled report by the EU Joint Research Council (JRC), under directive 2008/105/EC (the Environmental Quality Standards Directive, EQSD), has added diclofenac to its emerging pollutant watch list along with 17α-oestradiol (E2) and 17β-ethinylestradiol (EE2). The watch list is a list of ten substances that are to be closely monitored for

the next four years on an EU wide level in order to monitor and regulate their use. Diclofenac was added due to growing concerns on its concentration in water and its resulting impacts on wildlife, however there is little evidence to support this which is why it has been included in the watch list (Loos, 2015).

The potential for VMs to reach the environment largely depends on the target group and method of application. All aquaculture treatments are classed as high risk for their potential to reach the environment, as they are applied directly to the environment. Topical treatments that are applied to intensively reared animals are also high risk; this includes sheep dip and spray on solutions of antimicrobials. The potential to reach the environment from topical treatments is especially high when it is raining or the treated animals are near waterways soon after treatment, as wash off is likely to contain the parent compound (De Knecht *et al.*, 2009). Many topical treatments are used for the control of parasites; therefore they are specifically designed to target invertebrates (De Knecht *et al.*, 2009). Non-target invertebrates are especially at risk when exposed to these types of VMs. Medium risk treatments include those applied to herd animals either orally or intravenously and have a low or medium metabolism (low 80%-20%; medium <20%) and those VMs applied to individual or companion animals topically. VMs that have a low risk of entering the environment are those that are applied to herd animals intravenously or orally and have a high metabolism (>80%) and those that are applied to individual or companion animals orally or intravenously, no matter their metabolism.

1.1 Treatments in Aquaculture

The aquaculture of salmon is a fairly new farming practice, which has been in existence in the EU since the 1960s. The intensity of fish farming and levels of production in the EU have risen significantly over the past twenty years along with the reduction of large scale fishing (European Commission, 2014). This is partly in response to the decline of global fish stocks and a growing demand due to population increases (Jiang, 2010). However, carnivorous fish aquaculture such as salmon farming has been criticised for potentially damaging wild populations, as the feed often comes from wild stocks; the spread of disease is increased; and escaped fish may interact with wild populations, potentially damaging the gene pool (Krkošek *et al.*, 2006). Salmon farming is one of the biggest industries in Scotland, with 162,223 tonnes being produced in 2012 (Scottish Government, 2012) a 40.5 fold increase from 1984 where salmon production was just under 4000 tonnes (Whitmarsh and Wattage, 2006).

After Norway and Canada, Scotland is the third largest salmon producing nation (Burridge *et al.*, 2010) with the Scottish government hoping to increase production by a further 50% by 2020. Salmon can be kept at high densities e.g. sometimes up to 25 kg/m^3 in Norway (Oppedal *et al.*,

2011), which makes them more susceptible to infection, particularly by the sea lice *Lepeophtheirus salmonis* and *Caligus elongatus*.

Geographical information systems (GIS) are maps, which are used to plot data with spatial information to provide a graphical interpretation of that data. Examples of the use of GIS data include the mapping, emergence and spread of the Ebola virus in West Africa (Pigott *et al.*, 2014); determining inshore habitat types (Foster-Smith, 2010) and the modelling of particulate waste matter around aquaculture sites (Pérez *et al.*, 2002). The use of treatments to control the sea lice infestation has been of particular concern in Scottish aquaculture (Aaen *et al.*, 2015; Boxaspen, 2006; Sevatdal *et al.*, 2005). GIS can provide visual interpretations to assist with the analysis of treatments that are being applied as mixtures; which treatments are being used and where; the proximity of fish farms to special areas of conservation (SACs) and special protection areas (SPAs); the comparison of treatment use over several years; below surface currents; and the distance between sea lice treatment use and MECs in the marine sediment in Scottish salmon farms (see chapter 2).

1.2 Xenoestrogens in the Environment

The impact of xenoestrogens on the common roach *Rutilus rutilus* and the rainbow trout *O. mykiss* is well documented. In field studies it was found that 5% of a *R. rutilus* population downstream of sewage treatment plants (STP) were hermaphrodites (Jobling *et al.*, 2002). Other studies have concluded that the effluent from the STPs caused an increase in vitellogenin (VTG) production in male *O. mykiss* (Sumpter and Jobling, 1995). Other well-documented effects of xenoestrogens on fish in the environment include altered sexual development (Jobling *et al.*, 1998); reduced fertility (Jobling *et al.*, 2002); reduction in gonadosomatic growth index (Filby *et al.*, 2007); reduced osmoregulation (Carrera *et al.*, 2007; Lerner *et al.*, 2012); reduced immune functions (Casanova-Nakayama *et al.*, 2011); and altered embryonic development (Jobling *et al.*, 2003). Not only does an increase in oestrogenic substances cause change on a physiological level, but on a population level. Over a seven year study on the chronic addition of EE2 to a whole lake in Ontario, Canada, Kidd *et al.* (2007) found that population dynamics of the fathead minnow *Pimephales promelas* reduced from 180 (± 48) catch per unit effort (CPUE) in 1999 to 0.1 (± 0.001) CPUE in 2004 at concentrations of just 5 ng/L EE2. CPUE is a measure of abundance, in the Kidd *et al.* (2007) study CPUE was the mean number of *P. promelas* caught per day averaged over a 100 day period.

VTG production is often used as a biomarker of the oestrogenic activity of a substance in fish. VTG is the yolk precursor protein that is synthesised with an increase in oestrogen production. VTG is found in high levels in the plasma of gravid female fish, but is found in much lower levels in male fish (Sumpter and Jobling, 1995). As well as pharmaceuticals which have been specifically

developed to have an oestrogenic effect, such as the active ingredient in the contraceptive pill, EE2 and diethylstilboestrol (DES), it has been shown that compounds that were designed for other uses can produce oestrogenic effects on non-target organisms, primarily bisphenol A (BPA) (Ben-Jonathan and Steinmetz, 1998; Levy *et al.*, 2004) and dichlorodiphenyltrichloroethane (DDT) (Fu *et al.*, 2007) as well as some phytoestrogens such as genistein (Ng *et al.*, 2006). When investigating the oestrogenic effects of 200 pesticides against human oestrogen receptor (ER)α, human ERβ in an *in vitro* reporter assay using Chinese hamster ovaries Kojima *et al.* (2004) found that the synthetic pyrethroids, cypermethrin and permethrin, used in VM as ectoparasiticides, showed a positive oestrogenic response whereas Du *et al.* (2010) found the synthetic pyrethroid deltamethrin is a mild xenoestrogen and a major metabolite of several synthetic pyrethroids, 3-phenoxybenzioc acid (3PBA), was found to be ER antagonist. Cypermethrin and deltamethrin are used in agriculture primarily as sheep dips and also as aquaculture treatments, permethrin is used as a topical treatment for domestic dogs and cats.

It has been found that a number of chemicals used in industry and agriculture act as xenoestrogens. For instance BPA, often used in plastic and metal products, has been found to produce xenoestrogenic effects to a number of aquatic species such as the African clawed frog *Xenopus laevis* (Levy *et al.*, 2004); the rainbow trout *O. mykiss* (Ackermann *et al.*, 2002) and the zebrafish *Danio rerio* (Cosnefroy *et al.*, 2011). Other chemicals have been found to have similar effects such as nonylphenol (Madigou *et al.*, 2001); and DDT (Ackermann *et al.*, 2002; Cosnefroy *et al.*, 2011). It has also been reported that a number of VMs may have xenoestrogenic effects on non-target organisms (Kojima *et al.*, 2004) although there has been little work to support this.

In the aquatic environment, invertebrates such as molluscs and crustaceans also come into contact with xenoestrogens. Although many molluscs, such as the Pacific oyster *Crassostrea gigas* and the freshwater giant ramshorn snail *Marisa cornuarietis* have been found to have receptors similar to vertebrate ER *in vitro* studies have shown that they do not bind to oestrogens and xenoestrogens in a similar way to their vertebrate counterparts (Bannister *et al.*, 2013; Bannister *et al.*, 2007). However, *in vivo* studies have shown that oestrogens and xenoestrogens can have a physiological effect on molluscs. Oehlmann *et al.* (2000) showed that the known xenoestrogens bisphenol A (BPA) and octylphenol (OP) elicit a response on the female organs of *M. cornuarietis* and the dog whelk *Nucella lapillus*, including an increase in size of the pallial sex gland, formation of a second vagina and abnormality of the pallial oviduct. Pallial sex gland increases were also found in *N. lapillus* by Castro *et al.* (2007) when exposed to sewage effluent containing BPA and OP.

It is important to note that the biological pathway that a xenoestrogen takes can have impacts on several genes, and that these effects can vary between male and female, and with tissue.

Examples of genes affected by an increase in oestrogenic substances include thyroid hormone receptor-α (*thra*), growth hormone (*gh*) and insulin like growth factor (*igf1*), which may present complications not only affecting reproduction but other physiological changes such as growth and disturbances in osmoregulatory functions (Filby *et al.*, 2006; Lerner *et al.*, 2012). Alterations noted in male fish include the increase of VTG leading to reduced gonadal development and resulting in intersex characteristics, as well as reduced sperm motility and delayed sexual maturation (Jobling *et al.*, 2002; Jobling *et al.*, 1998; Kidd *et al.*, 2007). Changes in females include reduced fecundity and altered oogenesis (regressed and vacuolated oocytes) (Jobling *et al.*, 2002; Jobling *et al.*, 1998; Kidd *et al.*, 2007).

1.3 Using Molecular Docking as a Predictor of Ecotoxicology

Traditionally molecular docking has been used in drug development (Gschwend *et al.*, 1996), however it has been proposed more recently that molecular docking could be used to aid ecotoxicology testing (Raunio, 2011; Shyu *et al.*, 2011). While there are some commercially available programs such as FlexX and molegro there are also programs such as AutoDock 4, AutoDock Vina and DOCK that are available as open source software. Selected biomarker species are designated for traditional ecotoxicology testing, in aquatic environments these are generally an algae species (usually *Pseudokirchneriella subcapitata, Skeletonema costatum, Navicula pelliculosa, Anabaena flos-aquae*), an aquatic plant species (generally the duckweed *Lemna minor*) an invertebrate species (usually the water flea *D. magna*) and a fish species (e.g. the rainbow trout *O. mykiss* or the zebrafish *D. rerio*). These organisms might not always be representative of a given aquatic environment (Meredith-Williams *et al.*, 2012). It is proposed, in this study, that molecular docking programs could assist ecotoxicology testing by enhancing their relevance to the ecosystem that might be exposed to particular VMs through the ability to test species appropriate for the particular ecosystem.

Using these programs, this research is intended to provide an indication of which species may be more sensitive than others to certain VMs, and therefore which species may be appropriate target species when environmental risk assessments (ERA) are being designed. This represents a novel approach to ecotoxicology testing. Further wet work would need to be carried out if there are potential matches between species and compounds. Currently a mode of action (MOA) approach is being used i.e. where the MOA of the compound is considered as part of the ERA (ECETOC, 2007).

However, although certain pharmaceuticals and pesticides might have specific MOAs, it does not necessarily mean that they will only act on specific pathways and receptors. It is likely that they will act on other channels that may not have been considered (Hutchinson, 2002). By using high throughput screening of different receptors and different species links between compounds and

different channels can be made, further aiding the specificity of testing, and contributing to reducing the time and cost of traditional ecotoxicology testing. Therefore the focus of molecular docking in this instance will be on the oestrogen receptor alpha (ERα) of several aquatic species as endocrine disrupting chemicals (EDCs) and in particular xenoestrogens have been well documented in the aquatic environment (Bannister *et al.*, 2013; Bannister *et al.*, 2007; Ben-Jonathan and Steinmetz, 1998; Jobling *et al.*, 2003; Jobling *et al.*, 2002; Jobling *et al.*, 1998; Jobling and Tyler, 2003; Sumpter and Jobling, 1995; Urbatzka *et al.*, 2012).

There are limitations to using this technology in ecotoxicology testing. Firstly, 3 dimensional (3D) models of receptors need to be available. 3D coordinates of existing X-ray crystallography structures of receptors can be fed into the programs and their interactions with ligands of interest can be studied. However, as there are relatively few X-ray crystallography structures available, presently models are made using the existing X-ray crystallography structures as templates. Sequences of proteins with a similarity of >30% with an already existing 3D structure can be used as templates; however they must be used with caution and treated as theoretical proteins (Arnold *et al.*, 2006).

Once again, using sequences to build 3D structures has limitations in that only proteins that have been sequenced can be used and they must have a suitable 3D template available. Often, the crystal structure is not of the whole protein but of a specific domain. This is a fast developing technology, with more and more organisms sequenced and undoubtedly in the future these limitations will be reduced, enabling the use of 3D molecular docking to become more prevalent and relied upon.

1.4 Regulation of veterinary medicines in the EU

Environmental quality standards (EQS) are intended to protect non-target organisms from being exposed to levels of substances in the aquatic environment that may cause chronic or lethal effects. An EQS includes a maximum allowable concentration (MAC) that is calculated by determining the predicted no effect concentration (PNEC). The PNEC is a concentration of a substance that is thought to cause no effect in the environment. The PNEC is calculated by collecting ecotoxicology data from the 'most sensitive species' in the environment. Ecotoxicology tests are usually short-term acute tests, which rely on lethal concentration 50% (LC_{50}); this is the concentration where 50% of the population exposed is expected to suffer the lethal effect of the given compound. The effect concentration 50% (EC_{50}) is the concentration of the test compound at which a pre-determined effect on 50% of the population is achieved. This effect could refer to chronic effect (such as the concentration at which 50% of the population fail to produce offspring, compared with the control) or it could refer to a lethal effect (the concentration at which 50% of the population is unresponsive, this is usually used for invertebrates). By determining an EC_{50} or

LC_{50} of the most sensitive species and applying an assessment factor (AF) to it (usually 10 – 1000 times lower than the effect concentration), then it is assumed that the resulting concentration would cause no adverse effect on the wider community (European Commission, 2003). A downfall to the PNEC is that the most sensitive species may not always be the most sensitive and more 'robust' species may in fact suffer the effects of a substance more, such as the example of *Gyps* spp. vultures in section 1 of this chapter (Naidoo *et al.*, 2007; Taggart *et al.*, 2007). Calculation of a predicted environmental concentration (PEC) is more complicated than the PNEC. There is a different PEC depending on the environmental compartment in which the substance is to be released, for example in the aquatic environment there is the $PEC_{aquatic}$ and $PEC_{sediment,}$ and in the terrestrial environment there is the PEC_{soil}. A number of factors must be taken into consideration when calculating the PEC, these include projected use in kg; sources of entry (see table 1.1); environmental fate and physicochemical properties, such as log K_{ow} and solubility (see section 1.4 on regulation of VMs in the EU). If a PNEC is lower than a PEC then a substance may not be authorised for use or tougher regulation on licensing may be applied. However PECs are difficult to calculate, especially in sediment. Often the measured environmental concentration (MEC) of a substance can be substantially higher than the PEC (Boxall *et al.*, 2002). MECs are unavailable for a number of VMs, so it can be difficult to know whether or not they are having an effect on the environment and non-target organisms. Currently there is very little information on the bioavailability and adsorption of substances in sediments, this is especially concerning as, with the exception of azamethiphos and deltamethrin, the treatments used to control sea lice in fish farms disperse quickly from the water column and are likely to have a strong adsorption to sediment (see chapter 2, section 2.1.2 table 2.1).

In 2007 the registration, evaluation, authorisation and restriction of chemicals (REACH) was implemented by the European chemicals agency (ECHA) under regulation (EC) No 1907/2006 of the European Parliament and Council. REACH is an EU wide initiative which aims to manage the risk of chemicals by collecting safety information on chemicals used or produced within the EU. The majority of chemicals are regulated by REACH; however products exempt from REACH include human pharmaceuticals (under Directive 2001/83/EC) and VMs (under Regulation (EC) No 176/2004; Directive 2001/82/EC). Although all of the products discussed in this study are VMs it is important to note that some also fall under the category of plant protection products, which would fall under the regulation of REACH as well as the sustainable use of pesticides (Directive 2009/128/EC) and the WFD (Directive 200/60/EC). In particular endo and ectoparasiticides such as the synthetic pyrethroids cypermethrin and deltamethrin, and the organophosphates azamethiphos and diazinon would be subject to REACH regulation (HSE, 2012).

Medicinal products which do not fall under REACH and are instead covered by regulation (EC) No 726/2004 'Community procedures for the authorisation and supervision of medicinal products for human and veterinary use and establishing a European Medicines Agency' and more specifically for VMs, the directive 2001/82/EC 'Community code relating to veterinary medicinal products' applies. This regulation is covered by an EU-Japan-USA wide programme: 'International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products', otherwise known as VICH. VICH aims to harmonise the regulation of veterinary pharmaceuticals on an international scale. In terms of environmental impact assessments (EIA) there are two phases new VMs must pass through (VICH, 2000; VICH, 2006).

Phase I (VICH-GL6) of the EIA comprises of a series of questions on the properties of the VM to assess the possible impacts on the environment. Some VMs may pass through phase I without progressing to phase II, these are VMs which are used in low quantities and/or are substances made from natural products that degrade easily in the environment, for example vitamins, peptides and proteins (VICH, 2000). Questions include information on the estimated use, target species, whether the target species is to be treated in the aquatic or terrestrial environment, and estimates on PECs (VICH, 2000). A VM which is used for large numbers of animals at once, has an environmental introduction concentration ($EIC_{aquatic}$) of >1 µg/L or a PEC_{soil} of >100 µg/kg or is a ecto or endoparasiticide will most likely progress to phase II EIA which includes more complex physical-chemical analysis (table 1.2), environmental fate studies (table 1.3) and ecotoxcity testing (table 1.4).

Phase II (VICH-GL38) can be split into three branches of use depending on the target species main environment. The three branches are aquaculture, intensively reared animals and pasture animals. Phase II can also be split into tier A and tier B. Phase II focuses on gathering information to calculate a risk quotient (RQ). The RQ is based on calculated PEC and PNEC values. The RQ is calculated as set out in equation 1.1 (VICH, 2006).

Equation 1. 1

$$RQ = \frac{PEC}{PNEC}$$

Where the RQ is equal to the PEC over the PNEC. If the RQ is <1 then further testing is no longer needed, if the RQ ≥1 then further testing is required and the VM must go to tier B of testing.

Physical-chemical properties tests must be carried out on all VMs that progress to phase II (table 1.2). Physical-chemical properties can give vital information on how the test compound may interact in the environment and within biota (VICH, 2006). Water solubility along with n-octanol/water coefficient gives information on how hydrophobic or hydrophilic a compound is. Hydrophobic compounds will have low water solubility and are more likely to accumulate in the sediment or bioaccumulate in fatty tissues within the body of organisms. A compound with a log K_{ow} of ≥ 5 raises concerns over the likelihood of bioaccumulation (Montforts, 2006). The PEC is determined by the results from physical-chemical (table 1.2) and environmental fate tests (table 1.3). The PNEC is determined by the results from the EC_{50} or LC_{50} test. An assessment factor (AF) is applied to each test species, an AF of 1000 means that the PNEC would be 1000 times lower than the EC/LC_{50} value as determined from the acute toxicity test. After testing has established a PEC and PNEC then a RQ can be calculated (equation 1.1).

Table 1.2 Physical-Chemical property tests to be conducted on VMs in tier A phase II of VICH guidelines.

Study	**Description**	**Guideline**
Water Solubility	Maximum saturation mass of the VM in water at 20°C ± 0.5°C.	OECD 105 (OECD, 1995b)
Dissociation constant in water	The concentration at which a reversible spiting of one chemical compound into two or more smaller chemical species.	OECD 112 (OECD, 1981b)
UV-Visible absorption spectrum	The UV-Visible absorption spectrum gives information on the wavelengths needed for the photochemical degradation of the VM.	OECD 101 (OECD, 1981a)
Melting point/range	The temperature range needed for the VM to go from its solid state to its liquid state.	OECD 102 (OECD, 1995a)
Vapour pressure	The saturation pressure of a VM either in its liquid or solid form. This is also described as the equilibrium between the liquid (or solid) and its vapour.	OECD 104 (OECD, 2006a)
n-Octanol/water partition coefficient	The n-octanol/water coefficient determines the dissociation of the VM between two immiscible substances (in this case the fatty alcohol octanol and water). This can then establish the log K_{ow} which helps estimate a VMs bioaccumulative potential.	OECD 107 (OECD, 1995c) or 117 (OECD, 2004c)

Environmental fate tests need to be carried out on all VMs which progress to phase II (table 1.3). Photolysis and hydrolysis tests are optional, however they can help build a bigger profile on degradation and persistence in the environment (VICH, 2006).

Table 1.3 Environmental fate tests required in tier A of phase II of VICH

Study	**Description**	**Guideline**
Soil adsorption/desorption	Using a variety of soils with differing components (clay, organic carbon, sand) the partition coefficient can be determined for a number of environments, therefore determining a range.	OECD 106 (OECD, 2004a)
Soil biodegradation	The rate of transformation of the VM in aerobic and anaerobic soils where a half-life can be determined.	OECD 307 (OECD, 2002a)
Degradation in aquatic systems	VM is added to aquatic system and degradation in sediment is measured, half-lives can be determined.	OECD 308 (OECD, 2002b)
Photolysis	The amount of time taken for a VM to break down in water when exposed to differing degrees of light.	OECD 316 (OECD, 2008b)
Hydrolysis	The amount of time taken for VM to break down in water in three different pH values, 4, 7 and 9. The tests are carried out for 5 days at 50°C ± 0.5°C.	OECD 111 (OECD, 2004b)

Aquatic toxicology tests are usually only required for VMs which will directly enter waterways, for example VMs used in aquaculture (table 1.4). Further aquatic ecotoxicology testing may be needed for VMs intended for terrestrial use which progress to tier B (VICH, 2006).

Table 1.4 Aquatic ecotoxicology tests required for tier A phase II VICH.

Study	**Description**	**Guideline**
Freshwater algae inhibition	Freshwater algae or cyanobacteria are exposed to 72-hour EC_{50} test. A range of concentrations is tested and the rate of growth is measured against a control culture. PNEC is determined by an AF of 100.	OECD 201 (OECD, 2011)
Freshwater *Daphnia* immobilisation	Freshwater invertebrate, the water flea *Daphnia* sp. (most commonly *D. magna*) subjected to 48-hour EC_{50} test. Daphnids under 24 hours old are exposed to a range of concentrations; immobilisation is recorded and compared with controls. PNEC is determined by an AF of 1000.	OECD 202 (OECD, 2004d)
Freshwater acute fish	Freshwater fish undertake a 96-hour LC_{50} test. No species of fish is specified. Over a period of 96 hours fish are exposed to a range of concentrations of test VM, percentage of death is compared to controls, where 10% is the maximum death allowed in the control. PNEC is determined by an AF of 1000.	OECD 203 (OECD, 1992)
Marine algae inhibition	Similar to freshwater algae inhibition, but with marine specific media and the marine algae *Skeletonema costatum* and *Phaeodactylum tricornutum.* PNEC is determined by an AF of 100.	ISO 10253
Marine crustacean acute	One of the three copepod species *Acartia tonsa, Nitocra spinipies* or *Tisbe holothuriae* to be subjected to a range of concentrations of test VM for 96 hours. PNEC is determined by an AF of 1000.	ISO 14669
Marine acute fish	No official guideline is available for the acute test on marine fish. However it is assumed that the test is similar to the freshwater acute test, with marine species in marine media. PNEC is determined by an AF of 1000.	No current guideline, seek professional advice.

Terrestrial ecotoxicology tests are needed for VMs that are applied to terrestrial animals, for example topical treatments for herds such as sheep dip (table 1.5).

Table 1.5 Terrestrial toxicity tests required for tier A testing in phase II VICH EIA.

Study	**Description**	**Guideline**
Nitrogen transformation in soil	Soil is treated with the highest concentration of VM expected in the field. After 0, 7, 14 and 28 days of incubation soil a sample of soil is taken and a solvent added to extract nitrates. Results are compared to controls, if the difference between the control soil and the soil treated with the test VM is <25 % the test is carried on for 100 days. After 100 is the difference is still <25 % the VM progresses to tier B of testing.	OECD 216 (OECD, 2000)
Terrestrial plants	Seeds are planted in soil that has been spiked with various concentrations of the test VM. Seedling emergence and plant growth are measured and compared with the control. A number of crops are used in this study including tomato, barley and onion plants. An AF of 100 is applied to this test to determine a NOEC.	OECD 208 (OECD, 2006b)
Earthworm reproduction	The earthworm reproduction test uses either *Eisenia fetida* or *E. andrei*. Worms are exposed to soil, which has been spiked with varying concentrations of test VM. The test is carried out over 28 days and effects such as growth, number of cocoons produced and abnormalities are recorded and compared against the control.	OECD 222 (OECD, 2004e)
Dung fly larvae	Dung is spiked with the test VM at various concentrations, 10 eggs of *Scathophaga stercoraria* or 10 larvae of *Musca autumnalis* are also added to the dung. Five days after the emergence of the last adult from the control, the test is stopped. Effects on sex and number of emerged adults, and physical abnormalities are recorded and a NOEC/EC_x can be determined.	OECD 228 (OECD, 2008a)
Dung beetle larvae	Dung is spiked with the test VM at various concentrations; seven individuals of *Aphodius constans* in the 1st instar larval stage (≤ 7 days old) are added to the dung. Endpoints are EC_{50} and NOEC. Observations on survival and morphological changes are made once a week for three weeks until the end of the test (21 days).	Unclassified ENV/JM/MONO (2010)13 (OECD, 2010)

For VMs with a RQ of ≥1 (see equation 1.1) then tier B of phase II must be considered. Other instances where tier B of testing is required are when a VM has a log K_{ow} ≥4 or where the effect on soil micro-organisms is > 25% for terrestrial use VMs (table 1.5). Tier B of phase II considers more rigorous tests such as fish bioconcentration and chronic effect studies. Tier B of phase II is more focused in that tests only need to be carried out for those species in which the RQ is ≥1. Detailed below are the chronic studies used in tier B, phase II of VICH (table 1.6).

Table 1.6 Aquatic ecotoxicology testing in regard to tier B, phase II of VICH.

Study	**Description**	**Guideline**
Freshwater algae growth inhibition and marine algae growth inhibition	This is the same test as previously described in table 1.3, however in this tier the NOEC must be measured and an AF of 10 applies. The study must use the same algal species as in tier A.	OECD (2011) 201 (freshwater); ISO 10253 (marine)
D. magna reproduction	Young female *D. magna* are exposed to a range of concentrations of the VM over a period of 21 days. Surviving offspring are counted daily. Other features can also be recorded such as the sex ratio of offspring. The LOEC and NOEC are reported and an AF of 10 is applied.	OECD 211
Freshwater fish early life stage	At least 60 fertilised eggs are exposed to a range of concentrations of the test VM. The test lasts until all control eggs are free-feeding fish. The LOEC and NOEC are observed and an AF of 10 applies.	OECD 210
Freshwater sediment invertebrate species toxicity	Chironomid larvae are added to beakers containing sediment and water. Test subjects are exposed to either spiked sediment containing a range of concentrations of the test VM (OECD 218) or water containing a range of concentrations containing the test VM (OECD 219). The study lasts either 28 days (for *Chironomus riparius* and *C. yoshimatsui*) or 65 days (for *C. tentans*). Emergence time, survival and egg deposition are recorded as well as any behavioural abnormalities. The LOEC and NOEC are recorded and an AF of 10 applies	OECD 218 (primary entry to environment through sediment or soil) OECD 219 (primary entry to environment through water)
Marine species NOECs	Tier B, phase II also suggests the NOEC for crustacean chronic toxicity or reproduction, fish chronic toxicity and sediment invertebrate toxicity. As there are currently no official guidelines for these tests a description is not provided. An AF of 10 applies to all.	No current guidelines

VMs may need to go through re-evaluation if they pass through phase I but their intended use changes. For example, a substance that has been approved for use in domestic animals may pass through the EIA with relative ease, and without the need for further testing. If the use of that VM changes to include use in aquaculture or for pasture and intensively reared animals then progression to phase II testing is more likely to occur.

1.5 Reduction in animal testing

The paper "The Principles of Humane Experimental Technique" by Russell *et al.* (1959), first mentioned the 3Rs of animal testing, replacement, reduction and refinement. They suggested that where possible animals should be replaced with non-sentient alternatives. If testing was needed then a reduction in the number of animals to be used and refining the species used depending on the population to be exposed was essential. This principle was not implemented in the UK until the 1980s with the creation of the 'Animals (Scientific Procedures) Act 1986' (Baumans, 2004; Home Office, 2014). While the use of vertebrates has been reduced and replaced with invertebrate and *in vitro* models, the use of invertebrates appears to still be heavily relied on in ecotoxicology testing. For testing on 'sentient' beings, special licences are required, however for most invertebrates (excluding cephalopods) licencing is not needed and tests can be carried out with relative ease (Home Office, 2014).

Whilst nociception, the ability to recognise harmful stimuli, is a widely accepted response of both vertebrates and invertebrates the debate on whether invertebrates can experience pain is still a largely contentious issue (Sneddon *et al.*, 2014). A number of research articles have attempted to clarify pain in invertebrates, and it is now accepted that cephalopods, such as squid and octopi, do experience pain and therefore testing on these animals is limited and controlled, with protocols put in place in order to reduce discomfort when testing (Andrews *et al.*, 2013; Smith *et al.*, 2013).

Barr *et al.* (2008) exposed the rock shrimp *Palaemon elegans* to either pinching of the antenna, 10% acetic acid or a local anaesthetic treatment of 2% benzocaine. Tail flicking, a reflex behaviour, was observed as was grooming of the treated antenna. They found that a significant number of shrimp flicked their tail and groomed the antenna that had not been treated with anaesthetic after being subjected to either 10% acetic acid or pinching.

Although there is no way of conclusively determining whether invertebrates experience pain in a similar way to humans, the above studies suggest that there is at least a response which indicates pain when exposed to noxious stimuli. Therefore it has been proposed that the precautionary principle should also be applied to invertebrate testing with a reduction in testing, and more stringent controls over euthanasia techniques with regards to invertebrates (Sneddon *et al.*, 2014). While it is unlikely that animal testing will be totally eliminated in ecotoxicology testing,

the move towards more intelligent testing, which do not use the whole animal (*in vitro*) and the application of predictions using models (e.g. *in silico*) can help reduce the burden of testing, as well as potentially broadening our knowledge on the effects of chemicals on non-target organisms.

1.6 Quantitative structure activity relationships (QSARs) in ecotoxicology

Quantitative structure activity relationships (QSARs) are computer-aided prediction models used to determine the likely toxicity of a compound based on the already known toxicity of compounds with a similar structure (Schultz *et al.,* 2003). Under the Organisation for Economic Cooperation and Development (OECD) there are guidelines on which QSAR tests are to be taken into consideration. Implementation of QSARs amongst member states of the OECD began in the early 1990s, originally for use in aquatic ecotoxicology. QSARs are mathematical estimations on the effects of chemicals based on structure. The structure of the test compound is compared to a database of chemicals with pre-existing data on physical-chemical properties such as solubility, dissociation constant and n-octanol/water coefficient (table 1.2); environmental fate information such as biodegradation, soil sorption/desorption (see table 1.3) as well as ecotoxicity information (see tables 1.4, 1.5 and 1.6). Predictions on the test compounds likely effects on the environment and biota can be extrapolated from this information and a RQ can be calculated. If the new compound has a RQ ≥1 then testing via VICH guidelines should commence (see this chapter, section 1.4) (VICH, 2000; VICH, 2006). QSARs are now heavily relied on, since the regulation of chemicals has vastly improved and since the implementation of REACH, which requires all chemicals currently manufactured or used in the EU to be registered before 2018, QSARs are a time and cost effective method of determining the likely effects of the test chemical. Although QSAR information cannot be used alone, it is a good early stage method to be used in conjunction with other methods such as high throughput *in vitro* testing.

An advantage to using 3D QSARs such as molecular docking is that pre-existing data need not necessarily be used. Although pre-existing data is always an advantage, so long as the 3D structure of the compound is known and the 3D structure of the target protein is robust then predictions on binding can be made (Huey *et al.*, 2007; Morris *et al.*, 2009; Trott and Olson, 2010).

1.7 VMs of high concern in intensive farming in the UK

While prioritising the potential hazard the use of VMs in UK farming cause to the environment, Boxall *et al.* (2003) categorised 56 VMs as being high risk and high priority in the UK environment. Selection criteria for the prioritisation of these VMs are laid out below (figure 1.2). Of the high-risk VMs described, three are used in aquaculture to control sea lice in salmon farms (Boxall *et al.*, 2003). These three, cypermethrin, deltamethrin and emamectin benzoate are described in detail, along with their potential environmental impacts in chapter 2. Along with these three VMs a

fourth, azamethiphos, and a fifth, teflubenzuron are used for sea lice control in salmon aquaculture. These five aquaculture VMs and a major metabolite of the synthetic pyrethroids, 3-phenoxybenzoic acid, were tested for their mixture effects on the marine bacteria *Aliivibrio fischeri* (chapter 3) and in chapter 4 for their effects on the oestrogen receptor alpha (ERα) of ten species. Other VMs included in the prioritisation list (Boxall *et al.*, 2003) also were studied including sulfadiazine (an antibiotic used in aquaculture), diazinon (a sheep dip), fenbendazole, ivermectin, and amitraz. An in depth rationale behind choosing all of the VMs to be investigated in this project is set out in chapter 4 (section 4.2.1, table 4.2).

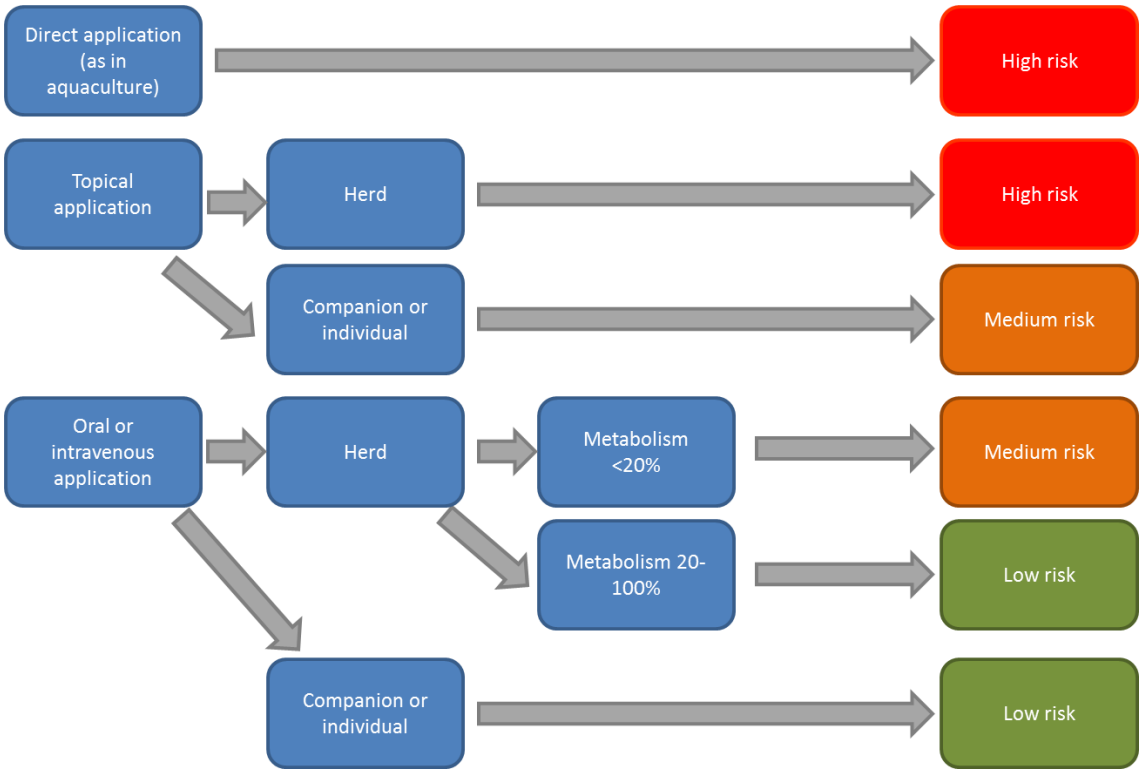


Figure 1.1 Decision tree for potential risk of VMs in the environment. Adapted from Boxall *et al.* (2003).

1.8 Conclusion

Ecological effects of the application of pesticides have been heavily researched (Cold and Forbes, 2004; Langan and Shaw, 2006; Margni *et al.*, 2002; Schäfer *et al.*, 2007). Apart from antibiotics (Blackwell *et al.*, 2009; Kay *et al.*, 2005; Morris and Masterton, 2002; Sarmah *et al.*, 2006), fish farm medicines (Kim *et al.*, 2008; Zuccato *et al.*, 2010) and sheep dips (Boucard *et al.*, 2008; Virtue and Clayton, 1997) the environmental impacts of VMs have not received as much attention. Consideration of factors including compound mixtures (Cleuvers, 2004) and multi species trials (Boleas *et al.*, 2005) are relatively under researched, this is an area which would benefit from further investigation.

Current legislative practise of ecotoxicology testing has been criticised for being dated and unrepresentative (Barry and Davies, 2004). A commentary on a strategic seminar held by the Partnership for European Environmental Research (PEER) in November 2010 by Artigas *et al.* (2012) concluded that for ecotoxicology testing to support environmental, social and economic welfare then tests should not only rely on laboratory and field based studies but should also include, where possible, computer modelling programmes and GIS technologies offering a more informed awareness of the environmental fate of VMs.

1.9 Hypotheses, Aim and Objectives

1.9.1 Hypotheses

1. Veterinary medicines used in intensive farming enter the environment as mixtures with the potential to have an additive effect, impacting negatively on non-target organisms.

2. Molecular docking is a viable tool for determining the environmental impacts of veterinary medicines on non-target organisms.

1.9.2 Aim

To provide informed recommendations to improve the current methods of conducting environmental risk assessments, including the assessment of chemical mixtures, and environmental risk management on veterinary medicines used in intensive farming within the EU.

1.9.3 Objectives

Five objectives were set out for the current project. They are:

1. Review the current knowledge base on VMs used in intensive farming including fish farm medicines in the environment (chapter 2; chapter 6).

2. Investigate the potential environmental impacts of sea lice treatments individually and as mixtures with the aid of GIS (chapter 2).

3. Examine the predictive efficacy of two equations on mixture toxicity to the marine bioluminescent bacteria *Aliivibrio fischeri* (chapter 3).

4. Determine potential effects of the selected VMs on non-target organisms using *in silico* techniques (chapter 4).

5. Compare *in silico* work with *in vitro* assay using the yeast oestrogen screen (YES) (chapter 5).

6. Examine the proposed EU plans to change ERAs to include mixture toxicity and multi species testing of compounds in environmental regulation (chapter 6).

1.10 Contribution to Knowledge

This project will evaluate the use of GIS as a tool to aid the assessment of sea lice treatment use in Scotland (between the years 2007 and 2011), their MECs (between 2008 and 2012) and potential impacts on the surrounding marine environment. The potential use of molecular docking as a tool in ecotoxicology testing of veterinary medicines will be determined and recommendations on its application made.

Chapter 2

The distribution and use of sea lice treatments in Scottish aquaculture

2.1 Introduction

The impacts of sea lice infestations on farmed salmon can range from minor skin irritation to stress induced mortality (Costello, 2006). Sea lice infestation not only causes a problem to farmed fish, but it is a growing concern that farmed fish pass on the parasite to wild populations of fish, causing a decline in wild salmon populations (Liu *et al.*, 2011). There has been an increase in sea lice infestations as fish farming has grown. Currently there are six treatments registered for use in Scottish salmon farms to try and combat the problem, these are azamethiphos, cypermethrin, deltamethrin, emamectin benzoate, hydrogen peroxide and teflubenzuron (table 2.1). Teflubenzuron is a selective treatment, which acts as a chitinase inhibitor (Burridge *et al.*, 2010; Tassou and Schulz, 2011), so must be applied before the adult stages of the parasite to achieve full effectiveness as a treatment (see figure 2.1 for the life cycle of *L. salmonis*). It is anticipated that if teflubenzuron is applied correctly then further treatment of sea lice should be obsolete for a full life cycle or one year (SEPA, 1999). The other treatments are less selective working on all life stages (table 2.1). However, there is a need for more selective treatments as resistance to the current treatments is increasing (Boxaspen, 2006). The concentration of sea lice treatment residues in Scottish marine environments are measured by Scottish Environmental Protection Agency (SEPA) every few years. In 2005 SEPA conducted a small sediment survey and took 51 samples from 33 fish farms. The samples were taken outside of the 25 m buffer zone or allowable zone of effects (AZE) surrounding fish farms. Out of the 51 samples taken 18 were positive for cypermethrin, two of these sites had a higher measured environmental concentration (MEC) than the lowest predicted no effect concentration (PNEC) of 2.2 µg/kg and two of the sites that tested positive for cypermethrin did not have consent for its use. Teflubenzuron tested positive in 25 of the 51 samples, none of the samples were above the lowest PNEC of 10 µg/kg. Three of the sites that tested positive did not possess a licence for discharging the substance (see chapter 1, section 1.4 on the regulation of VM use). Currently there is very little information on the bioavailability and adsorption of substances in sediments, this is especially concerning as, with the exception of azamethiphos and deltamethrin, the treatments used to control sea lice in fish farms disperse quickly from the water column and are likely to have a strong adsorption to sediment (table 2.1).

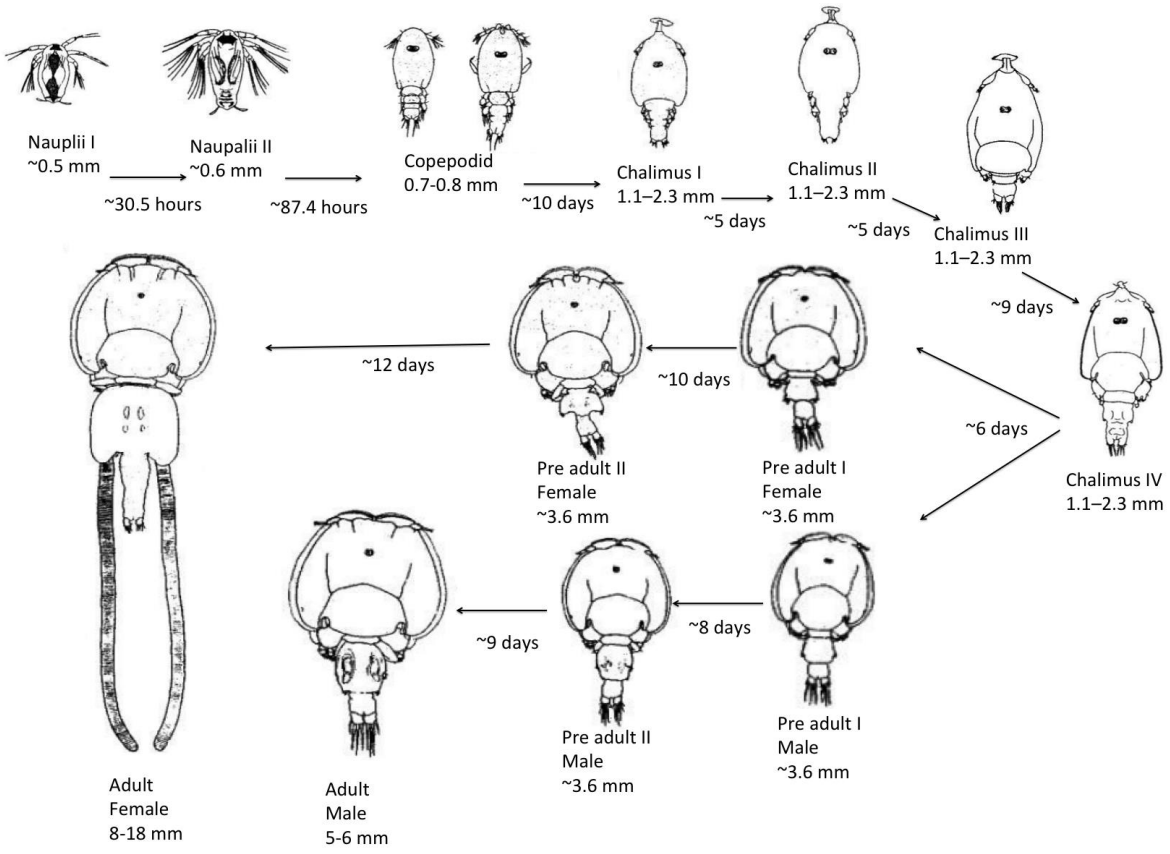


Figure 2.1 The life cycle of the sea lice *L. salmonis* with estimated timeline and size between each stage at 20°C. *Adapted from Schram (1993).*

Six treatments are currently used in Scottish aquaculture to control sea lice; these are azamethiphos, cypermethrin, deltamethrin, emamectin benzoate, teflubenzuron and hydrogen peroxide. Hydrogen peroxide has been excluded from this study due to the lack of information given on use. SEPA does not request information on hydrogen peroxide use from farms because it is not considered to be detrimental to non-target organisms, and with a log K_{ow} of -1.5 it is hydrolysed within and non-persistent in the water column and sediments (US EPA, 2007). Sea lice treatments are administered as either in-bath or in-feed. Table 2.1 outlines the environmental profile for each of these treatments while figure 2.2 provides the 2D structure for each treatment.

Table 2.1 Environmental profiles of the five sea lice treatments used in Scotland 2008-2011.

Environmental profile	**Azamethiphos**	**Cypermethrin**	**Deltamethrin**	**Emamectin benzoate**	**Teflubenzuron**
Log K_{ow}	1.05	6.3	4.6	5	5.39
Solubility in water 20°C	1.1 g/L	5-10 µg/L	2 µg/L	550 µg/L	3 µg/L
Mode of action on target species (sea lice)	Inhibitor of acetylcholinesterase (AChE)	Inhibition of sodium channel activation gate	Inhibition of sodium channel activation gate	Antagonist of γ,γ-aminobutyric acid (GABA)	Chitinase inhibitor
Half-life in sediment (days)	9 days (half-life water 10.8 days)	35-80 days	65	164-175 days	104-123 days
Recommended dose	IB 0.1 mg/L for 30 - 60 minutes	IB 5 µg/L for 60 minutes	IB 2 µg/L for 60 minutes	IF 50 µg/kg body weight (bw) per day, for 7 days	IF 10 mg/kg bw per day, for 7 days
PNEC (water column)	5 ng/L (72 hours)	N/A	N/A	0.22 ng/L	N/A
Annual average (water column)	N/A	0.05 ng/L	0.3 ng/L	N/A	6 ng/L
MAC (water column)	150 ng/L (24 hours); 250 ng/L (3 hours)	0.5 ng/L (24 hours); 16 ng/L (3 hours)	2 ng/L (24 hours); 9 ng/L (3 hours)	N/A	30 ng/L
Far field PNEC (sediment)	N/A	N/A	330 ng/kg	0.763 µg/kg wet weight	2 µg/kg dry weight
Near field (AZE) PNEC (sediment)	N/A	N/A	330 ng/kg	7.63 µg/kg wet weight	10 mg/kg dry weight

MAC = maximum acceptable concentration; PNEC = Predicted no effect concentration; IB = in-bath; IF = in-feed; N/A = not available; AZE = allowable zone of effect; Near field = within 25 m radius of aquaculture cage; Far field = 25 – 100 m radius of aquaculture cage. References: (Marsella *et al.*, 2000; SAMS, 2005; SEPA, 2008).

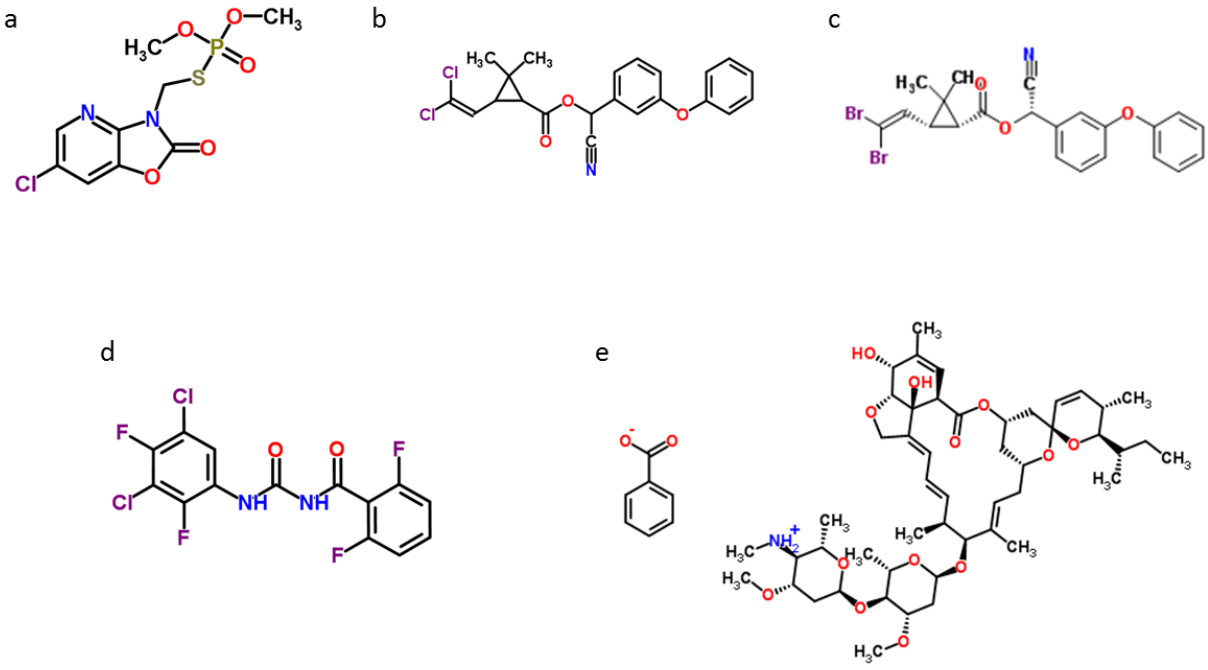


Figure 2.2 2D chemical structure of the five sea lice treatments; (a) azamethiphos; (b) cypermethrin; (c) deltametherin; (d) teflubenzuron; (e) emamectin benzoate.

2.1.1 Sediment transfer

A number of factors need to be considered when beginning to estimate the distribution of sea lice treatments from fish farm cages. These include the method of application (in-feed or in-bath); current speed and direction; settling velocity (especially in-feed); position and number of cages; bathymetry (underwater topography); amount of treatment; solubility and log K_{ow} (ability to bind to sediments) and persistence in sediment. For in-feed treatments it is estimated that 90% of feed will be eaten, while 10% will settle directly as uneaten feed (Hargrave 1994), this uneaten feed as well as faeces settles directly below the cage and travels along the sea floor by saltation when currents reach a critical value, dependent on the size and density of the particle (Rice *et al.*, 1996).

Chemicals with low water solubility and high lipophilicity readily bind to sediments and have a greater likelihood of becoming persistent in the environment (Jones and de Voogt, 1999). Persistent organic pollutants (POPs) are often chemicals with a log K_{ow} of ≥ 5 and have half-lives of several months or years (Kelly *et al.*, 2007). POPs have the capacity to be bioaccumulative and persist in sediments and organisms. Treatments used as sea lice control that share characteristics of POPs are a threat to the marine environment and can be especially detrimental to organisms in the benthos, cypermethrin, emamectin benzoate and teflubenzuron all have a log K_{ow} ≥ 5 and therefore pose an increased risk to benthic organisms (see table 2.1). Due to their lipophilic nature there is the added risk of bioaccumulation in fat stores within aquatic organisms becoming more concentrated as they move through trophic levels (Wania and Mackay, 1996).

The lethal and chronic effects of all five sea lice treatments have been reported as being in the µg/L and even ng/L range for invertebrates (see table 2.2). This highlights the importance of understanding the rates and concentrations that they are entering the marine environment, and to identify areas of risk and to establish common treatment mixtures and the knock on effect mixtures of these treatments might have on the surrounding environment.

Table 2.2 Lethal and chronic effects of the five sea lice treatments on non-target aquatic organisms.

Test organism	Test description	Effect concentration azamethiphos	Effect concentration cypermethrin	Effect concentration deltamethrin	Effect concentration emamectin benzoate	Effect concentration teflubenzuron	Reference
Echinogammarus marinus (marine amphipod*)	1 hr exposure 95 hr recovery EC_{50}	-	180 ng/L	47 ng/L	-	-	(Van Geest *et al.*, 2014)
E. marinus	24 hr exposure 72 hr recovery EC_{50}	-	20 ng/L	6.7 ng/L	-	-	(Van Geest *et al.*, 2014)
Corophium volutator (marine amphipod*)	10 d sediment LC_{50}	182 µg/kg (wet weight)	5 µg/kg (wet weight)	-	153 µg/kg (wet weight)	-	(Mayor *et al.*, 2008)
Hediste diversicolor (ragworm)	10 d sediment LC_{50}	-	-	-	1368 µg/kg (wet weight)	-	(Mayor *et al.*, 2008)
Homarus americanus Stage I (American lobster)	24 hr LC_{50}	8.9 ng/L	-	0.8 ng/L	-	-	(Burridge *et al.*, 2014)
H. americanus adult	24 hr LC_{50}	2.8 ng/L	-	15 ng/L	-	-	(Burridge *et al.*, 2014)
Crangon septemspinosa (sand shrimp)	24 hr LC_{50}	191 ng/L	-	27 ng/L	-	-	(Burridge *et al.*, 2014)
Mysid sp. (opossum shrimp)	24 hr LC_{50}	12.5 ng/L	-	1.4 ng/L	-	-	(Burridge *et al.*, 2014)
Daphnia magna (water flea)	48 hr EC_{50}	0.67 µg/L	0.3 µg/L	0.56 µg/L	1 µg/L	1.2 µg/L	(Koyanagi *et al.*, 1998; University of Hertfordshire, 2013)
Chironomus riparius (non-biting midge/bloodworm)	Sediment EC_{50} fecundity	-	-	-	-	112.7 µg/kg (dry weight)	(Tassou and Schulz, 2011)
C. riparius	Sediment EC_{50} fertility	-	-	-	-	74.5 µg/kg (dry weight)	(Tassou and Schulz, 2011)

EC_{50} = Effect concentration 50%; LC_{50} = Lethal concentration 50%; dashes represent no available data; * = no common name available.

2.1.2 Marine Environment in Scotland and the designation of SPAs and SACs

The UK coastal environment is under a number of EU and UK regulation and protection. The three main pieces of legislation that are of relevance within this chapter are the EU habitats directive (92/43/EEC) in which the designation of special areas of conservation (SACs) are applied; the EU birds directive (2009/147/EC) in which the designation of special protection areas (SPAs) are applied; and the EU water framework directive (WFD) (2000/60/EC). SPAs are areas in which rare or vulnerable species of birds (under Annex I of the EU birds directive 2009/147/EC) habituate and therefore are in need of protection (European Parliment, 2009). SACs are habitats that support protected and priority habitats (listed under Annex I of the EU habitats directive 92/43/EEC), such as reefs and submerged sea caves, and species (listed under Annex II), such as the common seal *Phoca vitulina* and the otter *Lutra lutra* (McLeod *et al.*, 2005). The Scottish environment has 243 designated SACs, with 38 of these falling into the marine environment. There are 161 SPAs in Scotland, with 49 sites having marine components.

2.1.3 Chapter objectives

In order to address objectives 1 and 2 (chapter 1; section 1.9.3) the objectives for this chapter are to:

- Examine the changing practice in sea lice treatments in Scottish salmon farms between 2007 – 2011 using SEPA data (Natural Scotland, 2015b).

- Establish any relationship between sea lice treatment use on salmon farms between 2007 - 2011 to sediment MECs taken 2008 – 2012.

- Assess the potential impact of sea lice treatments on the surrounding aquatic environment including SPAs and SACs.

2.2 Methods

2.2.1 Data sources and preparation for use in the Geographical Information System ArcGIS

Information on sea lice treatment use in Scottish salmon farms was provided by SEPA access to information. The information obtained from SEPA is used by them for monitoring purposes. The information supplied included the geographical coordinates of salmon farms in Scottish waters; maximum allowed biomass at individual salmon farm; the company, name and code of the farm; and monthly information on the amount in grams of sea lice chemicals for the years 2008-2011 (Natural Scotland, 2015b). The sea lice treatments that were included in the database were cypermethrin, deltamethrin, azamethiphos, emamectin benzoate and teflubenzuron. Geographical coordinates were provided as national grid reference (NGR), these were converted to latitude and longitude coordinates using the batch conversion tool on the ordnance survey

website (Ordnance Survey, 2013) for use in ArcGIS 10.1 (ESRI, 2012). Maps throughout this chapter were created using ArcGIS® software provided by Esri. ArcGIS® and ArcMap™ are the intellectual property of Esri and are used herein under license. For more information about Esri® software, visit www.esri.com.

Total treatment use in grams per farm was calculated for each year from the SEPA data. ArcGIS 10.1 (ESRI, 2012) was used to visualise the locations of the farms and distribution of chemicals and changes with year. ArcGIS 10.1 uses layers of information that can then be added and modified on maps. Layers for each treatment were the amount of use in grams per farm per year and the average MECs at a single site (n = 3) and were created using the information provided by SEPA. Layers containing information on marine SACs and SPAs were downloaded from the Joint Nature Conservation Committee (JNCC) and were added to the maps (JNCC, 2012). Maps were made to show the treatment use, mixtures of treatments and their proximity to SACs and SPAs for the whole of the Scottish coastline.

Information on sediment MECs was obtained from the SEPA website (SEPA, 2013). These included measurements for teflubenzuron and emamectin benzoate for the years 2008-2012. Measurements were taken either within the 25 m AZE or between 100 m and 1000 m away from the site of a fish farm. ArcGIS 10.1 was used to plot the locations of the sampling sites for sediment MEC determinations for each year. The number of sites sampled per year varied between 9 sites in 2009 and 2010 and 19 sites in 2012 (with three sample replicates per site).

Data on monthly ocean current speed and direction was downloaded in the form of a NetCDF file from the open source website MyOcean http://marine.copernicus.eu/ (European Commission, 2015), an EU initiative for the monitoring and forecasting of ocean activity. Ocean current depth was available from surface currents then every 25 m until a 200 m depth. It was decided that a depth of 50 m would be represented on the maps, as much of the Scottish coastal area is between 50 and 100 m deep (Foster-Smith, 2010). Annual means were calculated from monthly means using ArcGIS 10.1. Information on zonal velocity and meridional velocity was taken from this data, and using equation 2.1 the direction of flow could be calculated and added to maps in the form of arrows.

Equation 2.1

$$\left(\frac{180}{\pi}\right) \times \operatorname{atan2}(z, m)$$

Where z is the zonal (east/west) velocity and m is the meridional (north/south) velocity.

2.3 Results

2.3.1 Total use of sea lice treatments on Scottish salmon farms

Cypermethrin was applied year round from 2007-2010 where use decreased in the second half of 2011 (figure 2.3a). Deltamethrin was applied year round from the middle of 2008-2011 (figure 2.3a). Azamethiphos was applied year round from 2008-2011 and emamectin benzoate was applied year round for all years 2007-2011 (figure 2.3b). Teflubenzuron was applied mainly in the winter months for all years except 2008 (figure 2.3b).

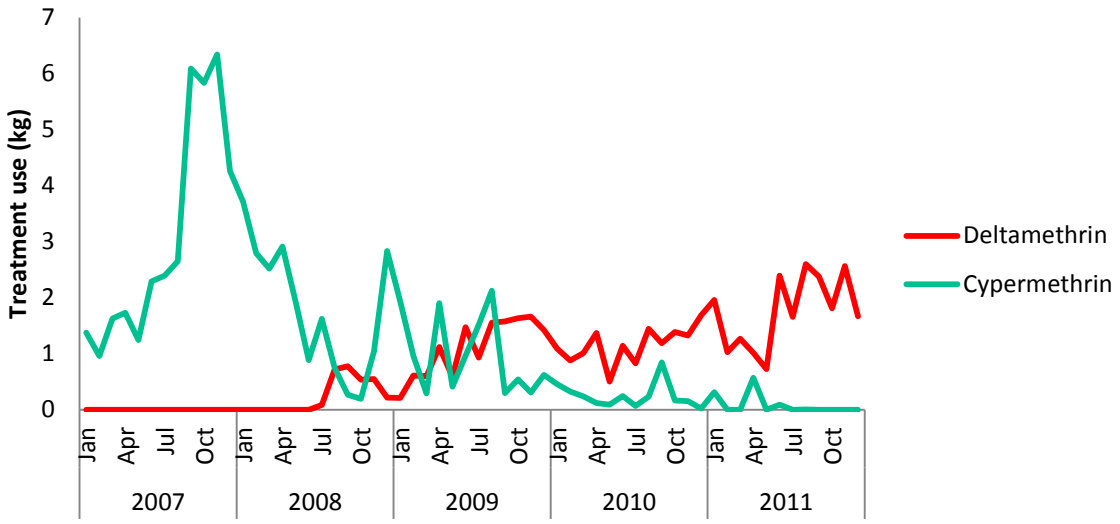


Figure 2.3a The total monthly use in kg of cypermethrin and deltamethrin between 2007 and 2011 for all Scottish salmon farms. Source SEPA (2012).

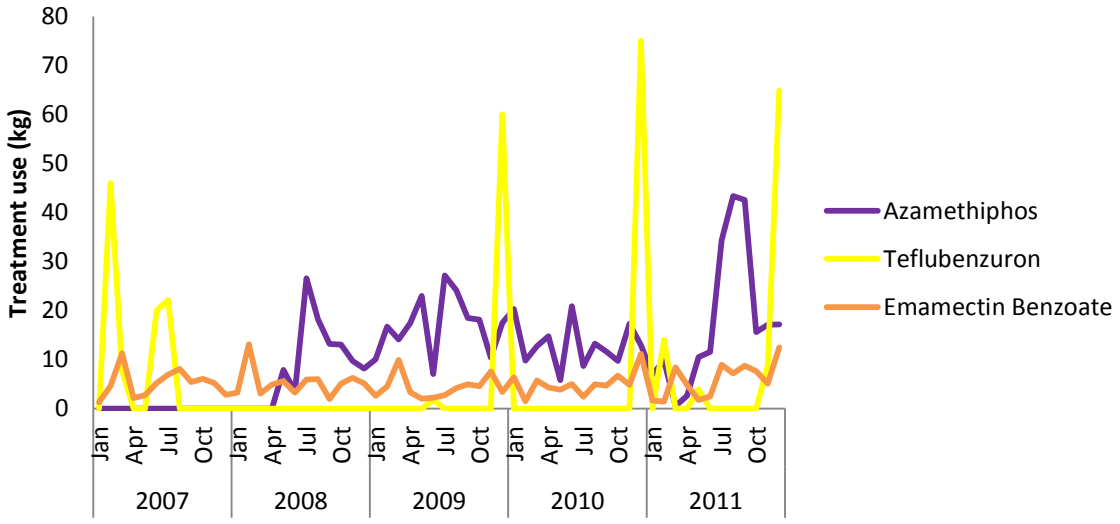


Figure 2.3b The total monthly use in kg of azamethiphos, teflubenzuron and emamectin benzoate between 2007 and 2011 for all Scottish salmon farms. Source SEPA (2012).

Use of all of the sea lice chemicals increased from 2002 to 2014 except for cypermethrin, which declined in use over the twelve-year period. Cypermethrin use declined between 2007 and 2011 and has not been used on Scottish salmon farms since 2011. Total use peaked in 2012 when 514.7 kg of all sea lice treatments were used. Overall the VM used in the highest quantity throughout the four years was azamethiphos, with use more than doubling over the four years from 100.2 kg in 2008 to 211.9 kg in 2011. Teflubenzuron showed the biggest increase in use from not being used at all in 2008 to 91.6 kg applied in 2011. Emamectin benzoate was the only VM which was used in every year and was the third most used VM (in kg) over the 12 year period (Table 2.3).

Table 2.3 The total use of each of the five treatments in grams of active ingredient per year for all the salmon farms monitored by SEPA. Data that is in italics has been represented using GIS (figures 2.4-2.18). Data obtained from Natural Scotland (2015b) database.

Year	**Cypermethrin (kg)**	**Deltamethrin (kg)**	**Emamectin Benzoate (kg)**	**Teflubenzuron (kg)**	**Azamethiphos (kg)**	**Total use* (kg)**
2002	12	0	12.6	72.7	45.6	142.9
2003	9	0	29	36	30.2	104.2
2004	7.9	0	45.3	0	7.6	60.8
2005	5.7	0	33.6	0	0	39.3
2006	9.1	0	36	0	0	45.1
2007	*36.8*	*0*	*61.7*	*95.8*	*0*	194.3
2008	*21.4*	*2.9*	*63.5*	*0*	*100.2*	188.7
2009	*11.9*	*13.4*	*51.8*	*61.8*	*203.9*	342.8
2010	*3*	*13.8*	*61.4*	*75*	*157.6*	310.8
2011	*1*	*21.1*	*70.5*	*91.6*	*211.9*	396.1
2012	0	21.1	73.4	224.8	195.4	514.7
2013	0	12.4	59.5	262	153.7	487.6
2014	0	17.5	63.6	0	253.3	334.4
Total use** (kg)	117.8	102.2	661.9	919.7	1360.1	3161.7

* = Total use of all treatments per year.

** = Total use of each treatment over 12 years.

The majority of the farms are located on the west coast of Scotland and across the Shetland and Orkney islands. Patterns of treatment use changed each year, confirming that farms are likely to use a mixture of chemicals rather than use only one (figures 2.4 - 2.6), for example deltamethrin was barely used in 2007 and 2008 (figure 2.4 and 2.5a; table 2.3) but use increased in 2009 through to 2012 (figures 2.5b, 2.6a and b; table 2.3). Treatments were used in conjunction with one another, with there being 401 occasions over the four years where more than one treatment was used in the same month on the same farm, on two occasions in 2008 four treatments, azamethiphos; cypermethrin; deltamethrin; and emamectin benzoate, were used on the same farm in the same month, on the farms CAG1 (UK NGR NM64425884) and GCD1 (UK NGR NM67606070). Both of these farms were located on the SAC, Sunart (UK NGR NM665605), a conservation area due to its reefs and population of the otter *L. lutra* (JNCC, 2015b). Teflubenzuron was not used in 2008 (figure 2.5a) and was the least frequently used treatment for the control of sea lice in the years 2007 (figure 2.4), 2009 (figure 2.5b), 2010 (figure 2.6a) and 2011 (figure 2.6b).

In 2007, fourteen salmon farms that were located on seven different SACs used the treatments emamectin benzoate and/or cypermethrin (figure 2.3, appendix A; table A.1); three SACs (Sunart; Loch Duich and Loch Laxford) located in med-West Scotland; had three farms on them, using a mixture of emamectin benzoate and cypermethrin.

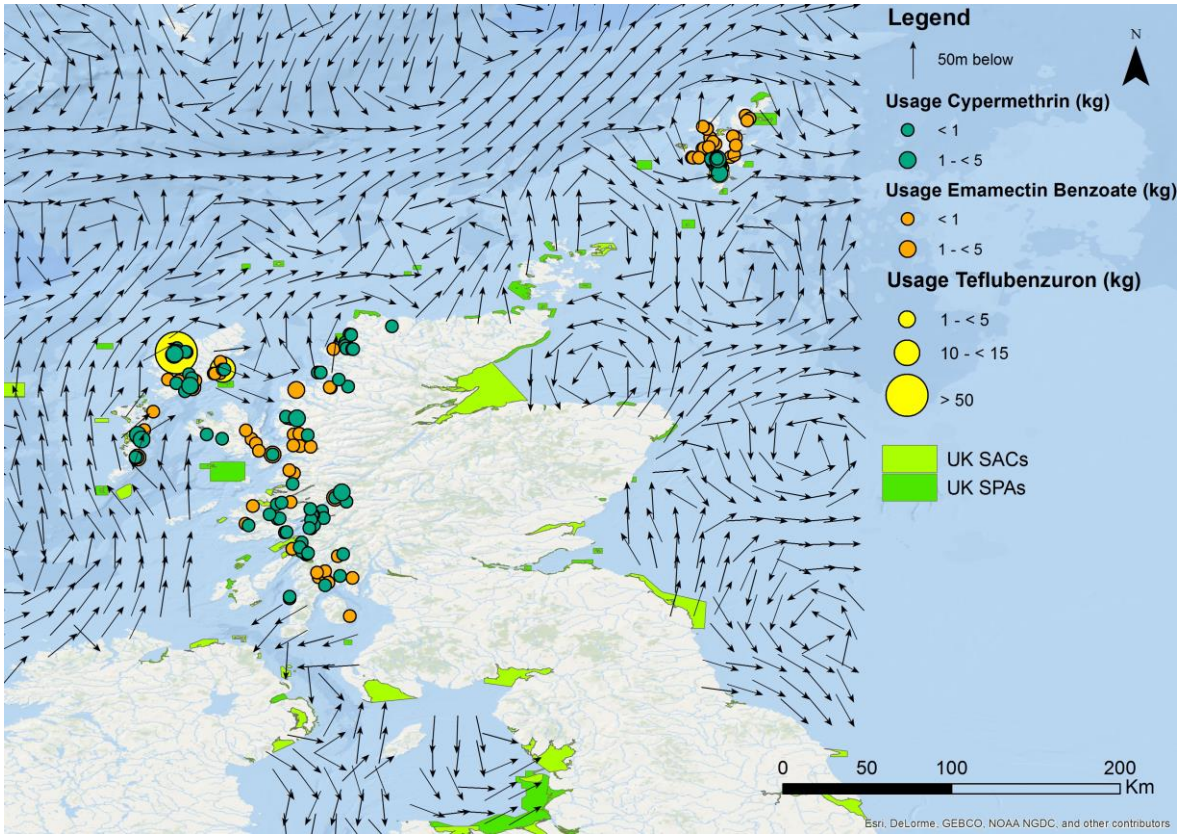


Figure 2.4 Total use in the year 2007 of three sea lice treatments, cypermethrin (teal); emamectin benzoate (orange) and teflubenzuron (yellow). Special protection areas (SPAs) are represented by the dark green areas and special areas of conservation (SACs) are represented by the light green areas. Circles increase as treatment use (in grams) increases. Black arrows represent the direction of ocean currents 50 m below the surface (annual average). Background map source: Esri®

In 2008, eleven salmon farms located on five different SACs used one or a combination of the treatments azamethiphos, cypermethrin, deltamethrin and emamectin benzoate (figure 2.5a and appendix A; table A.2). In 2009, twelve salmon farms that were located on seven different SACs used one or a combination of the four treatments azamethiphos, cypermethrin, deltamethrin and emamectin benzoate (figure 2.5b and appendix A).

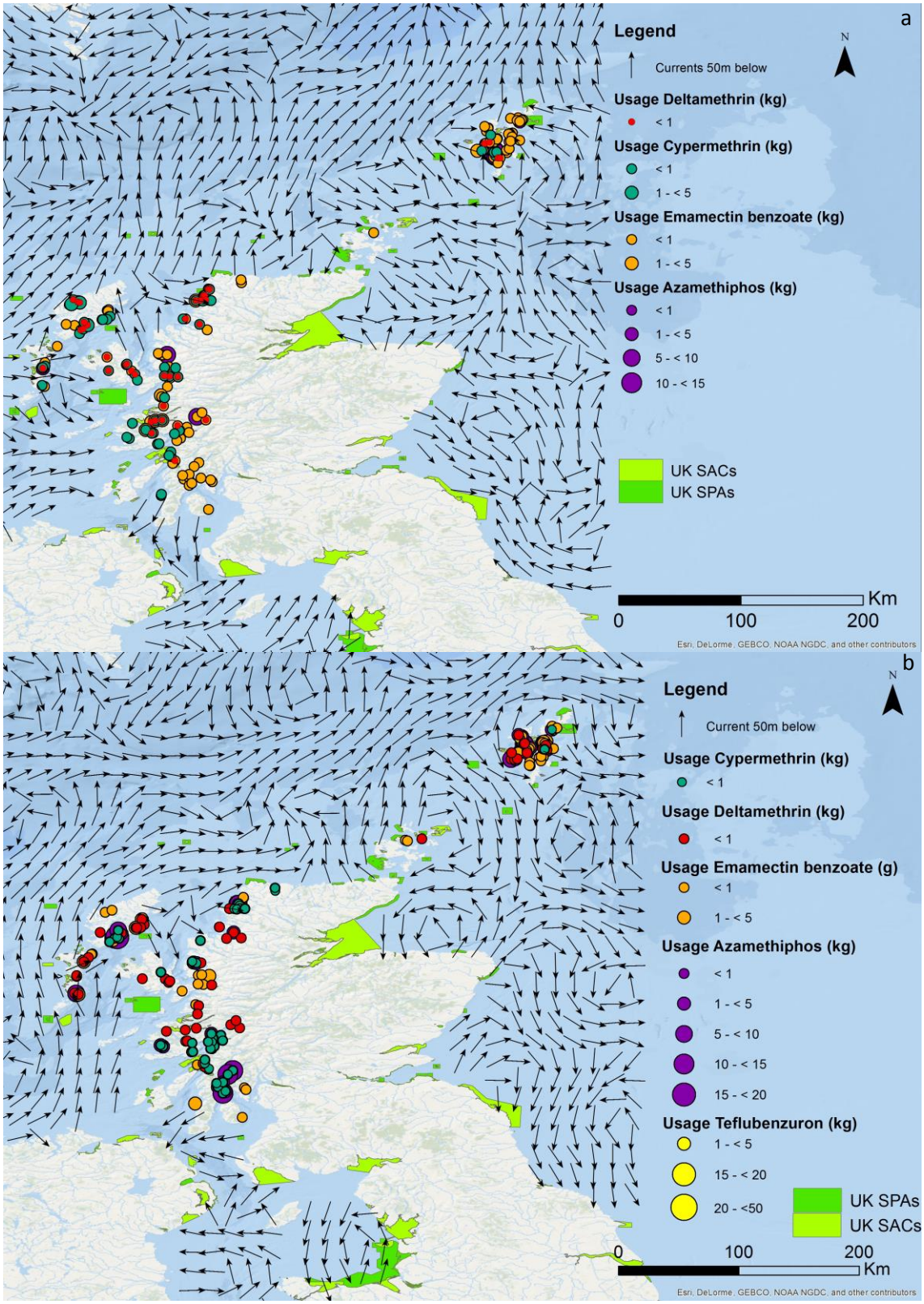


Figure 2.5 Total treatment use in 2008 (a) and 2009 (b) for the five sea lice treatments, azamethiphos (purple); cypermethrin (teal); deltamethrin (red); emamectin benzoate (orange) and teflubenzuron (yellow). SPAs are represented by the dark green areas and SACs are represented by the light green areas. Circles increase as treatment use (in grams) increases. Black arrows represent annual average direction of ocean current at 50m below sea level for 2008 (a) and 2009 (b). Background map source: Esri®

In 2010, twelve salmon farms located on seven separate SACs used either one or a combination of the treatments azamethiphos, cypermethrin, deltamethrin and emamectin benzoate (see figure 2.6a; appendix A; table A.4). One farm (CAG1), located on the SAC Sunart used azamethiphos, cypermethrin, deltamethrin and emamectin benzoate within the year. In 2011, eleven fish farms, located on seven separate SACs, used one or a combination of the treatments azamethiphos, cypermethrin, deltamethrin, emamectin benzoate and teflubenzuron (figure 2.6b; appendix A; table A.5). One farm in 2011 used azamethiphos, deltamethrin, emamectin benzoate and teflubenzuron within the same year, this was the farm FOI1 (UK NGR NC19904920) located on the SAC Loch Laxford (UK NGR NC198501), an area of conservation due to its reef and shallow inlet habitats (JNCC, 2015a).

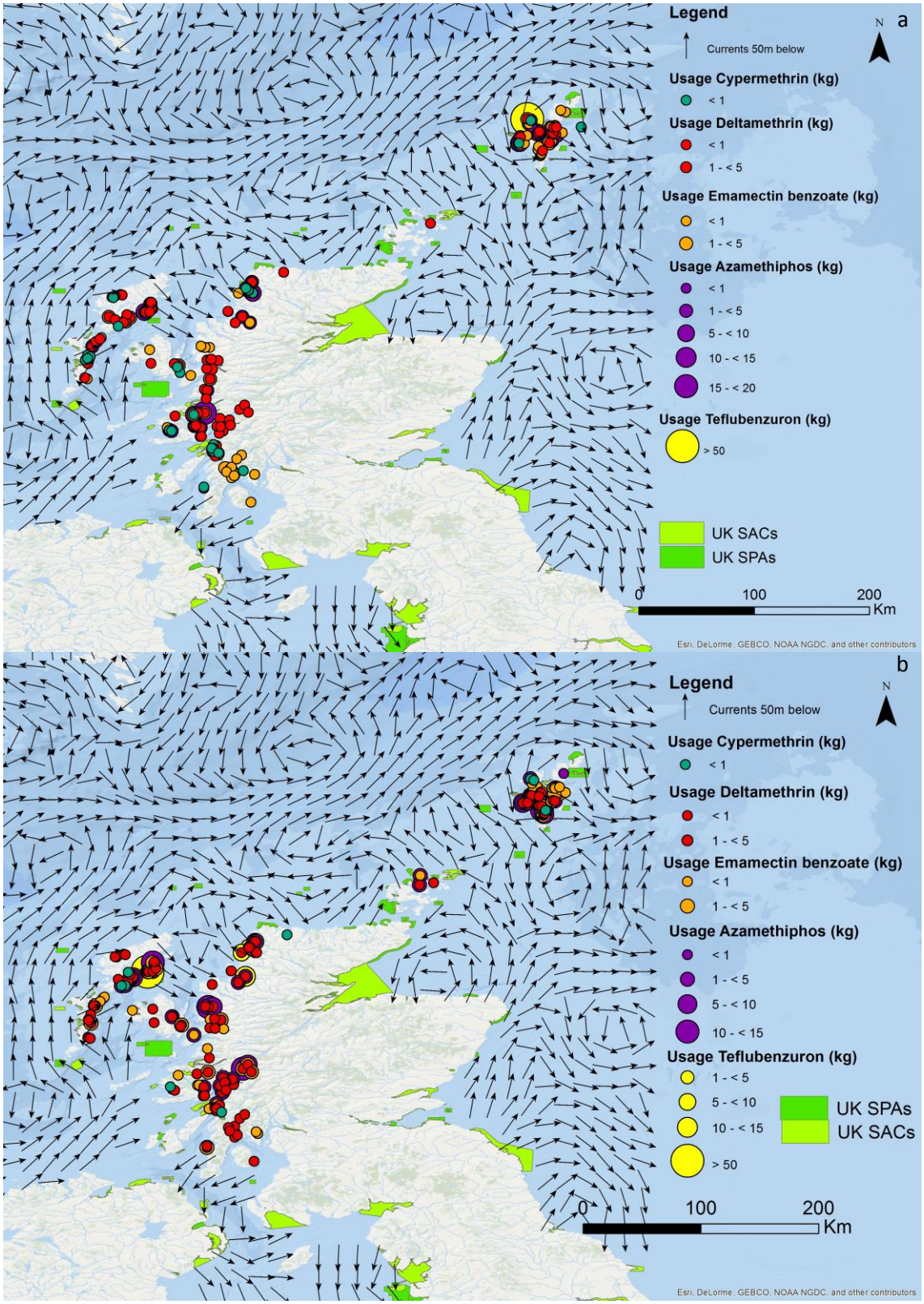


Figure 2.6 Total treatment use in 2010 (a) and 2011 (b) for the five sea lice treatments, azamethiphos (purple); cypermethrin (teal); deltamethrin (red); emamectin benzoate (orange) and teflubenzuron (yellow). SPAs are represented by the dark green areas and SACs are represented by the light green areas. Circles increase as treatment use (in grams) increases. Black arrows represent annual average direction of ocean current at 50m below sea level for 2010 (a) and 2011 (b). Background map source: Esri®

2.3.2 Sediment MECs

Sediment MECs are presented for two of the five fish farm medicines, teflubenzuron (table 2.4) and emamectin benzoate (table 2.5) for the years 2008-2012. Sediment MECs were not available for azamethiphos as it was not included in the monitoring, this is because azamethiphos does not bind readily to sediment due to its hydrophilic nature (table 2.1); deltamethrin and cypermethrin sediment MECs were all below the limit of detection (LOD) and therefore were not included. The LOD for teflubenzuron was 0.2 µg/kg (dry weight) whereas the LOD for emamectin benzoate was variable as measurements were taken from wet samples. Emamectin benzoate LOD varied between 0.08 and 0.69 µg/kg (wet weight).

Table 2.4 Mean teflubenzuron measured environmental concentration (MEC) (n = 3) for each site and the sites distance from nearest salmon farm (that used teflubenzuron the previous year) for the years 2008-2012 MEC data source: SEPA (2013).

Year	MEC (µg/kg) Dry weight	Distance from nearest farm (km)	No. of farms within 2km
2008	0.4	59.8	0
	0.18	59.3	0
	0.27	59.1	0
	2	71.5	0
	154.2	71.1	0
	1.34	70.9	0
	170.1	94.8	0
	6.36	95.1	0
	0.45	71.5	0
	0.16	72	0
	0.86	178.5	0
	0.27	245.5	0
2009	0.53	N/A	0
	0.31	N/A	0
2010	0.17	15.5	0
2011	0.27	35	0
2012	402.67	0.316	1
	698.33	0.261	1
	333	0.162	1
	7.105	0.709	1
	43.13	1	1
	15.21	0.887	1
	2.24	30	0
	0.19	8.7	0
	0.62	8	0

N/A = not applicable as no farms had decalred teflubenzuron use the previous year.

Table 2.5 Mean emamectin benzoate measured environmental concentration (MEC) (n = 3) for each site and the sites distance from nearest salmon farm (that used emamectin benzoate the previous year) for the years 2008 - 2012. MEC data source: SEPA (2013).

Year	MEC (µg/kg) wet weight	Distance from nearest farm (km)	No. of farms within 2km
2008	4.97	0.118	3
	1.47	0.126	3
	2.27	0.111	1
	0.31	0.916	1
	0.9	0.355	1
	0.28	0.58	1
	1.79	0.415	1
	0.35	0.478	1
	0.2	0.782	1
2009	1.35	23.5	0
	7.19	0.25	1
	1.045	0	1
	8.03	0.122	1
	1.39	1.5	1
	6.06	0.2	1
	17.4	0.165	1
2010	0.31	1.3	1
	13	0.47	2
	0.57	1.1	2
	14.2	0.127	1
	7.96	0.208	2
	1.73	0.111	1
2011	1.29	0.111	2
	0.23	0.498	2
	4.75	0	1
	0.32	0.568	1
	10.66	0.794	1
	25.08	0.223	1
	23.83	0.111	2
	1.61	0.166	2
	0.92	12.1	0
	0.71	22	0
2012	1.08	0.316	2
	1.51	0.261	2
	1.57	0.162	2
	0.93	0.296	2
	1.86	0.117	2
	5.98	0.236	2
	20.7	0	2
	0.96	0.223	2
	9.84	0.223	3
	0.39	0.274	3
	0.18	0.339	2
	0.09	0.659	3
	2.03	0.26	2
	0.15	0.162	2
	1.96	0.629	2

The ocean current data could not be used to draw conclusions on the likely movement of sediment from farms to sediment MECs as the measurements for currents were taken 16 km apart, and measurements were 16 km away from the coastline, which is where most of the farms are located.

In 2008 sediment samples from nine sites showed detectable MECs for emamectin benzoate, of which five were over the MAC of 0.763 µg/kg (table 2.5). The five sites that had a MEC greater than the MAC were within 500 m of the nearest salmon farm that had used emamectin benzoate in the previous year (figures 2.7c and d; and 2.8c and d). Twelve sites tested positive for teflubenzuron in 2008 (table 2.4; figures 2.9c and d and 2.10c and d). All twelve sites where samples were taken were several kilometres away from the nearest salmon farm that had used teflubenzuron the previous year. The majority of sites that were positive for teflubenzuron in 2008 were found below the MAC of 2 µg/kg; however two samples were over 75 times higher than the MAC at 154.2 µg/kg and 170.1 µg/kg (figure 2.9d).

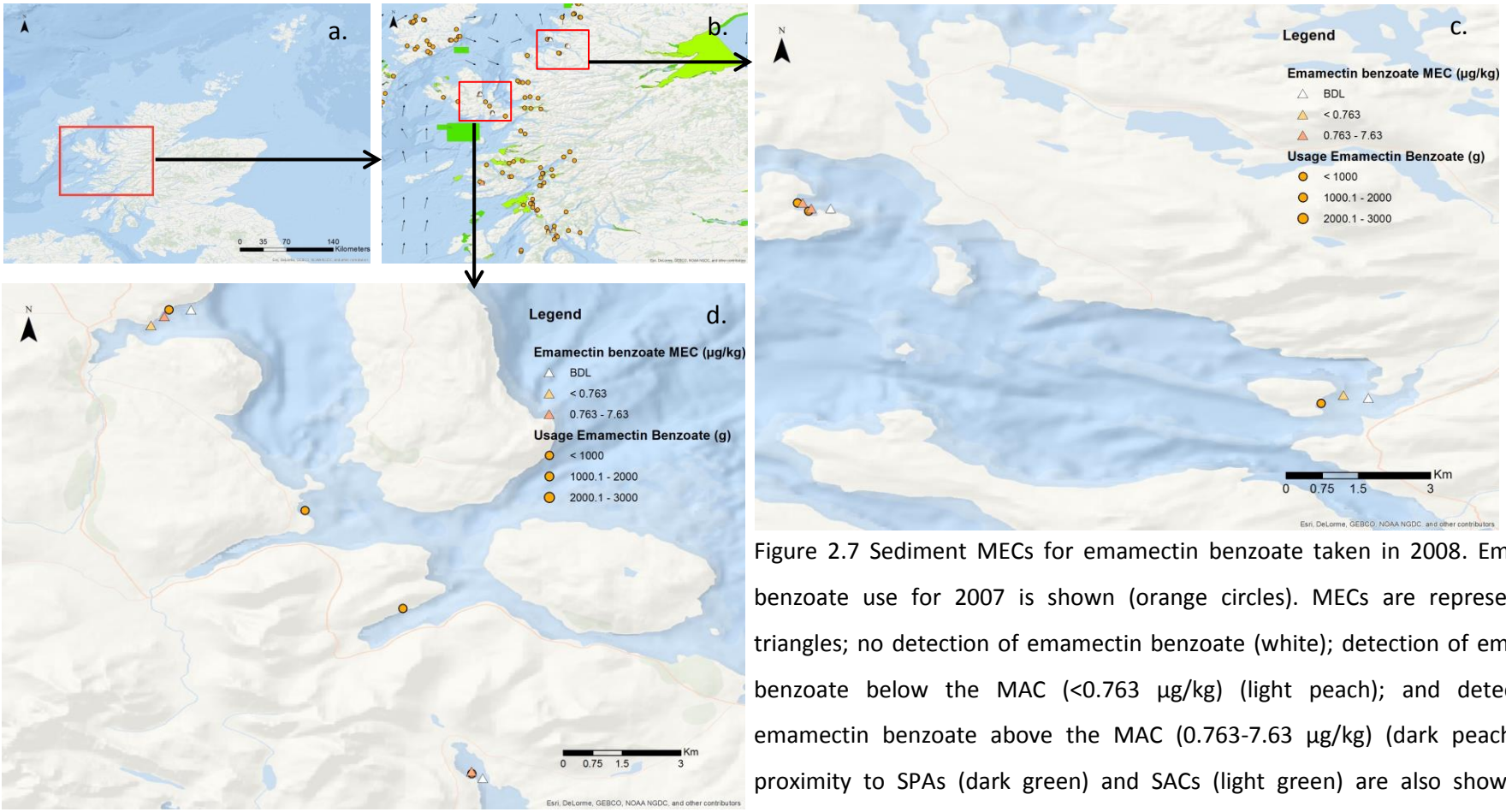


Figure 2.7 Sediment MECs for emamectin benzoate taken in 2008. Emamectin benzoate use for 2007 is shown (orange circles). MECs are represented by triangles; no detection of emamectin benzoate (white); detection of emamectin benzoate below the MAC (<0.763 µg/kg) (light peach); and detection of emamectin benzoate above the MAC (0.763-7.63 µg/kg) (dark peach). Their proximity to SPAs (dark green) and SACs (light green) are also shown. Black arrows represent current direction (50 m below surface). Background map source: Esri®

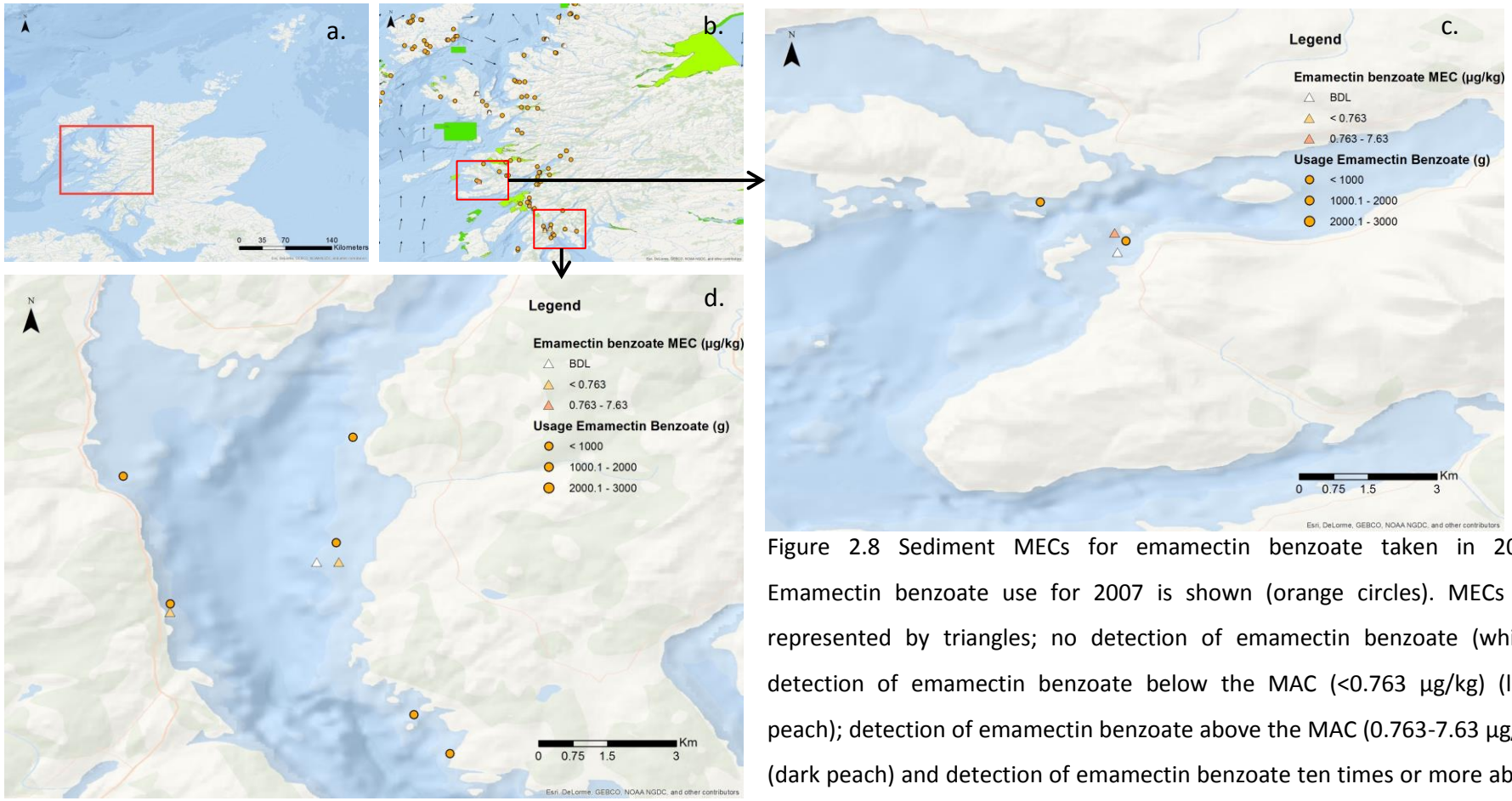


Figure 2.8 Sediment MECs for emamectin benzoate taken in 2008. Emamectin benzoate use for 2007 is shown (orange circles). MECs are represented by triangles; no detection of emamectin benzoate (white); detection of emamectin benzoate below the MAC (<0.763 µg/kg) (light peach); detection of emamectin benzoate above the MAC (0.763-7.63 µg/kg) (dark peach) and detection of emamectin benzoate ten times or more above the MAC (>7.63 µg/kg) (red). Their proximity to SPAs (dark green) and SACs (light green) are also shown. Background map source: Esri®

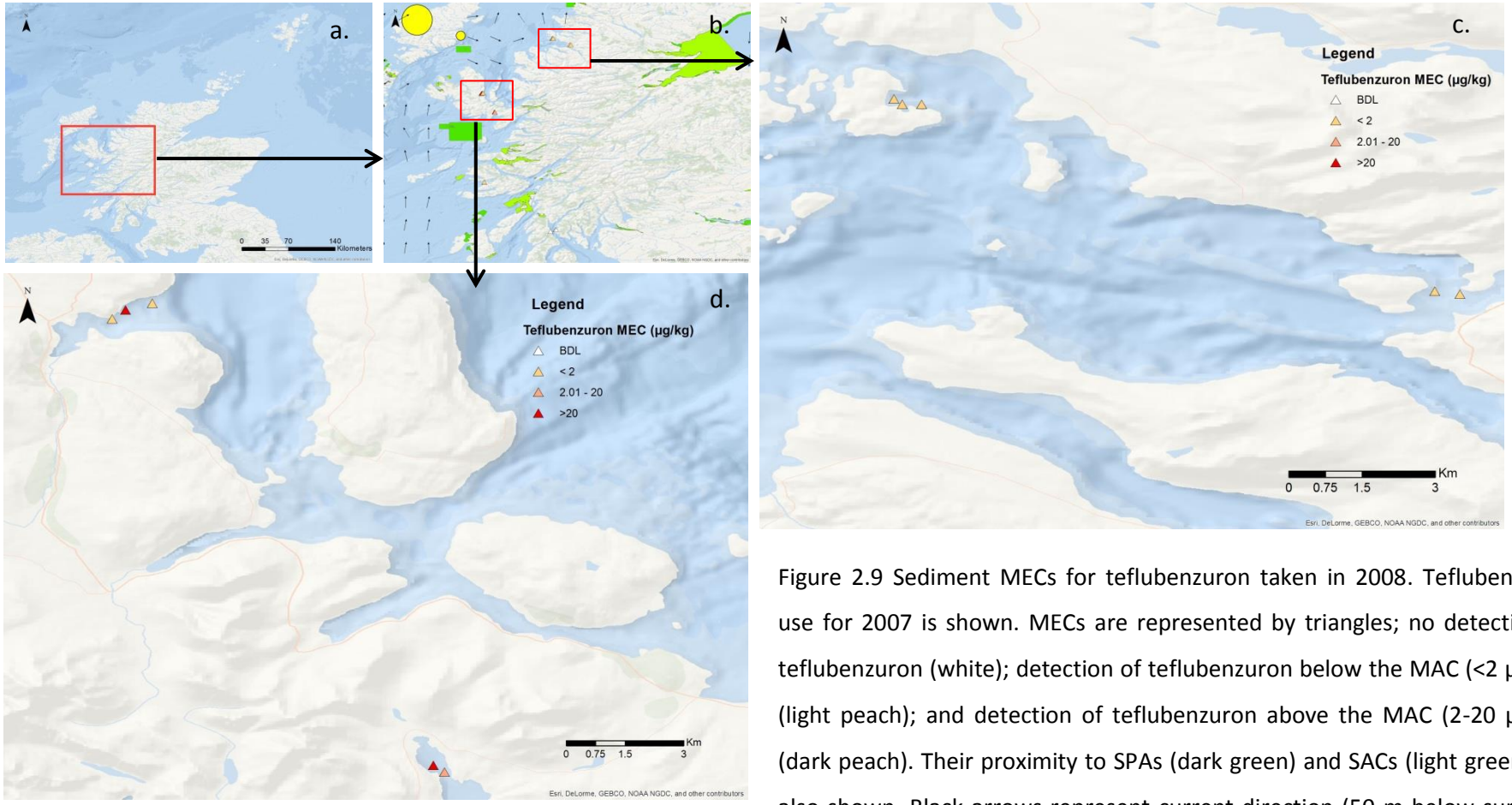


Figure 2.9 Sediment MECs for teflubenzuron taken in 2008. Teflubenzuron use for 2007 is shown. MECs are represented by triangles; no detection of teflubenzuron (white); detection of teflubenzuron below the MAC (<2 µg/kg) (light peach); and detection of teflubenzuron above the MAC (2-20 µg/kg) (dark peach). Their proximity to SPAs (dark green) and SACs (light green) are also shown. Black arrows represent current direction (50 m below surface). Background map source: Esri®

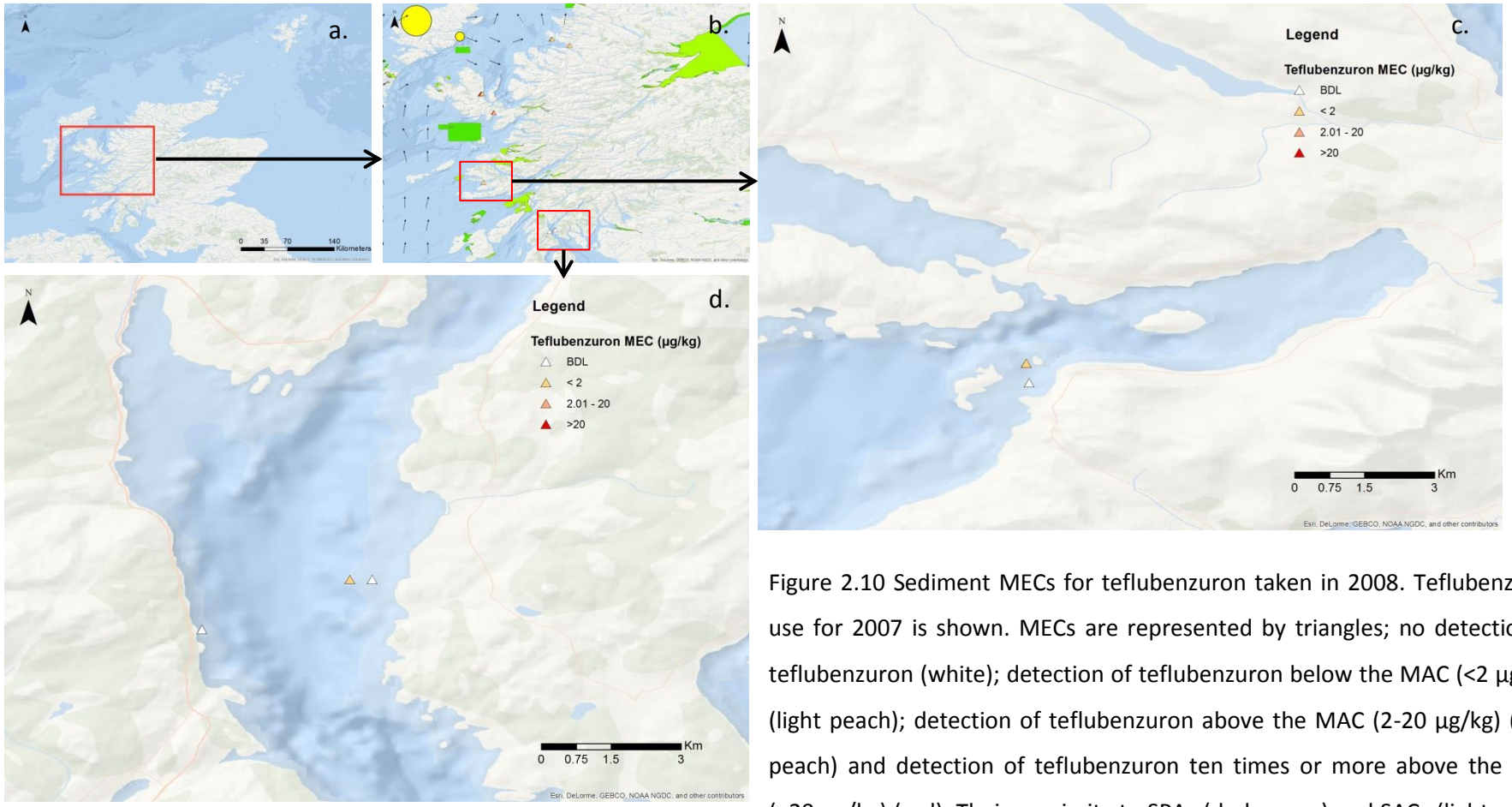


Figure 2.10 Sediment MECs for teflubenzuron taken in 2008. Teflubenzuron use for 2007 is shown. MECs are represented by triangles; no detection of teflubenzuron (white); detection of teflubenzuron below the MAC (<2 µg/kg) (light peach); detection of teflubenzuron above the MAC (2-20 µg/kg) (dark peach) and detection of teflubenzuron ten times or more above the MAC (>20 µg/kg) (red). Their proximity to SPAs (dark green) and SACs (light green) are also shown. Black arrows represent current direction (50 m below surface). Background map source: Esri®

In 2009 nine sites had detectable emamectin benzoate sediment MECs. Of these nine sites, seven contained emamectin benzoate in detectable concentrations (figures 2.11c, d and e). Two sites contained emamectin benzoate at concentrations more than ten times higher than the MAC; the two sites were 100-250 m from the nearest fish farm that had used emamectin benzoate the previous year (figure 2.11e). In 2009 two sites had a detectable concentration of teflubenzuron; the concentration of both of the sediment MECs from the two sites was below the MAC of 2 µg/kg. However there was no recorded use of teflubenzuron in the previous year (figure 2.12; table 2.3).

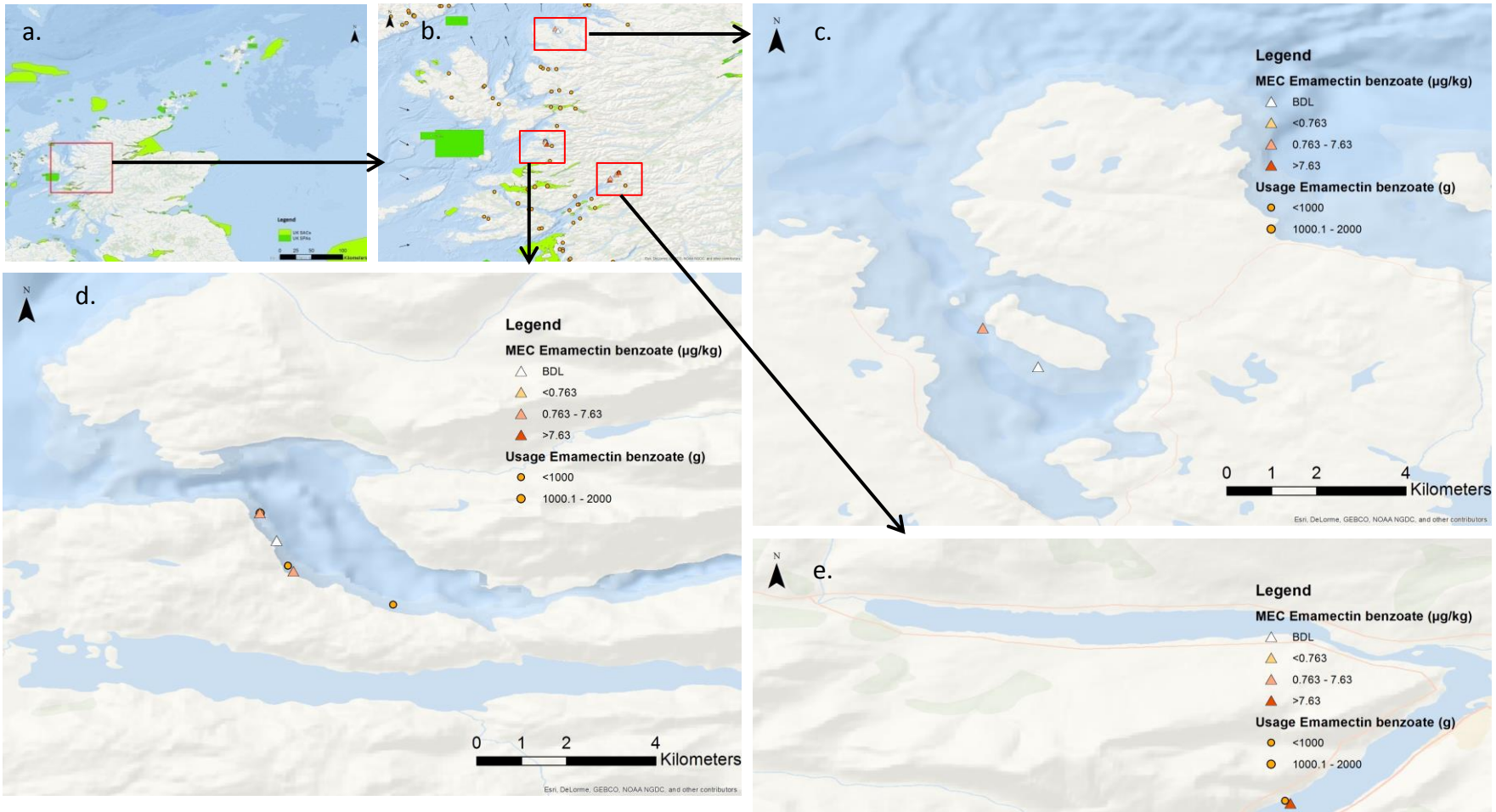


Figure 2.11 Sediment MECs for emamectin benzoate taken in 2009. Emamectin benzoate use for 2008 is shown (orange circles). MECs are represented by triangles; no detection of emamectin benzoate (white); detection of emamectin benzoate below the MAC (<0.763 µg/kg) (light peach); detection of emamectin benzoate above the MAC (0.763-7631 µg/kg) (dark peach) and detection of emamectin benzoate ten times or more above the MAC (>7.63 µg/kg) (red). Their proximity to SPAs (dark green) and SACs (light green) are also shown. Black arrows represent current direction (50 m below surface). Background map source: Esri®

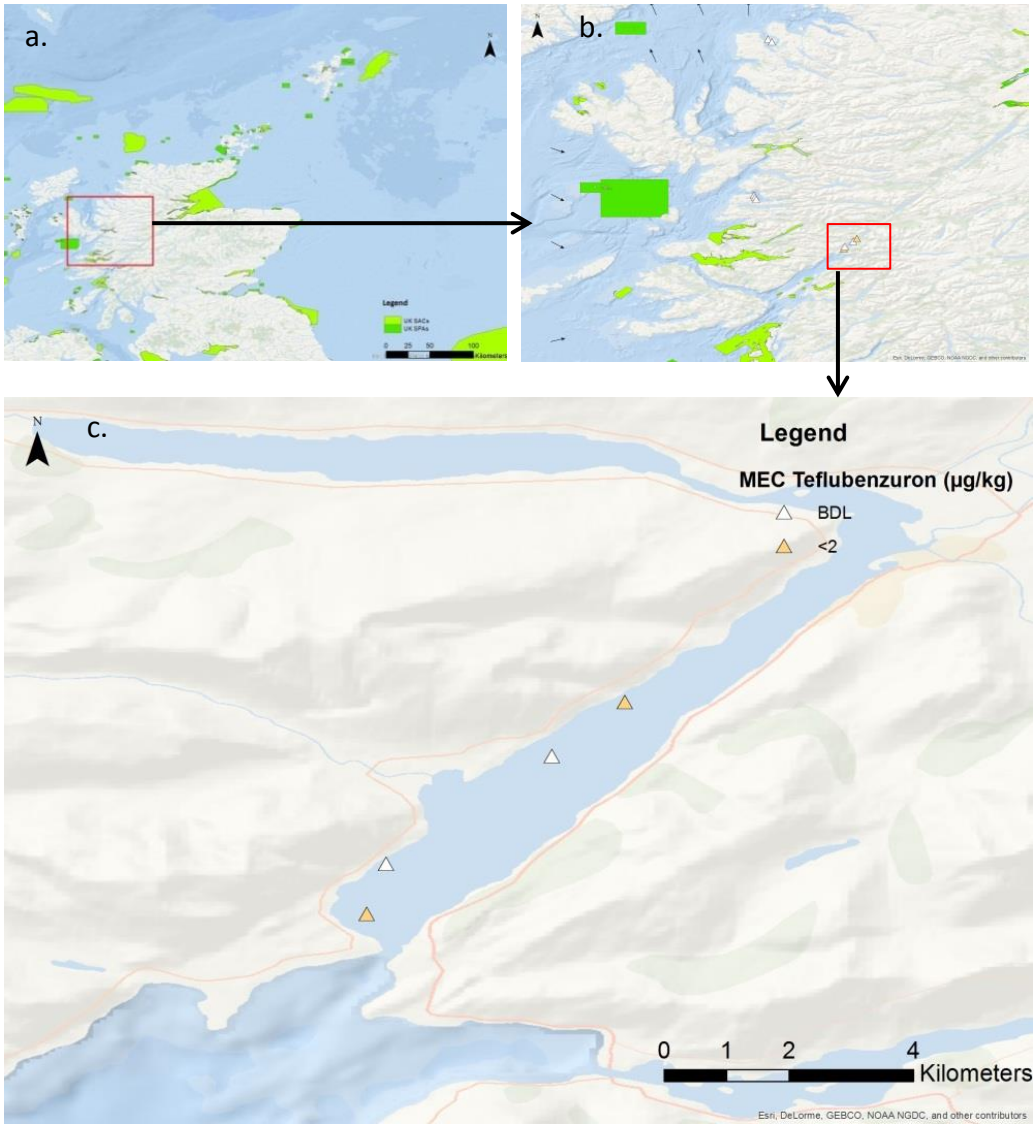


Figure 2.12 Sediment MECs for teflubenzuron taken in 2009. Usage is not shown as there was no teflubenzuron use in 2008. MECs are represented by triangles; no detection of teflubenzuron (white); and detection of teflubenzuron below the MAC (<2 µg/kg) (light peach). Their proximity to SPAs (dark green) and SACs (light green) are also shown. Black arrows represent current direction (50 m below surface). Background map source: Esri®

Emamectin benzoate was detected in 6 of the sample sites in 2010 (figure 2.13c;d and e). Four of those sites had sediment levels of emamectin benzoate at a higher concentration than the MAC (>0.763 µg/kg), with three being higher than the MAC by at least ten times. Teflubenzuron was detected at one of nine sites in 2010 (figure 2.14c) at a concentration of 0.17 µg/kg, which is lower than the MAC (2 µg/kg) however this site was more than 16 km away from two farms, which in the previous year used 17.3 kg and 42.7 kg of teflubenzuron. There are some fish farms on the Shetland Islands using emamectin benzoate treatments that are located on the SAC Yell Sound Coast, which is a protected habitat supporting populations of the European otter *L. lutra* and the UK biodiversity action plan (BAP) priority species, the common seal *P. vitulina* (JNCC, 2013). There are also seven farms that are within 2 km of the Yell Sound Coast SAC.

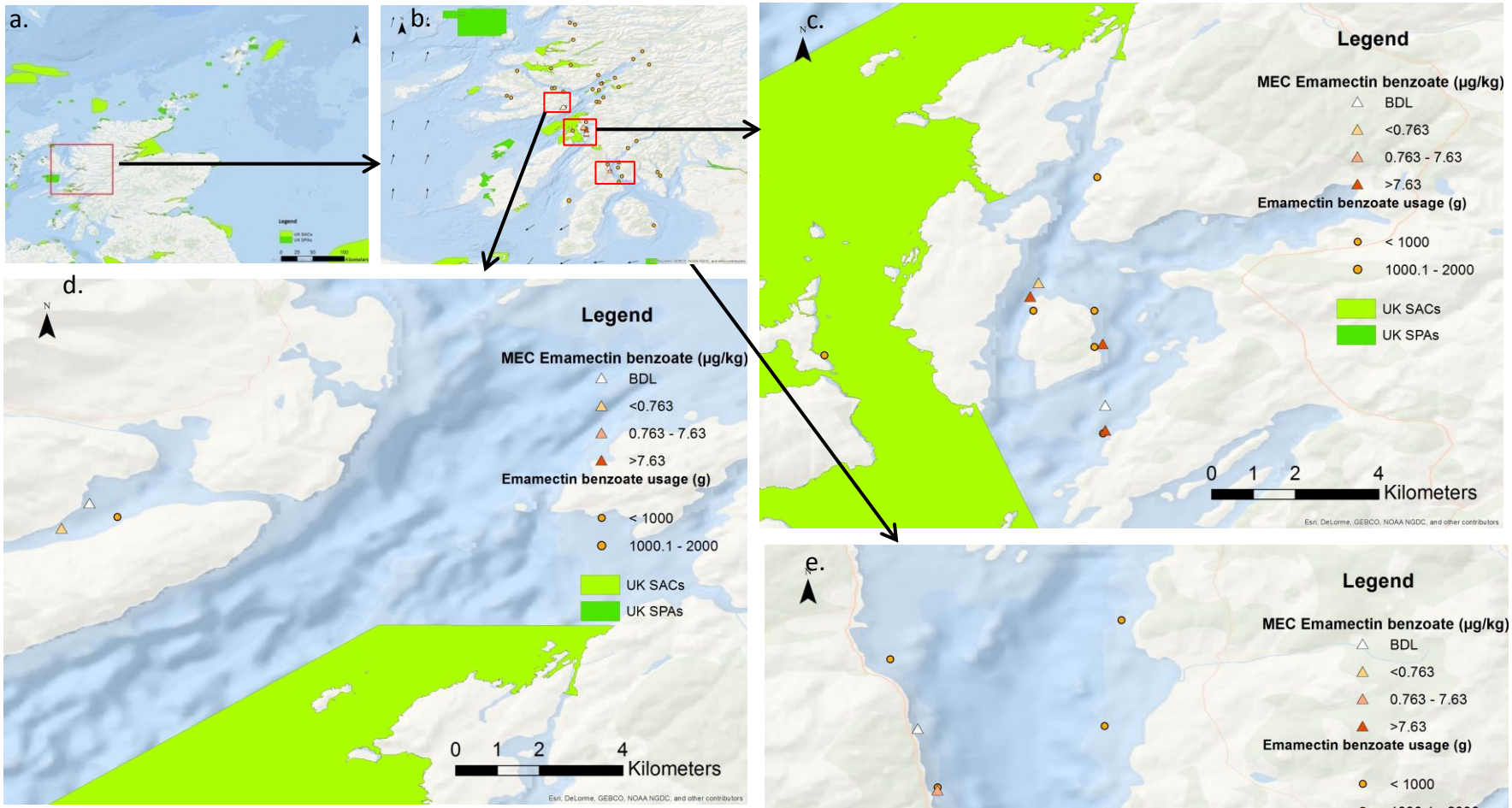


Figure 2.13 Sediment MECs for emamectin benzoate taken in 2010. Emamectin benzoate use for 2009 is shown (orange circles). MECs are represented by triangles; no detection of emamectin benzoate (white); detection of emamectin benzoate below the MAC (<0.763 µg/kg) (light peach); detection of emamectin benzoate above the MAC (0.763-7.63 µg/kg) (dark peach) and detection of emamectin benzoate ten times or more above the MAC (>7.1 µg/kg) (red). Their proximity to SPAs (dark green) and SACs (light green) are also shown. Black arrows represent current direction (50 m below surface). Background map source: Esri®

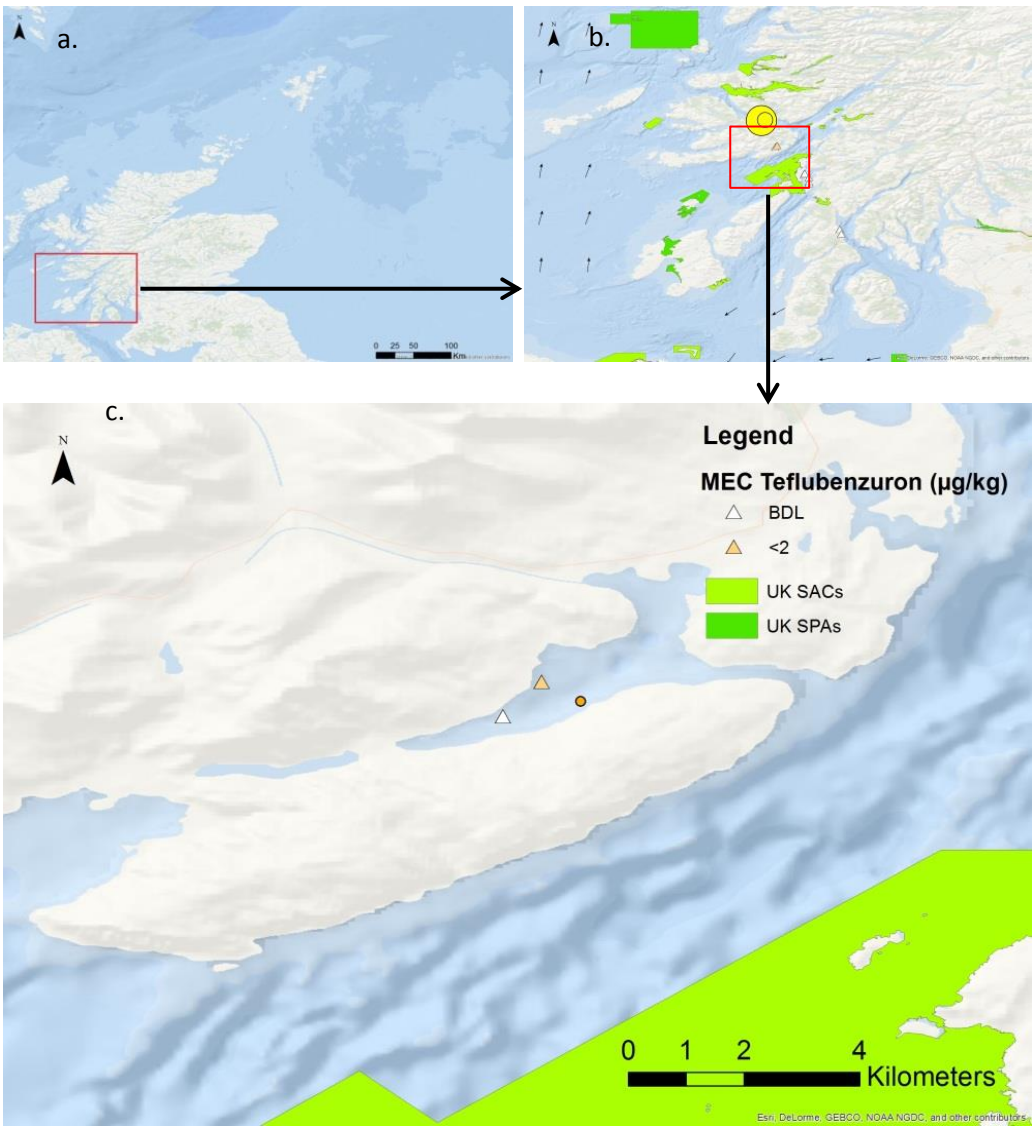


Figure 2.14 Sediment MECs for teflubenzuron taken in 2010. MECs are represented by triangles; no detection of teflubenzuron (white); and detection of teflubenzuron below the MAC (<2 µg/kg) (light peach). Their proximity to SPAs (dark green) and SACs (light green) are also shown. Black arrows represent current direction (50 m below surface). Background map source: Esri®

In 2011 ten sites had a detectable MEC of emamectin benzoate, out of a possible 18. Seven of these sites were equal to or higher than the MAC. The samples in 2011 were taken from the Shetland islands and the total use of emamectin benzoate for the surrounding farms for the previous year 2010, varied between 0 g and 1.55 kg (figure 2.15). One site, that was ~800 m away from the nearest farm that used emamectin benzoate had an MEC of >7.63 µg/kg. In 2011 a measurable concentration of teflubenzuron (figure 2.16c) was found at one of 18 sites, this concentration was lower than the MAC at 0.27 µg/kg. The closest fish farm to this site that used teflubenzuron was 35 km away and used 75 kg of teflubenzuron in 2010, and 17.59 kg in 2009.

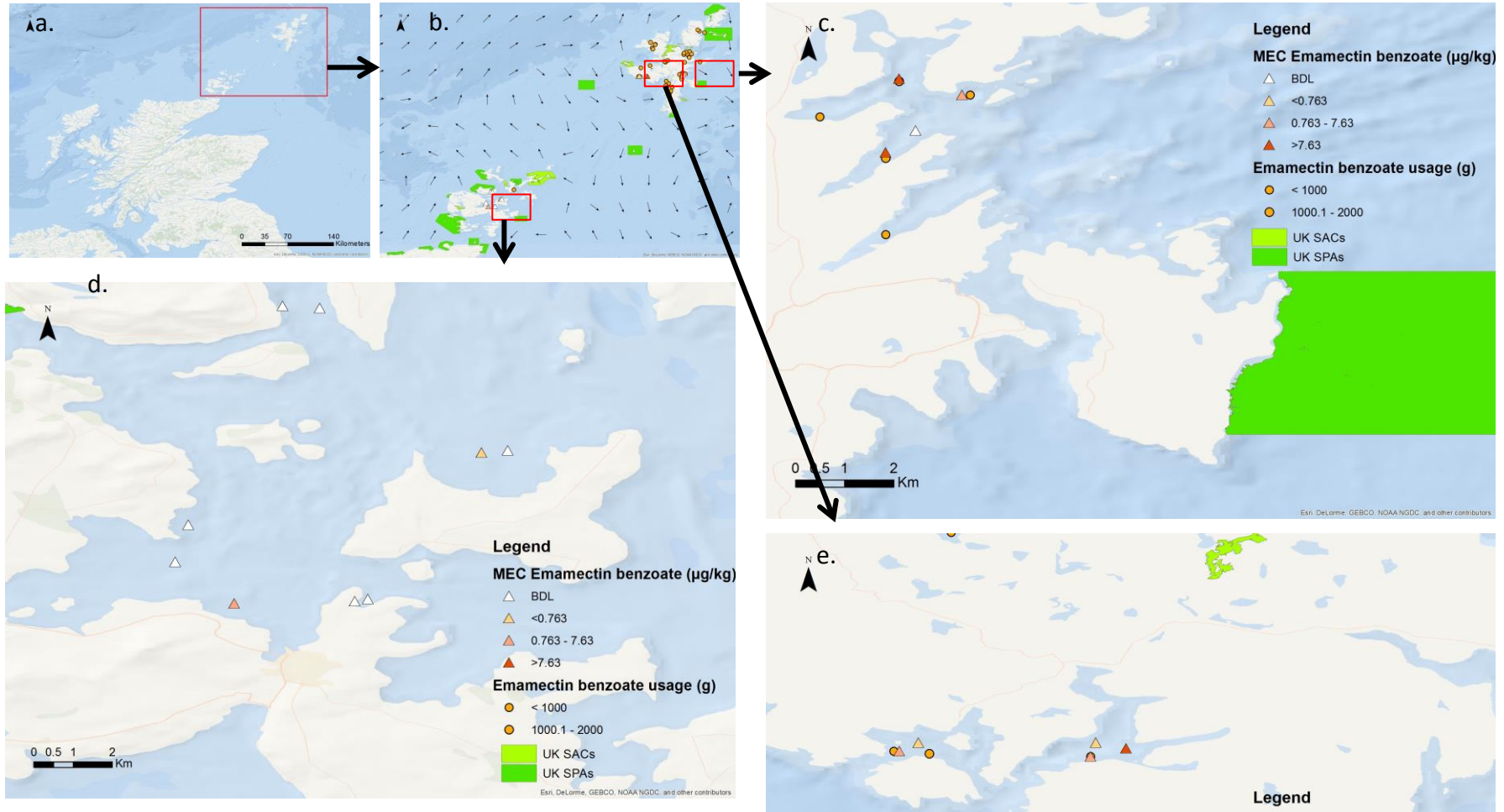


Figure 2.15 Sediment MECs for emamectin benzoate taken in 2011. Emamectin benzoate use for 2010 is shown (orange circles). MECs are represented by triangles; no detection of emamectin benzoate (white); detection of emamectin benzoate below the MAC (<0.763 µg/kg) (light peach); detection of emamectin benzoate above the MAC (0.763-7.63 µg/kg) (dark peach) and detection of emamectin benzoate ten times or more above the MAC (>7.1 µg/kg) (red). Their proximity to SPAs (dark green) and SACs (light green) are also shown. Black arrows represent current direction (50 m below surface). Background map source: Esri®

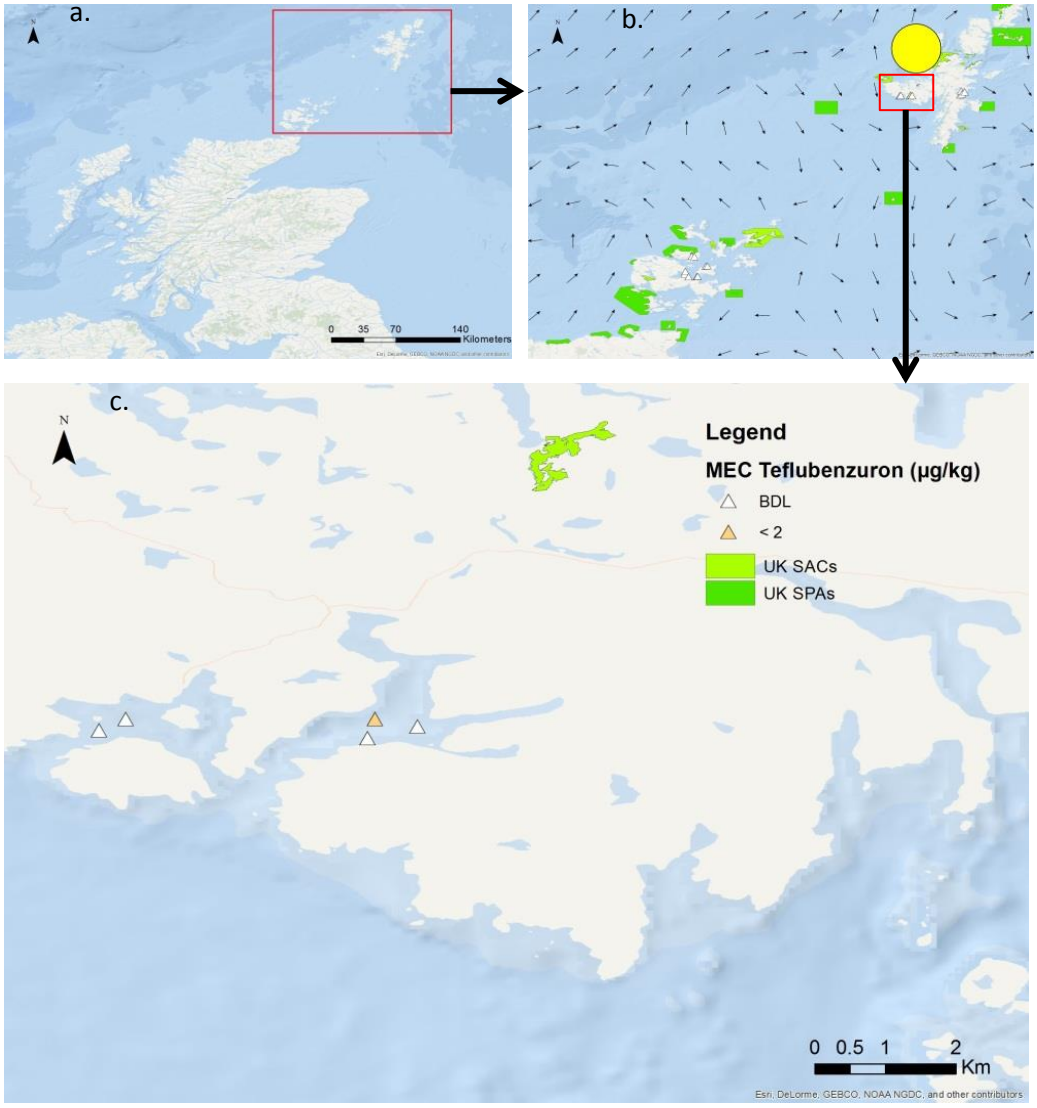


Figure 2.16 Sediment MECs for teflubenzuron taken in 2011. MECs are represented by triangles; no detection of teflubenzuron (white); and detection of teflubenzuron below the MAC (<2 µg/kg) (light peach). Their proximity to SPAs (dark green) and SACs (light green) are also shown. Black arrows represent current direction (50 m below surface). Background map source: Esri®

Emamectin benzoate was detected in 17 of the 19 sites in 2012 (figure 2.17c; d and e). The nearest farm to one of these sampling sites that used the treatment was ~3950 m away. The 2012 teflubenzuron MECs (figure 2.18c; d and e) showed there were ten samples containing detectable levels of teflubenzuron in sediment across the 19 sites. Of these samples, seven were higher than the MAC, with three having a concentration between 333-698.33 µg/kg. The three sites which had a concentration between 333-698.33 µg/kg were within 400 m of a farm which used 54 kg of teflubenzuron in December 2011. One site which had a concentration of 43.13 µg/kg was ~1 km away from a farm that had used 54 kg of teflubenzuron in 2011.

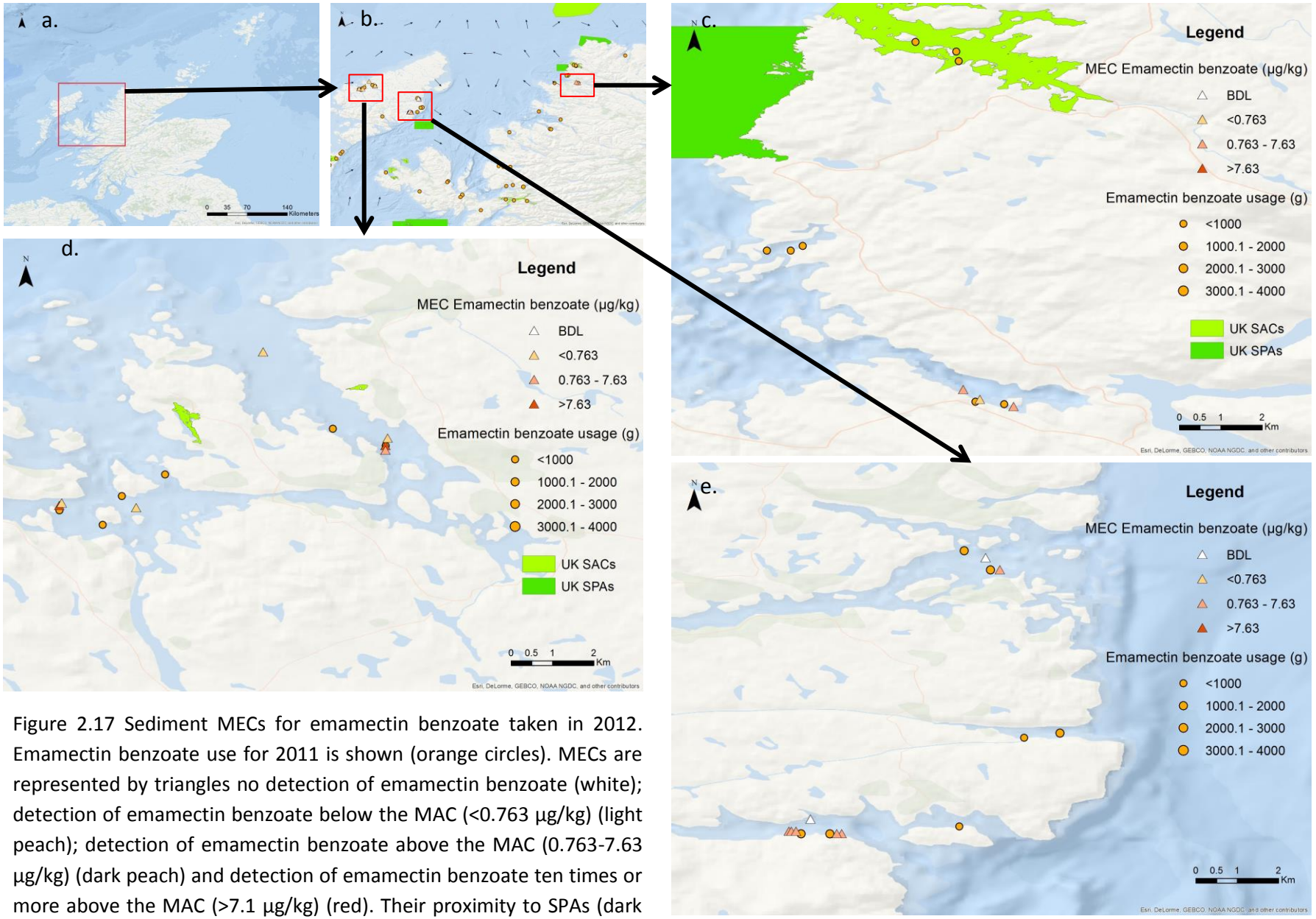


Figure 2.17 Sediment MECs for emamectin benzoate taken in 2012. Emamectin benzoate use for 2011 is shown (orange circles). MECs are represented by triangles no detection of emamectin benzoate (white); detection of emamectin benzoate below the MAC (<0.763 µg/kg) (light peach); detection of emamectin benzoate above the MAC (0.763-7.63 µg/kg) (dark peach) and detection of emamectin benzoate ten times or more above the MAC (>7.1 µg/kg) (red). Their proximity to SPAs (dark green) and SACs (light green) are also shown. Black arrows represent current direction (50 m below surface). Background map source: Esri®

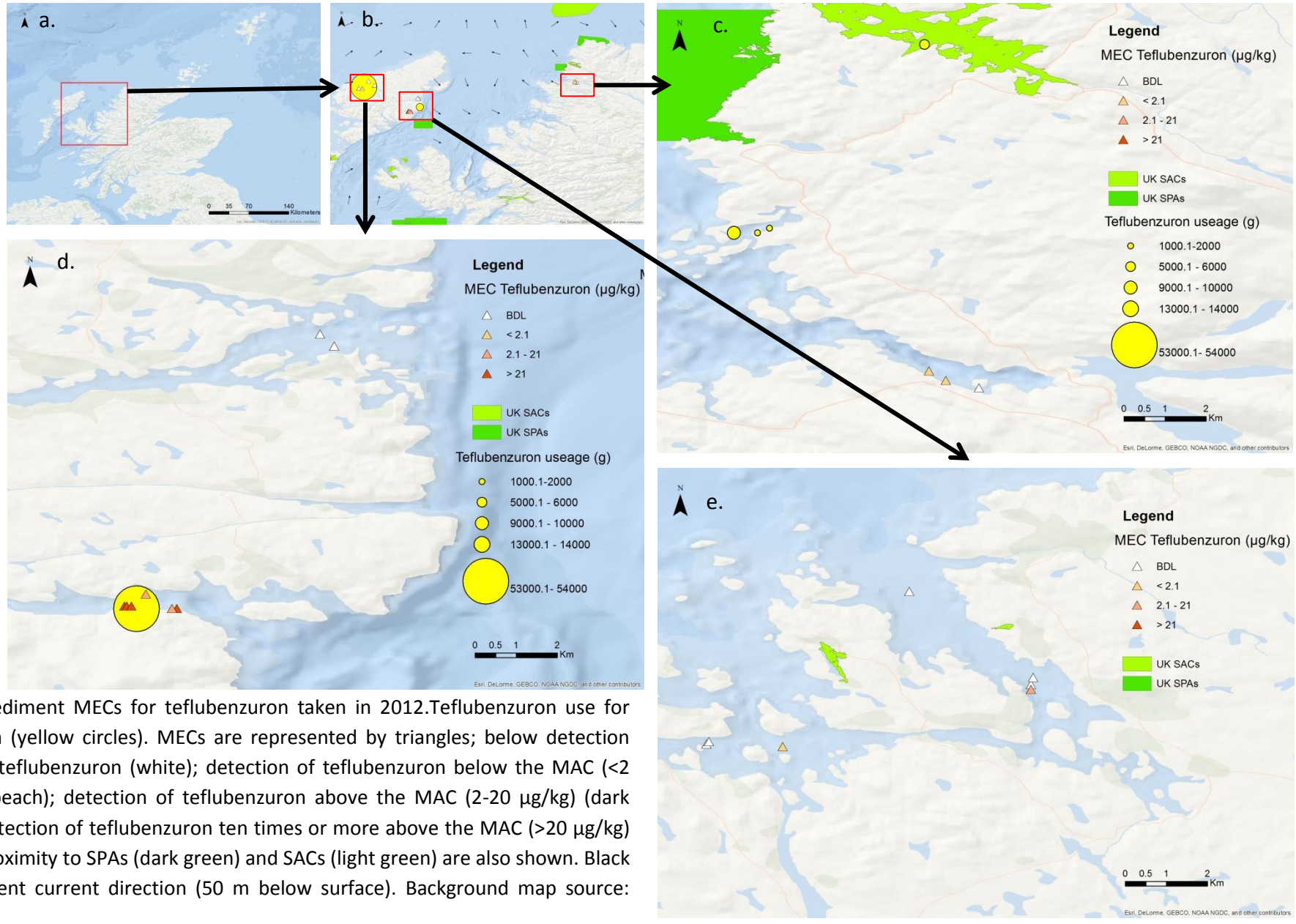


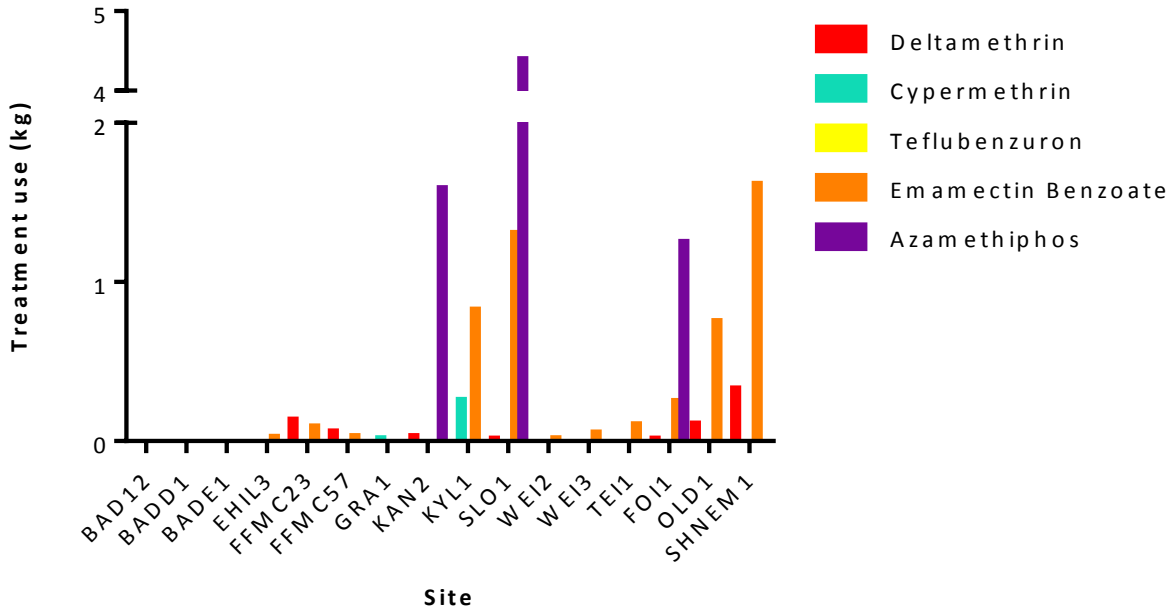
Figure 2.18 Sediment MECs for teflubenzuron taken in 2012.Teflubenzuron use for 2011 is shown (yellow circles). MECs are represented by triangles; below detection limit (BDL) of teflubenzuron (white); detection of teflubenzuron below the MAC (<2 µg/kg) (light peach); detection of teflubenzuron above the MAC (2-20 µg/kg) (dark peach) and detection of teflubenzuron ten times or more above the MAC (>20 µg/kg) (red). Their proximity to SPAs (dark green) and SACs (light green) are also shown. Black arrows represent current direction (50 m below surface). Background map source: Esri®

Between 2008 and 2012, 47 out of a total 70 sediment samples tested positive for emamectin benzoate (table 2.5), while 25 tested positive for teflubenzuron (table 2.4). Of the 47 positive emamectin benzoate samples, 35 were above the MAC of 0.763 µg/kg, with a further 11 samples exceeding concentrations ten times higher than the MAC. Of the 25 positive teflubenzuron samples, 11 were higher than the MAC of 2 µg/kg, with six exceeding the MAC by at least ten times. Four of the six sites were all within 2 km of the nearest fish farm that had used teflubenzuron in the preceding year (figure 2.18; table 2.4). The majority of sites which had measurable concentrations of emamectin benzoate were within 2 km of a fish farm that had used emamectin benzoate in the previous year (n = 47). There were three sites with detectable levels of emamectin benzoate where the nearest farm was over 2 km away, with two of these sites having concentrations above the MAC. Six sites with detectable concentrations of teflubenzuron were within 2 km of the nearest farm to have used the treatment in the last year. Of the seven sites that were more than 2 km away from the nearest farm that had used the treatment, one was over the MAC with a concentration of 2.24 µg/kg (table 2.4, figure 2.17d).

2.3.3 Timing of teflubenzuron application

Teflubenzuron is a treatment that is used to break the life cycle of sea lice and if used correctly should prevent the use of additional sea lice treatments for a further year (SEPA, 1999). However, the SEPA data shows the salmon farms around the coast of Scotland that have used teflubenzuron were no less likely to use other sea lice treatments up to ten months after applying teflubenzuron than ten months before using teflubenzuron (figure 2.19). Overall use of treatments increased from 15.6 kg treatment use in the ten months before the application of teflubenzuron, to 27.2 kg treatment use in the ten months after teflubenzuron application. The use of cypermethrin, teflubenzuron, emamectin benzoate and azamethiphos increased after the first application of teflubenzuron, with the increase in emamectin benzoate being significantly higher after the first application of teflubenzuron ($P = 0.017$). Deltamethrin was the only sea lice treatment whose use decreased after the first application of teflubenzuron (figure 2.19).

a.



b.

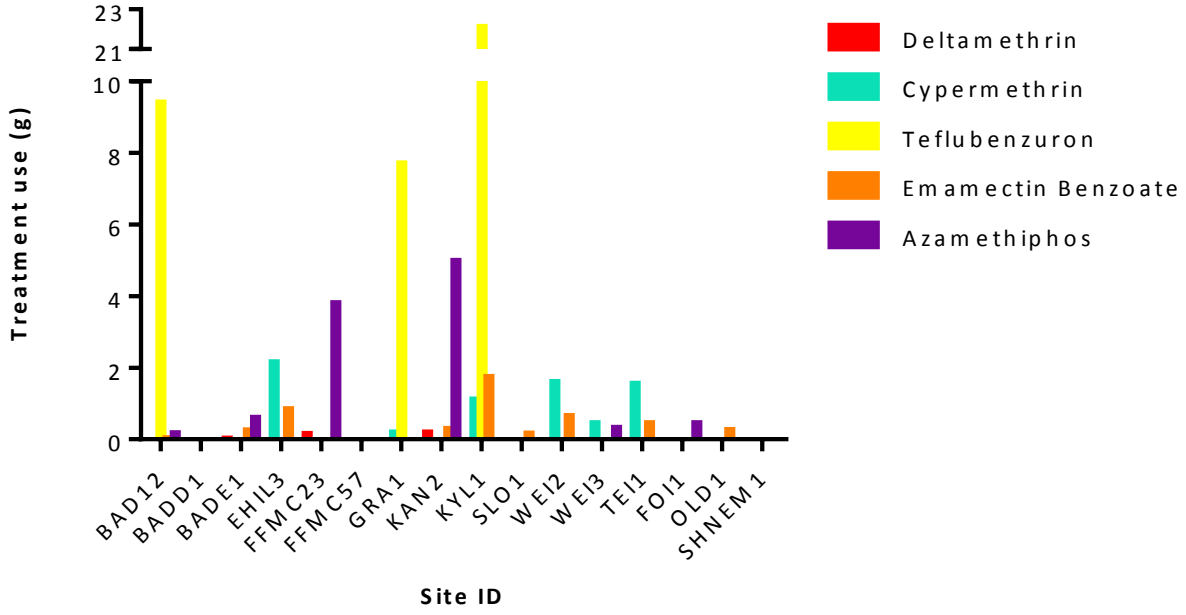


Figure 2.19 Total treatment use on Scottish salmon farms (a) ten months before and (b) ten months after teflubenzuron application. Timing of application of teflubenzuron varied between farms, application of treatment was for the years 2007-2012.

2.4 Discussion

2.4.1 Chemical Use on Scottish Salmon Farms

The general use of sea lice chemicals around the Scottish coast increased between 2002 and 2014 (table 2.3; figures 2.4 – 2.6). To avoid resistance in sea lice the veterinary medicines directorate (VMD) recommends that treatments are used in cycles with breaks of use for a few years (VMD, 2013). This appears to be the case for azamethiphos, cypermethrin, deltamethrin and teflubenzuron; however emamectin benzoate was used every year for twelve years (table 2.3). Resistance to pyrethroids, emamectin benzoate and azamethiphos have been documented in sea lice while resistance in teflubenzuron has not been reported (Aaen *et al.*, 2015; Sevatdal *et al.*, 2005). There are a number of fish farms using emamectin benzoate, deltamethrin and azamethiphos from 2009 onwards (figure 2.5b; figure 2.6a and b; figure 2.15) in and around the Yell Sound Coast SAC, around the Shetland islands (UK NGR HU467755). This is a protected habitat due to the populations of the otter *L. lutra* and the common seal *P. vitulina* that it supports. The marine environment is especially valuable for the algal beds that *L. lutra* feed on (JNCC, 2013). The 72hr EC_{50} for emamectin benzoate on the algal species *P. subcapitata* is 0.072 mg/l compared with the PEC for emamectin benzoate in aquaculture of 4.16 pg/l it is unlikely that it will have a chronic effect on algal species (Willis and Ling, 2003). Sediment MECs on the other hand are higher than the aquatic PEC, with 33 measurements exceeding the MAC of 0.76 µg/kg (table 2.1; table 2.5; figures 2.7; 2.8; 2.11; 2.13; 2.15). The LC_{50} of the amphipod crustacean *C. volutator* has been reported at 153 µg/kg (table 2.2) (Mayor *et al.*, 2008). The LC_{50} of *C. volutator* is just over 6 times the highest recorded sediment MEC for emamectin benzoate measured in 2011 (UK NGR HU449484; figure 2.15). Even when applying an AF of 10 on the emamectin benzoate LC_{50} of *C. volutator* would mean this concentration is above the PNEC for this species. The 72hr EC_{50} for *P. subcapitata* exposed to deltamethrin is 9.1 mg/l, the PEC for deltamethrin as an aquaculture treatment is unavailable, however given the hydrophobicity of deltamethrin and the MEC of deltamethrin being reported as <10 ng/L (Langford *et al.*, 2014), environmental concentrations are unlikely to cause chronic or acute effects on *P. subcapitata*. There is no information on the ecotoxicology of azamethiphos on algal species. The SPAs are protected due to their ability to support migratory or breeding birds under Annex I of the EC Birds Directive (European Parliment, 2009). Some habitats support marine birds such as the red crested loon *Gavia stellata* which feeds on fish, molluscs and other invertebrates. The sediment MECs consistently measured above the sediment MAC for teflubenzuron and emamectin benzoate (table 2.3 and 2.4); both of these VMs have low reported EC_{50} and LC_{50} for invertebrates (table 2.2) and it is therefore possible that changes in marine species composition due to chronic or lethal effects of VMs on invertebrates could affect species diversity and richness, causing a secondary effect on birds using these areas to feed (Suryan *et al.*, 2006).

There have been observed occurrences of intersex invertebrates in bodies of water containing EDCs. Intersexuality is defined as either the masculinity of female individuals or the feminisation of male individuals; it has been documented in both vertebrates and invertebrates (Ford *et al.*, 2007; Jobling *et al.*, 2003; Jobling *et al.*, 2002; Oehlmann *et al.*, 2000; Schulte-Oehlmann *et al.*, 2000). Intersexuality of invertebrates has been known to occur in the presence of EDCs such as the anti-fouling agent tributyltin, a xenoandrogen and bisphenol A, a xenoestrogen. Effects of intersex can include the growth of male sex organs in female individuals in the presence of xenoandrogens (Schulte-Oehlmann *et al.*, 2000) and the growth of female sex organs in males individuals (Oehlmann *et al.*, 2000). In a field study on the marine amphipod *E. marinus* at a salmon farm near the SAC Sunart, (Ford *et al.*, 2007) found a higher incidence of intersex females (16% of total sample) and a lower proportion of males (27% of total sample) compared with a site 2.5 km away which found 0% intersex females and 47% males. The fish farm in question, had used both cypermethrin and emamectin benzoate, although (Ford *et al.*, 2012) do not explicitly link the higher proportion of intersex individuals to the use of cypermethrin and emamectin benzoate it is a possibility that these treatments are having an effect on the endocrinology of *E. marinus.* In a study on the abundance of zooplankton around a farm on Loch Sunart treated with emamectin benzoate and cypermethrin, Willis *et al.* (2005) reported that there were no adverse effects on the abundance of zooplankton over the 31 month test period. The amount of active ingredient that was added the farm was 78 g of cypermethrin and 315 – 316.6 g of emamectin benzoate (which was applied on three occasions). These rates of application are much lower than the highest reported use of cypermethrin and emamectin benzoate in Scotland between the years 2007 and 2011. The highest amount of cypermethrin used was 840 g, while the highest amount of emamectin benzoate used was reported as being 14.7 kg (Natural Scotland, 2015a). There are three salmon farms located on the SAC Sunart, which is protected due to its reef habitats and the occurrence of the otter *L. lutra.* These farms repeatedly used sea lice treatments over the years 2007-2011, often occurring in mixtures of emamectin benzoate and cypermethrin, as well as occasions where azamethiphos was used as well as emamectin benzoate and cypermethrin (see appendix A). Repeated applications of mixtures of these treatments could lead to endocrine disrupting effects not only on *E. gammarus* but also on other invertebrates which reside in the reefs such as the rare tubeworm *Placostegus tridentatus* which has been found at Loch Sunart, leading to a possible reduction in fecundity and fertility, which could ultimately result in population decline.

In 2010 four sites had sediment MECs that were over the sediment MAC for emamectin benzoate (see table 2.5; figure 2.13). Three of these sites were within 5 km of the SAC Firth of Lorn. The Firth of Lorn is a designated area of conservation area due to its diverse species of reefs which are home to the gravel brittle star *Ophiopsila annulosa,* a benthic starfish and *Leptometra celtica* a

filter feeding echinoderm, which is a Scottish natural heritage priority marine feature (Scottish Natural Heritage, 2015). The salmon farm, FFMC53 (UK NGR NM71400781), is situated directly on the Firth of Lorn, in 2007 and 2009 FFMC53 used emamectin benzoate and 2011 FFMC53 used emamectin benzoate and azamethiphos (appendix A; tables A.1; A3; and A5). Emamectin benzoate is known to persist in the sediment with a half-life of 164-175 days (table 2.1), as *L. celtica* is a filter feeder it may be exposed to suspended sediment that has bound to emamectin benzoate, the highest recorded sediment MEC for emamectin benzoate was 25.08 µg/kg in 2011 this was 223 meters away from the nearest farm (table 2.5; figure 2.15). The half-life for azamethiphos in water is 10.8 days (table 2.1); *L. celtica* could also be exposed to concentrations of azamethiphos. The highest water MEC data for azamethiphos, found by Langford (2015) was 26 ng/L, directly below a fish farm that had used azamethiphos the previous week (table 3.1). It is likely that *L. celtica* will be exposed to emamectin benzoate and azamethiphos at concentrations that could cause chronic effects, and will be particularly vulnerable due to the specific nature of these VMs to target invertebrates.

Dispersion tests using the dye rhodamine as a tracking tool show that azamethiphos and cypermethrin have the potential to travel up to 3km away from point source, within 5.5 hours of exposure (Ernst *et al.*, 2001). Cypermethrin is notoriously toxic to invertebrates at very low concentrations (table 2.2). Due to the high toxicity of cypermethrin, especially on crustaceans and molluscs it is likely that usage causes a toxic plume, of up to 2km, capable of mass mortality immediately following release. In total 139 farms used cypermethrin on 622 occasions, either as a single treatment or in combination with other treatments between 2007 and 2011 (cypermethrin was not used in 2012, see table 2.2). Overall cypermethrin use decreased from 308 applications in 2007 to just 14 applications in 2011 (figure 2.4 and 2.6a; appendix B).

2.4.2 Teflubenzuron Use

Teflubenzuron is a treatment that was used on a total of 16 farms, on 20 occasions between 2007 and 2011 (figure 2.4, 2.5b; figure 2.6). Although it was the treatment that was used the least frequently the total amount in kg used was the second highest of all treatments. Teflubenzuron is a chitinase inhibitor and is therefore only effective when applied to salmon infected with juvenile lice in the moulting stages (Burridge *et al.*, 2010; Tassou and Schulz, 2011). Controlling sea lice infestation with teflubenzuron requires careful timing since correct use of the treatment should break the lifecycle and prevent infestation for a whole year (SEPA, 1999). However, this is difficult to achieve as the moulting stage between juvenile and adult largely depends on the temperature of the water. It was suggested by SEPA (1999) that salmon farmers apply teflubenzuron between May and October. The data, however, indicates that teflubenzuron was applied in February, March, June and July in 2007; June and December 2009; December 2010; and February, May,

November and December 2011 (figure 2.3b). In the ten months following teflubenzuron treatment, all of the salmon farms investigated continued to use one or more of the other four treatments (figure 2.19). It is possible that the application of teflubenzuron was too late or too early. It is also is recommended that before applying teflubenzuron another treatment should be used directly before to first remove adult lice. This appears not to be the case, which could be the reason why most farms have used other treatments in the ten months after teflubenzuron (figure 2.19). Field trials by SEPA (1999), on 'worst case scenario' test of Calicide, a commercial teflubenzuron product, used 19.6 kg of product over seven days to treat 294.6 tonnes of salmon. It was found that there was an adverse effect on benthic fauna up to 50 m from cages but it was not thought to be detrimental to the species richness and diversity of the area, and it was concluded that after the 115 day half-life community structure would be rebuilt. The site SLO1, located in a Loch on the North West side of the Shetland Islands (UK NGR HU28508270) used a total of 75 kg of teflubenzuron as a sea lice treatment in December 2010 with a maximum allowed biomass of 1500 tonnes (figures 2.6b and 2.16a). In the SEPA (1999) report on teflubenzuron persistence, the field example that it was based on was one application of the VM, however there were three farms that used teflubenzuron in the ten months following the original application of teflubenzuron (see figure 2.19). These sites were BAD12 (UK NGR NC14834061), which used 8.8 kg of teflubenzuron of 6 months after the first application of 0.87 kg; GRA1 (UK NGR NB40501430), which used 7.7 kg of teflubenzuron 1 month after the first application of 6 kg; and KYL1 (UK NGR NB40501430), which used 40 kg of teflubenzuron 5 months after the first application of 22.1 kg. The calculation of the half-life of teflubenzuron did not take into account repeat applications. Considering the longevity of teflubenzuron in sediment, repeat applications should be taken into account as accumulation can occur which would in turn increase the sediment MEC (SEPA, 1999).

Metabolism of teflubenzuron by Atlantic salmon is low, with only around 10% of teflubenzuron absorbed by salmon, the remaining 90% enters the environment through excretion predominantly as parent compound (Jenkins, 1995; Ritchie, 1997; SEPA, 1999). According to preliminary studies by Samuelsen *et al.* (2014), faecal matter from *S. salar* showed concentrations of teflubenzuron that were 2 times that of the original administered dose. Teflubenzuron also is administered as an in feed treatment, meaning any uneaten food will enter directly into the environment, providing food for wild organisms in the surrounding area (Telfer *et al.,* 2006). Teflubenzuron adsorbs strongly to sediment as the log K_{ow} is high at 5.39 and its water solubility is low at 20 µg/l at 20°C (table 2.1). It is, therefore, likely to remain in the benthos, affecting benthic organisms such as crustaceans and other marine invertebrates. The EC_{50} for fecundity in *C. riparius* is 112.7 µg/kg (table 2.2), however teflubenzuron was measured in sediment at concentrations exceeding 112.7 µg/kg on two and three occasions in 2008 and 2012 respectively.

C. riparius is a sediment dwelling invertebrate which is found in rivers in the 1st instar larval stage. There is no ecotoxicology data for teflubenzuron available for marine species. It has been said that crustaceans are particularly sensitive to teflubenzuron during the moulting stages (SEPA, 1999). It is therefore likely that in the locations where teflubenzuron was measured above 112.7 µg/kg, in 2012 at concentrations as high as 698.33 µg/kg, there will be a detrimental effect on crustaceans. This may lead to a knock on effect on species dynamics through the trophic level in the areas affected (Jonsson *et al.*, 2006). Chronic ecotoxicology studies on the European lobster *Homarus gammarus* by Samuelsen *et al.* (2014) fed pellets containing 10 mg or 20 mg of teflubenzuron per kg bodyweight (bw) for seven days and with a rest period of three months showed that almost 50% of exposed juveniles experienced mortality or deformities including delay in moulting, stiff or twisted of joints due to over calcification, damage to tail fans and puffy or swollen carapace resulting in exposure of gills. The majority of mortalities were during the moulting stages, which was to be expected given the MOA of teflubenzuron (a chitinase inhibitor). Of the two doses administered during this study there was no significant difference in mortality between low and high dose (41% and 38% respectively). This study highlights that the recommended treatment dose of teflubenzuron is significant enough to cause detrimental effects on *H. gammarus* with the possibility that exposure to concentrations found in the sediment around Scotland of up to 698.33 µg/kg (see table 2.2) could pose the risk of significant alterations to populations of this and potentially other crustaceans exposed in the environment.

2.4.3 Sediment MECs of Teflubenzuron and Emamectin benzoate

GIS has been used to predict the distribution of particulate waste for individual farms in order to determine the settling of treatments and establish a tailored AZE; dependent on the strength and direction of currents (Pérez *et al.*, 2002). In general however, there are two AZE for aquaculture sites, a near field which is a 25 m buffer zone around the fish farm and a far field AZE which is a 100 m buffer zone around the fish farm. Each AZE has a different MAC depending on the treatment used. The near and far field MAC for emamectin benzoate is 7.63 µg/kg and 0.763 µg/kg respectively, while the near and far field MAC for teflubenzuron is 10 mg/kg and 2 µg/kg respectively (table 2.1). The majority of samples in the SEPA data set were taken more than 100 m away from the nearest fish farm that had last used either emamectin benzoate or teflubenzuron. There were 19 sites with detectable concentrations of teflubenzuron that were more than 2 km from the nearest farm that had last used this treatment, while 3 sites with a detectable concentration of emamectin benzoate were 2 km from the nearest farm that had used the treatment. In 2008 three samples had levels of teflubenzuron that ranged from 6.36 – 170.1 µg/kg, but the nearest farm that had used teflubenzuron was 71.1-94.8 km away from the three sites. These levels of teflubenzuron are consistent with samples that are within 1 km a farm that has used teflubenzuron in the past year (table 2.4) (SEPA, 1999). The most probable explanation

for such high concentrations of treatments occurring at long distances from nearest farm that has declared use, is that some fish farms have not been forthcoming in their use of teflubenzuron and emamectin benzoate, this information indicates that it is possible that the source of this pollution is from salmon farms that are closer to the sampling sites, rather than travelling from over 70 km away.

Both emamectin benzoate and teflubenzuron are used in terrestrial agriculture as pesticides, there is the possibility that the source of pollution is from terrestrial agriculture, especially as many of the MECs are coastal and near estuaries. Terrestrial sources of pollution from agriculture, transport and industry have previously been detected in marine sediments (Brodie *et al.*, 2012). Emamectin benzoate is approved as a plant protection product in the EU for peppers, cucumbers and melons (European Commission, 2013) and teflubenzuron is approved for use on tomato plants to control the whitefly *Trialeurodes vaporariorum* and the beet armyworm caterpillar *Spodoptera exigua* (European Commission, 2010). However, records on terrestrial use of teflubenzuron show that in Scotland over the years 2007-2010 teflubenzuron was used at a rate of <1 kg per year over a total of 15 ha (FERA, 2015). The exact location for the terrestrial use of teflubenzuron is not provided, however due to the low amount used as a plant protection product it is unlikely that sediments with a detectable level of teflubenzuron were polluted by terrestrial sources. No information was present for emamectin benzoate usage, however due to the nature of terrestrial use it is also likely to have been used in low quantities in terrestrial agriculture in Scotland. Given this it is unlikely that terrestrial sources contributed to sediment MECs of emamectin benzoate. All samples were taken within 1 km of an aquaculture site, there is the possibility that the source of pollution is coming from nearby salmon farms that have not declared their use of emamectin benzoate or teflubenzuron.

In-feed treatments generally persist in the environment more than in-bath treatments. It is estimated that 90% of feed will be eaten, while 10% will settle directly as uneaten feed (Hargrave, 1994), this uneaten feed as well as faeces settles directly below the cage and travels along the sea floor by saltation when currents reach a critical value (Rice *et al.*, 1996). This leaves benthic organisms vulnerable to exposure to these treatments not only if they are below the cages but potentially over some distance. This seems to be indicated by the SEPA data since the fish farms are often some distance from the sampling site but the treatments are still detectable (figures 2.9c and d; 2.10c and d; 2.12c and 2.16c). A reason for detection of teflubenzuron and emamectin benzoate in samples that were far away from using fish farms may be two fold. First the persistence of these treatments in the sediment might be longer than expected and secondly treatments are not bound to sediment in the immediate vicinity of the fish farm as quickly as thought (see table 2.1 for log K_{ow} relating to accumulation in sediment) but travel much further

distances in the water column than previously expected. Both teflubenzuron and emamectin benzoate are provided as in feed treatments. Information on the in bath treatments, deltamethrin and cypermethrin, were included in the SEPA report on sediment MECs, however all of the samples did not have detectable concentrations of these two pyrethroids. While cypermethrin and deltamethrin generally have a higher toxicity to the majority of tested species (table 2.2) they appear to be less persistent in the environment than their in feed counterparts. In feed treatments are not only risky to non-target organisms due to their presence in the sediment (figures 2.9 – 2.18), it is also known that organisms in the wild will travel in order to scavenge uneaten feed from aquaculture sites (Telfer *et al.*, 2006).

The half-life of teflubenzuron has been provided by SEPA as being 115 days, however other studies have stipulated that it may be as long as 170 days (Samuelsen *et al.*, 2015). Field studies undertaken in Loch Eil, Scotland (SEPA, 1999) (separate studies from the data analysed above) have shown that measureable concentrations of teflubenzuron have been found up to 1 km away in the direction of currents from treated fish farms. It was estimated that 98% of teflubenzuron was degraded or dispersed from the site of treatment after 645 days (SAMS, 2005; SEPA, 1999).

Teflubenzuron was detected at measurable concentrations in two sites (UK NGR NN019645; NN059705) in 2009 (figure 2.12). Teflubenzuron was not used in 2008 and in 2007 a total of 95.769 kg of teflubenzuron was used. Teflubenzuron use in 2007 was applied 8 times in 6 salmon farms in the North-West of Scotland, around Stornoway (table 2.2; figure 2.4). This is still over 160 km far away from the sites in Loch Leven, situated in a Loch in mid-West Scotland, which found teflubenzuron in detectable concentrations; it is highly unlikely that the farms around Stornoway are the source. The last known use of teflubenzuron within 10 km away from the two sites was at a salmon farm in Loch Leven, which used 16 kg in December 2002 (CALL1; UK NGR NN0805980). The two sediment samples had a concentration that was lower than the MAC at 0.53 and 0.31 µg/kg but nevertheless apparently detectable concentrations were found more than 6 years, almost 10 km away, after treatment on fish farms (Natural Scotland, 2015b). Field studies from Norwegian fish farms treated with teflubenzuron by Langford (2011) show that teflubenzuron was detected in sediment samples 'several months' (exact dates are not mentioned in the report) after application of either 80 kg or 225 kg to farms at concentrations of 7.2 – 66 µg/kg and 8.3 – 269.2 µg/kg respectively, these concentrations are similar to what has been presented in the current work (table 2.4). This suggests either that teflubenzuron is more persistent than other studies suggest (Samuelsen *et al.*, 2015; SEPA, 1999) or perhaps that the source of contamination is from farms that have not declared their use.

There are a number of farms using emamectin benzoate less than 2 km away from SPAs and SACs in the Argyll and Bute region, with some salmon farms using emamectin benzoate which are

directly located on SACs and SPAs (see figure 2.17c and section 2.4.1). Both teflubenzuron and emamectin benzoate also have the potential to travel long distances as they were detected in samples taken more than 20 km away (figure 2.9 and 2.11c). If this is the case, rather than the lack of declaration (as mentioned previously above) then it is more than likely that several SACs and SPAs will be exposed to sea lice treatments, with possible knock on effects on community structure and population dynamics.

A potential explanation of the distance travelled by teflubenzuron and emamectin benzoate could be the presence of phytoplankton in the water column. During spring and summer blooms higher than normal densities of phytoplankton may be exposed to and accumulate teflubenzuron. Phytoplankton have the potential to travel considerable distances in water currents, before dying and sinking to the benthos carrying any accumulated load of sea lice treatments. This could result in benthic organisms being exposed to elevated concentrations of teflubenzuron some distance from the treatment site (SAMS, 2005). However, further investigation is needed to determine whether this could be a contributing factor in the trace amounts of teflubenzuron in sediment at considerable distance form fish farms that were found in the monitoring data.

MEC samples were taken at different locations in the years 2008-2012. Monitoring of sediments to detect these contaminants is undertaken by SEPA and Marine Scotland Science (MSS). Under the WFD, there are currently no environmental quality standards (EQS) for sediment, in place of an EQS it has been suggested that the effects range low (ERL) take its place whilst sediment EQS is being adopted (Webster *et al.,* 2013). Despite this, monitoring of sediments appears to be in different locations each year (figures 2.7-2.18). A proposal by Webster *et al.* (2013) suggests that sites where sediments have concentrations above ERL monitoring should be annual. Webster *et al*. (2013) also suggested that in sites where sediments were found to have concentrations below the limit of detection (BDL) should occur every six years, and for sites that had concentrations above the BDL but below the ERL sampling should occur every three years. There was a total of 70 sites sampled for sediment MECs of teflubenzuron and emamectin benzoate, over the five year period 2008 - 2012 according to the proposed monitoring by Webster *et al.* (2013) 40 of these sites would need revisiting every year, with 16 requiring 3 yearly sampling and 14 sites only requiring 6 yearly sampling. Hopefully this will bring a more comprehensive assessment to the sediment quality of sites around salmon farms; however it is clear that this proposed sampling would put a lot more pressure on SEPA to increase sediment monitoring as more than half of the sites sampled had sediment MEC above the sediment MAC for emamectin benzoate and/or teflubenzuron. Additionally, more frequent sampling in the same sites could give insight into degradation times of both teflubenzuron and emamectin benzoate.

2.5 Conclusion

A number of sites where sediment MEC samples were taken and show a detectable concentration of either teflubenzuron or emamectin benzoate were >2 km from the nearest salmon farm that is known to have used either treatment in the previous year. The most likely explanation for this is that salmon farms declared use of treatments on a voluntary basis, some farms may have not been forthcoming with information or there are inaccuracies in the data that was provided by SEPA. Sediment MECs of teflubenzuron have been reported as being at concentrations that are higher than MACs and in some cases in concentrations high enough to cause lethal and chronic effects of *C. riparius* and *H. gammarus* (table 2.2 and 2.4) (Park and Kwak, 2012; Samuelsen *et al.*, 2014). With resistance of sea lice to treatments rising and the growth in salmon production in Scotland it is inevitable that reliance on treatments will also increase. All treatments were used on at least one occasion on SACs. Particularly vulnerable SACs are those containing reef habitats that support important and rare invertebrates. Monitoring of sediment MECs around fish farms is in need of being more consistent, as in the years 2008-2012 monitoring occurred at different sites each year, the new proposals for monitoring by Webster *et al.* (2013) should address this problem.

Chapter 3

Aliivibrio fischeri assay – a measure of acute toxicity and mixture effects

3.1 Introduction

Aliivibrio fischeri (previously classified as *Vibrio fischeri*) (Urbanczyk *et al.*, 2007) is a Gram-negative bioluminescent marine bacterium with a wide global distribution. It is a bacterium of ecological importance due to its symbiotic relationship with marine animals, including the highly reported and researched Hawaiian bobtail squid *Euprymna scolopes* (Ruby and McFall-Ngai, 1999; Soto *et al.*, 2012). In their free living state *A. fischeri* are not bioluminescent, it is only when they are found in high densities, such as within another organism, that they begin to emit luminescence through quorum sensing (James *et al.*, 2000). This luminescence can be triggered when grown in certain laboratory conditions and thus has resulted in *A. fischeri* being used as a model organism for a number of assays (Whitehead *et al.*, 2001).

One such assay is using *A. fischeri* as a bio-indicator for water quality (Costa *et al.*, 2015; Hernando *et al.*, 2007; Ma *et al.*, 2015; Parvez *et al.*, 2008; Villa *et al.*, 2012; Yan *et al.*, 2015). A standard protocol has been developed by the International Organisation for Standardization (ISO) in 1998 (ISO 11348). The assay has a wide scope and can be applied to field samples as well as laboratory samples. The *A. fischeri* assay can be conducted using freeze-dried bacteria; liquid dried bacteria or freshly prepared bacteria.

3.1.1 Luminescence in *A. fischeri* and application as a bioassay

Quorum sensing is an intercellular process in communities of bacteria involving auto induction of a particular gene. In Gram-negative bacteria it is most often regulated by *N*-acylated homoserine lactones (AHLs). AHLs are signalling molecules that assist in the communication of gene expression between bacterial cells. AHLs are produced within the cell and excreted into the environment to be picked up by neighbouring cells, so a higher number of cells results in a higher concentration of AHLs (Kuttler and Hense, 2008).

In *A. fischeri* luminescence, a quorum sensing event, is regulated by the *lux* genes, which are ordered into two operons, the left (O_L) and the right (O_R). On O_R is the *luxI* gene which produces the *A. fischeri* specific AHL, *N*-3-oxohexanoyl-L-homoserine lactone (OHHL). O_R also contains the genes *luxCDABEG* which are the genes responsible for encoding the luminescence enzymes (James *et al.*, 2000). O_L contains the *luxR* gene, which encodes the transcriptional activator protein, luxR, which when bound to OHHL binds to the *lux* box to transcribe *luxCDABEG* causing

subsequent elevation in bioluminescence and increased OHHL production (Whitehead *et al.*, 2001). The luxI protein produces the signal molecules OHHL, however in order for the OHHL transcription activator protein luxR to bind to the *lux* box a certain threshold must be reached, if that threshold is not reached then transcription of *luxCDABEG* is not operated and the cell is not luminescent (figure 3.1 a). When OHHLs are present in higher concentration from neighbouring cells, they can then bind to the luxR protein. LuxR in turn induces the *lux* box to transcribe the *luxCDABEG* operon, regulating bioluminescence. When the *lux* box is bound to luxR it also transcribes luxI which in turn produces more OHHLs which are transmitted into the surrounding environment further increasing the signal to surrounding molecules to produce more luminescence (figure 3.1 b).

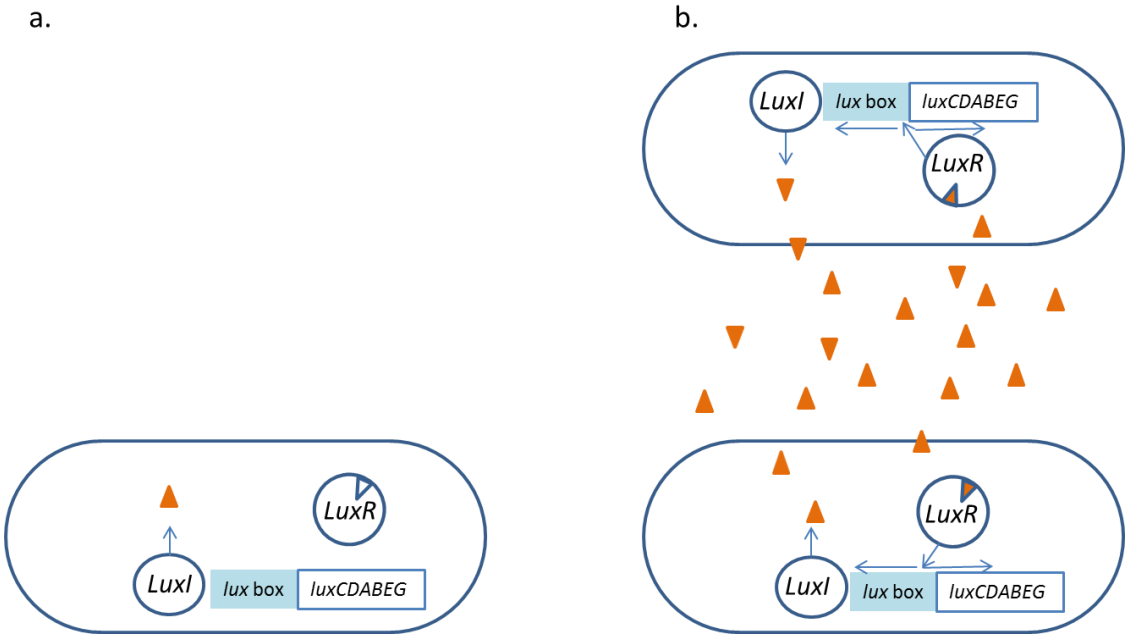


Figure 3.1 Quorum sensing in *A. fischeri* when found in low densities (a.) and in high densities (b.). Orange triangles represent OHHLs. Adapted from Whitehead *et al.* (2001).

Loss of luminescence occurs when cells die. Since high densities of active *A. fischeri* emit luminescence, and this luminescence decreases with numbers of active *A. fischeri* loss can be quantified using a luminometer (Whitehead *et al.*, 1979). Luminescence of *A. fischeri,* caused by quorum sensing, is therefore what makes *A. fischeri* an ideal organism for studying the acute effects of chemicals. The use of *A. fischeri* has been used as a bio-indicator for water health and quality (see figure 3.2 for example of *A. fischeri* bioluminescence).

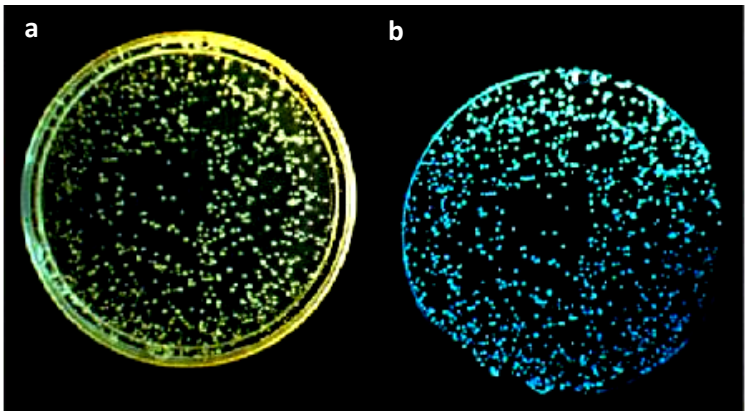


Figure 3.2 *A. fischeri* colonies grown on agar plates shown under (a) light and (b) in the dark *Source: (Pierson III et al., 2009)*

3.1.2 Predicting mixture effects

Ecotoxicology testing generally focuses on acute single compound testing. Realistically however, aquatic organisms are exposed to a cocktail of pollutants, which are released in pulses rather than uniformly as in laboratory controlled systems (Janssens and Stoks, 2012; Rasmussen *et al.*, 2013). Testing for the outcome of an infinite combination of mixtures has been a problem. An alternative to laboriously testing different combinations of mixtures in varying ratios is to use prediction models based on effects of single compound data. The two most commonly used models are concentration addition (CA) (equation 3.1), originally known as Loewe additivity (Loewe, 1953) and independent action (IA) (equation 3.2), otherwise known as Bliss independent action (Bliss, 1939; Villa *et al.*, 2012).

Equation 3.1 – CA

$$D_{mix} = \sum_{i=1}^{n} aD_i$$

Where D_{mix} is the predicted effect concentration of the mixture, and aD_i is the effect concentration of each individual compound (SCHER *et al.*, 2012).

Whereas the IA model assumes compounds have a dissimilar MOA and therefore act independently. The equation is as follows:

Equation 3.2 – IA

$$P_{mix} = 1 - (1 - P_1)(1 - P_2)(1 - P_3) \ldots . (1 - P_n)$$

Where P_{mix} is the total predicted effect from the mixture, and P_1, P_2 etc. is effect of each individual component of the mixture.

Both CA and IA assume that the chemicals act independently and therefore neither model accounts for chemicals that may have a synergistic effect or those that might have an antagonist effect. Cedergreen *et al.* (2008), compared the predictability of IA and CA for compounds with different MOAs on seven test systems including the water flea *D. magna,* the micro algae *P. subcapitata,* the bioluminescent bacteria *Aliivibrio fischeri* and the duckweed *L. minor.* Pooling data from various studies they were able to analyse the predictive value of these models for 98 chemical mixtures, over 158 data sets. They concluded that IA could successfully predict the outcome of 47% of the mixtures, where CA could predict 36%. It appears that these models are not sufficiently accurate to predict the effects of mixtures with differing MOAs. *In vitro* systems could provide a more comprehensive understanding of mixture toxicity.

The *A. fischeri* assay is a rapid way of determining the acute toxic effects of VMs on bacterial cells. Although environmental concentrations of the VMs tested are unlikely to reach acute levels, due to the immediate results obtained the *A. fischeri* assay has been deemed suitable to test for mixture toxicity. The acute test for toxicity on *A. fischeri* was decided to be the most efficient and cost effective assay to test for the reliability of the two prediction equations CA and IA.

3.1.3 Chapter objectives

In order to address objective 3 (chapter 1; section 1.9.3), the objectives of using the *A. fischeri* assay were:

- To determine the acute toxicity of the five VMs azamethiphos, cypermethrin, deltamethrin, emamectin benzoate and teflubenzuron used in UK aquaculture as a means of sea lice infestation removal.
- To compare acute toxicity of a common degradation product, 3PBA, to two of its parent compounds (cypermethrin and deltamethrin).
- To predict the effect of mixtures on acute toxicity using single compound data as a reference point using the CA and IA equations and compare to measured mixture effects.

3.2 Materials and Methods

3.2.1 Acute *Aliivibrio fischeri* assay for selected VMs and degradation products

Solutions were filter sterilised using a 0.22 µm filter (Merek Millipore, Darmstadt, Germany) or by autoclaving at 121°C for 15 minutes. A single colony of *A. fischeri* (NCIMB, Aberdeen, UK) was grown in oceanibulbus broth (see appendix D for components) at 20°C in a shaking incubator (150 rpm) for 24 hours until the optical density $(OD)_{600}$ was ~2.5. A 5 ml aliquot of this culture was inoculated in 50ml of oceanibulbus broth and grown at 20°C in a shaking incubator (150 rpm) for 24 hours until OD_{600} was ~2.5. Cells were harvested by centrifuging for 10 minutes at 3000 rpm at

15 °C and washed with 10ml filter sterilised 2% mass per volume (w/v) NaCl (Sigma-Aldrich, Poole, UK). The wash step was repeated once. Cells were then diluted in 2% (w/v) NaCl to an OD_{600} of ~5.

All test chemicals were purchased from Sigma-Aldrich (Poole, UK), unless otherwise stated. Cypermethrin, deltamethrin, azamethiphos, teflubenzuron, emamectin benzoate and the 3-phenoxybenzoaic acid (3PBA) were first dissolved in dimethyl sulfoxide (DMSO) (Fisher, Loughborough, UK) to a concentration of 20 mg/ml apart from deltamethrin which was dissolved to a concentration of 10 mg/ml. Test chemicals were then serially diluted in 2% (w/v) NaCl to a concentration ranging 400 – 0.1 mg/ml.

A positive control standard curve of phenol was performed with each assay at a concentration of 50 - 1 mg/ml to ensure validity of *A. fischeri* cells. Phenol controls were treated in the same way as test VMs. DMSO, concentration in wells never exceeded 2%; controls also contained 2% DMSO.

The test VM was added to each well, of a white opaque 96-well plate (ThermoScientific, Loughborough, UK) at a volume of 150 µl, along with 150 µl of *A. fischeri* to give a final OD_{600} of 2.5 in each well. Plates were incubated at 15 °C for 15 minutes. Blanks contained 294 µl 2% (w/v) NaCl and 6 µl DMSO; controls contained 150 µl *A. fischeri* 144 µl of 2% (w/v) NaCl and 6 µl DMSO. Luminescence was measured using a plate reader (FLUOstar OPTIMA, BMG LABTECH, Germany) using a transparent lens with a gain of 4095 across a wavelength range of 230 – 900 nm. Luminescence was measured in relative light units (RLU) minus the blank measurement and presented as a percentage to the DMSO control. The experiment was repeated three times. Results were given as relative light units (RLU), and expressed as a percentage from the mean using equation 3.3.

Equation 3.3

$$X = (T \div C) \times 100$$

Where X is the percentage from control, T is the measured value in RLU and C is the control value in RLU.

3.2.2 Prediction of mixture toxicity

VMs were initially tested individually, once the results had been analysed by determining the EC_{50} using sigmoidal dose response curves (see section 3.2.4) mixtures of chemicals were tested. Three compounds were used for the mixture toxicity tests. These were 3PBA, emamectin benzoate and azamethiphos. This was because an EC_{50} for cypermethrin, deltamethrin and teflubenzuron could not be calculated for *A. fischeri* (see table 3.1).

Four mixtures were investigated. Mixture A consisted of 1:1 ratio of azamethiphos and 3PBA at a concentration range of 0.05 - 200 mg/L of each substance. Mixture B consisted of equal parts of azamethiphos and emamectin benzoate at a concentration range of 0.05 - 200 mg/L of each VM. Mixture C consisted of equal parts of 3PBA and emamectin benzoate at a concentration range of 0.05 - 200 mg/L of each substance. Mixture D consisted of equal parts of azamethiphos, 3PBA and emamectin benzoate at a concentration range of 0.05 - 100 mg/L. Equations 3.1 and 3.2 (section 3.1.2) were used to determine whether the toxicity of single VMs could be used to make predictions on mixture effects. The effectiveness of CA and IA were compared to measured effects of mixtures using statistical analysis (section 3.2.4).

3.2.3 Determination of *A. fischeri* sensitivity to emamectin benzoate using plate counts

Due to elevated bioluminescence in the previous assay, plate counts of *A. fischeri* exposed to different concentrations of emamectin benzoate were carried out. *A. fischeri* was cultured and washed in 2% (w/v) NaCl as described previously (section 3.2.2). To each 90 mm petri dish (Sterilin Ltd, Newport, UK) 20 ml of spiked Oceanobulbus agar (see appendix D for constituents) was added, with concentrations of emamectin benzoate between 0.1 - 400 mg/L. Phenol was used as a positive control at a concentration of 0.1 - 50 mg/L. Filter sterilised emamecin benzoate and phenol were added to agar after autoclaving and immediately before pouring into plate (maximum temperature 45°C). Negative controls contained only 20 ml of Oceanobulbus agar. Petri dishes were inoculated with 100 µl of culture, which was spread with a sterile glass plate spreader, at a cell density of 1500 cells/ml. Petri dishes were incubated at 16 °C for 72 hours after which colony counts were performed. Results were expressed as cells/ml. Each concentration had a replication of three. The experiment was repeated twice.

3.2.4 Statistical methods

GraphPad (GraphPad Prism, 2010) was used to create sigmoidal dose response curves, from which the EC_{50} of each compound and each mixture could be determined. Predicted mixture curves were compared using nonlinear regression. One-way analysis of variance (ANOVA) with Tukey's post hoc test was performed for each concentration of each test chemical to determine significant differences ($P \leq 0.05$) from the control (Tukey, 1949). Predictions on mixture toxicity were made using both the CA (equation 3.1) and IA (equation 3.2) models. Pearson's correlation was used to determine the accuracy of CA and IA.

3.3 Results

3.3.1 Single compound toxicity to *A. fischeri* and calculated EC_{50}

Six compounds were analysed for single compound acute toxicity. These were 3PBA, azamethiphos, cypermethrin, deltamethrin, emamectin benzoate and teflubenzuron. Concentration ranged between 0.1 - 400 mg/L for all VMs except deltamethrin which ranged between 0.2 – 200 mg/L (due to the low solubility of deltamethrin in DMSO). Cypermethrin, deltamethrin and teflubenzuron appeared to have no effect on the bioluminescence on *A. fischeri* even at the highest concentration of 400 mg/L for cypermethrin and teflubenzuron, and 200 mg/L for deltamethrin (figure 3.5). Therefore these compounds were not included when investigating mixtures. At higher concentrations, 200 – 400 mg/ml, emamectin benzoate showed a significant increase in bioluminescence ($P < 0.05$) when compared with DMSO controls (section 3.3.2; figure 3.7). Lower concentrations of emamectin benzoate, 0.5 - 150 mg/ml, showed no difference in luminescence when compared with the control (section 3.3.2; figure 3.7). The EC_{50} for each tested VM was calculated and compared with reported MECs (table 3.1). Azamethiphos and 3PBA caused sufficient loss of luminescence at the concentrations tested and therefore a EC_{50} was able to be calculated for these two VMs. Cypermethrin, deltamethrin, emamectin benzoate and teflubenzuron did not cause more than 50% cell death at the highest concentrations and so an EC_{50} was not calculated for these VMs. Rather than causing a reduction in luminescence emamectin benzoate appeared to encourage luminescence (section 3.3.2; figure 3.7). As some of the tested VMs caused an increase in luminescence from the control, some dose response curves start below 0.

Table 3.1 Comparison of EC_{50} mg/L for the six compounds for *A. fischeri* and the highest reported aquatic MEC.

Compound	***A. fischeri* EC_{50} mg/L (this study)**	**Highest reported aquatic MEC**	**Reference (for aquatic MEC)**
Azamethiphos	41.25 ± 0.04	26 ng/L (Norway)	(Langford, 2015)
Cypermethrin	>400	85.1 µg/L (UK)	(Boxall *et al.*, 2002)
Deltamethrin	>200	n.d	n.d
Emamectin benzoate	>400	25.08 µg/kg (sediment)	(Natural Scotland 2015b)
Teflubenzuron	>400	12.9 ng/L (Norway)	(Langford *et al.*, 2014)
3PBA	281 ± 0.02	n.d	n.d
Phenol (positive control)	17.38 ± 0.03	N/A	N/A

n.d = no data. N/A = not applicable

Azamethiphos and 3PBA had an acute toxic effect on *A. fischeri.* These were the only compounds where an EC_{50} could be calculated. Azamethiphos produced an EC_{50} of 41.25 mg/L (figure 3.3). One-way ANOVA with Tukey's post hoc indicates that a significant increase in *A. fischeri* cell inhibition by azamethiphos begins at 10 mg/L ($P < 0.001$) and cell inhibition is significantly increased right through to 400 mg/L ($P < 0.001$).

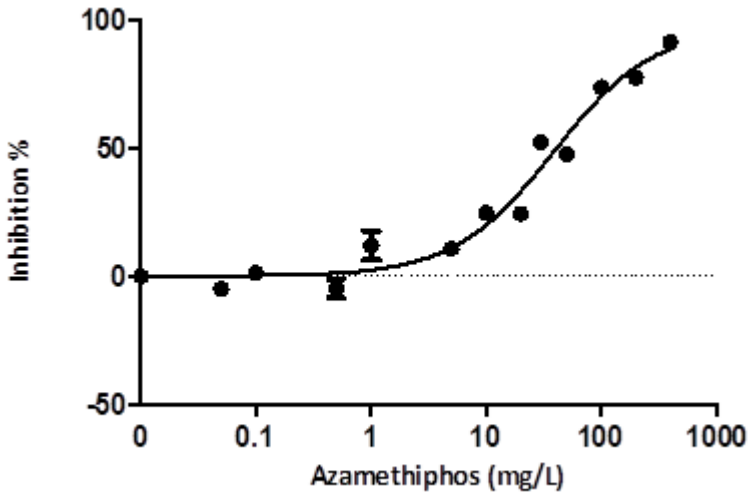


Figure 3.3 The acute toxicity of azamethiphos on *A. fischeri*, after 30 minutes incubation (15 °C), shown as percentage of cell death against the control (% inhibition). Bars show standard error of the mean (n = 3).

The degradation product 3PBA caused greater toxicity to *A. fischeri* than its parent compounds cypermethrin and deltamethrin (figures 3.4 and 3.5). The EC_{50} for 3PBA was 281 mg/L whereas the EC_{50} for cypermethrin and deltamethrin could not be determined since the highest concentration

for both VMs did not cause 50% of inhibition. One-way ANOVA with Tukey's post hoc indicates that a significant increase in *A. fischeri* cell inhibition by 3PBA begins at 150 mg/L (P = 0.008) and cell inhibition is significantly increased right through to 400 mg/L (P <0.001). Bioluminescence was significantly higher in *A. fischeri* exposed to 10 mg/L of 3PBA than *A. fischeri* control (P = 0.01).

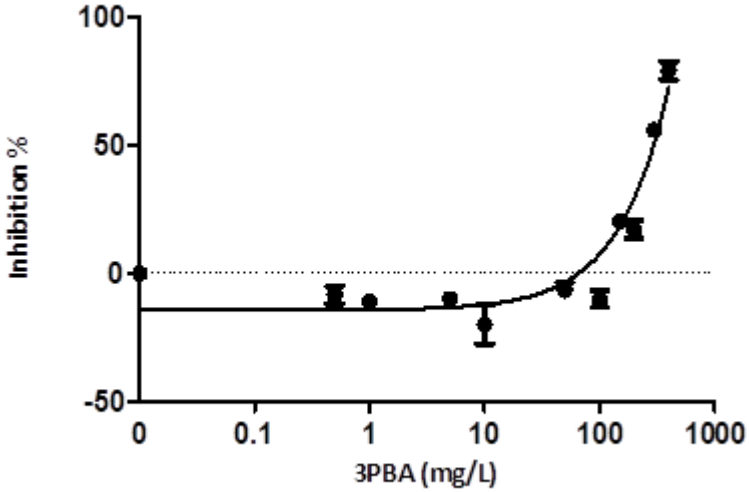


Figure 3.4 The acute toxicity of 3PBA on *A. fischeri*, after 30 minutes incubation (15 °C), shown as a percentage of cell death from the control (% inhibition). Bars show standard error (n = 3).

Cypermethrin, deltamethrin and teflubenzuron did not cause significantly higher cell inhibition on *A. fischeri* than controls apart from teflubenzuron at a concentration of 0.4 mg/L which caused an average of 38.8 (± 0.93) cell inhibition.

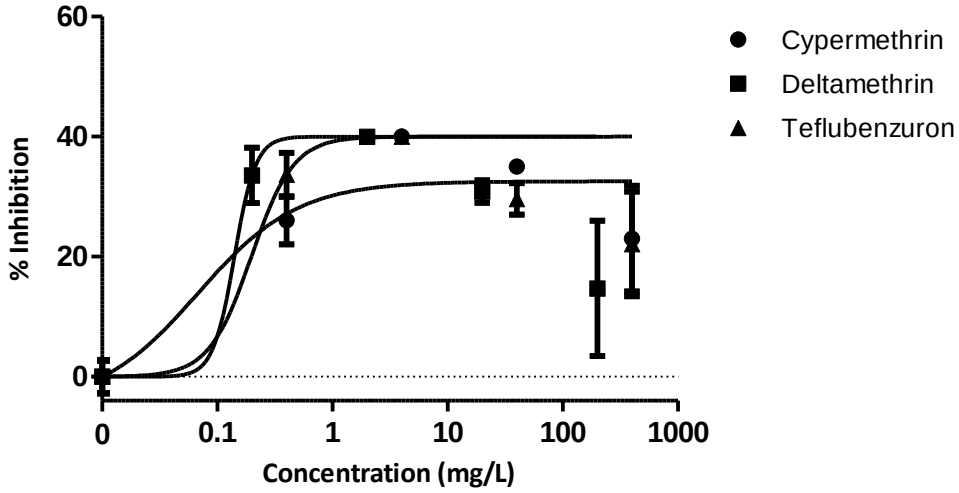


Figure 3.5 Dose response curves for cypermethrin, deltamethrin and teflubenzuron on *A fischeri*, after 30 minutes incubation (15 °C), shown as percentage cell death. Bars show standard error (n = 3).

Phenol was used as a positive control, phenol has a reported EC_{50} to *A. fischeri* of 22 – 40.2 mg/L(Pintar *et al.*, 2008). In this case phenol produced an EC_{50} of 17.38 mg/L (figure 3.6).

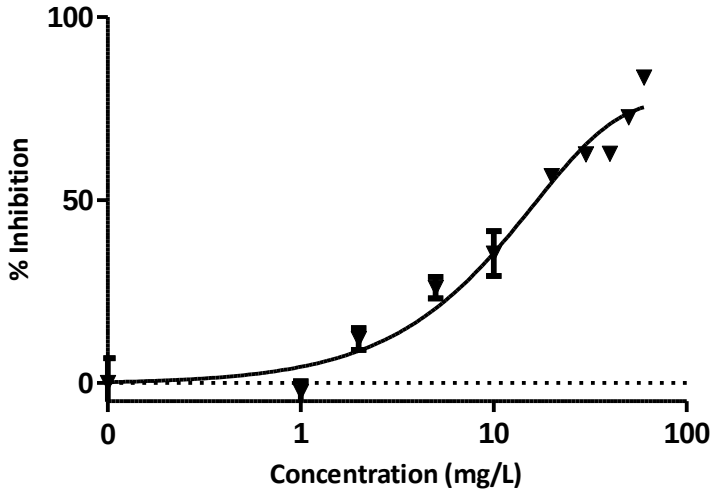


Figure 3.6 Dose response curves for the positive control phenol on *A fischeri*, after 30 minutes incubation (15 °C), shown as percentage cell death. Bars show standard error (n = 3).

3.3.2 Emamectin benzoate increases in luminescence

A. fischeri was exposed to emamectin benzoate in concentrations between 0.5 mg/L – 400 mg/L. When exposed to 200 mg/L, 300 mg/L and 400 mg/L *A. fischeri* showed a significantly higher expression of bioluminescence than the control (figure 3.7). For luminescence to exceed the control is unexpected as luminescence is associated with growth. If emamectin benzoate had no effect on luminescence then results should not have significantly increased compared with the control.

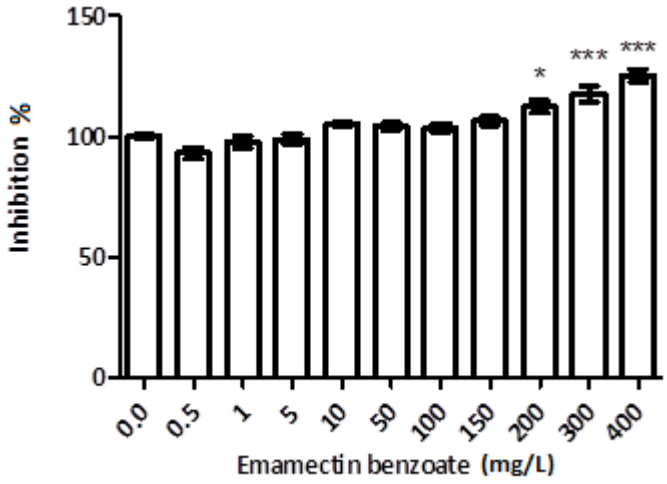


Figure 3.7 *A. fischeri* cell inhibition when exposed to concentrations of emamectin benzoate (0.5 - 400 mg/L) after 30 minutes incubation (15 °C), shown as percentage cell death. Bars show standard error of the mean (n = 3). Values were tested for significance from the mean using one-way ANOVA * = P <0.05 *** = P <0.0001.

Due to this unexpected increase in bioluminescence of *A. fischeri* when exposed to emamectin benzoate, the possibility of chemiluminescence was investigated. A 96 well plate without *A. fischeri* was prepared at all concentrations of emamectin benzoate dissolved in DMSO and diluted in 2% (w/v) NaCl. The outcome of this test was that emamectin benzoate does not exhibit any chemiluminescent properties.

As it was discovered that emamectin benzoate is not chemiluminescent, plate counts were carried out in order to establish whether the increase in bioluminescence of *A. fischeri* in the original assay was as a result of enhanced growth due to the addition of emamectin benzoate.

Due to the unexpected increase in bioluminescence of *A. fischeri* when exposed to emamectin benzoate, plate counts were carried out in order to establish actual cell number and whether there was a negative growth response of *A. fischeri* when exposed to different concentrations of emamectin benzoate. Despite an increase in bioluminescence of *A. fischeri* when exposed to concentrations of emamectin benzoate between 200 – 400 mg/L in the original assay (figure 3.7), this was not reflected in the plate count assays. Agar plates containing 400 mg/L, 200 mg/L and 100 mg/L, *A. fischeri* showed a significant decrease in the number of colonies grown compared to the negative DMSO control. Exposure to 50 mg/L and 1 mg/L emamectin benzoate cell numbers were not significantly different from the control (figure 3.8).

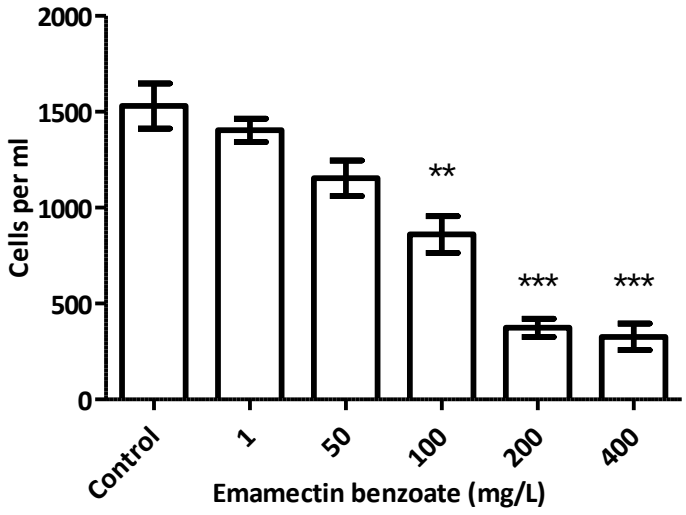


Figure 3.8 Plate counts of *A. fischeri* exposed to emamectin benzoate. Cells/ml when exposed to concentrations of emamectin benzoate ranging between 400 – 1 mg/L, incubation was 72 hours at 15 °C. DMSO was used as positive control and phenol as negative control. Bars show standard error (n = 3). Values were compared to the mean to test for significance using one-way ANOVA ** = <0.01 significance from control; *** <0.001 significance from control.

3.3.3 Mixture effects on *A. fischeri* and comparison with prediction equations

Four mixtures were investigated, these were mixture A which was equal parts 3PBA and azamethiphos (figure 3.9), mixture B which was equal parts azamethiphos and emamectin benzoate (figure 3.10), mixture C which was equal parts 3PBA and emamectin benzoate (figure 3.11) and mixture D which was equal parts 3PBA, azamethiphos and emamectin benzoate (figure 3.12). Measured toxicity of the four mixtures was compared with predicted toxicity using CA (equation 3.1) and IA (equation 3.2). IA estimates gave lower EC_{50} results than observed findings for all four mixtures (figures 3.9 – 3.12; table 3.2). CA estimates were higher than measured EC_{50} results for mixture B (figure 3.10) and mixture D (figure 3.12). An EC_{50} was not calculated for mixture C as the given range estimated by CA did not reach the 50% mark.

A. fischeri was exposed to mixture A, which was a 1:1 ratio of azamethiphos and 3PBA, at total concentrations of 0.1 – 400 mg/L (0.05 – 200 mg/L of each VM). Results from a one-way ANOVA with Tukey's post-hoc test shows that a significant increase in *A. fischeri* cell inhibition from the control started at 60 mg/L ($P < 0.001$) total concentration through to 400 mg/L total concentration of mixture A ($P < 0.001$) (figure 3.9). One way ANOVA of curves shows there is a significant difference between measured curve and the predicted curves calculated using CA (equation 3.1) and IA (equation 3.20) ($P < 0.0001$).

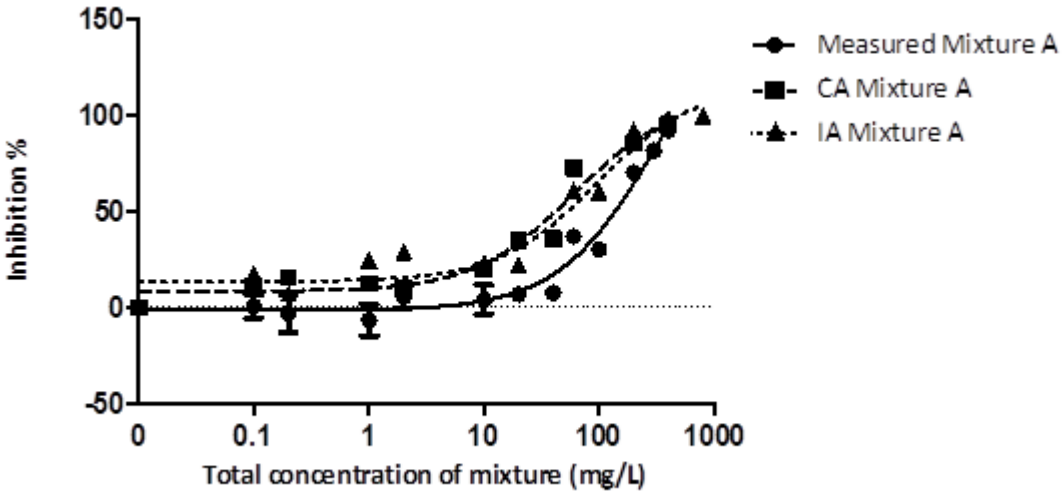


Figure 3.9 Acute toxicity of mixture A (1:1 ratio of azamethiphos and 3PBA; 0.1 – 400 mg/L total concentration) on *A. fischeri,* after 30 minutes incubation (15 °C), compared with the predicted standard curves of mixture A using CA (equation 3.1) and IA (equation 3.2). Cell death was measured by percentage of death when compared to control. Bars show standard error (n=3) (IA and CA were calculated so error is not shown).

A. fischeri was exposed to mixture B, which was a 1:1 ratio of azamethiphos and emamectin benzoate, at total concentrations of 0.1 – 400 mg/L (0.05 – 200 mg/L of each VM). Results from a one-way ANOVA with Tukey's post-hoc test shows that a significant increase in *A. fischeri* cell inhibition from the control started at 100 mg/L (P = 0.005) total concentration through to 400 mg/L total concentration of mixture B (P <0.001). Bioluminescence was significantly higher in *A. fischeri* exposed to mixture B beginning at a concentration of 0.1 mg/L (P <0.001) through to 20 mg/L (P <0.001) (figure 3.10). One way ANOVA of curves shows there is a significant difference between measured curve and the predicted curves calculated using CA (equation 3.1) and IA (equation 3.21) (P <0.0001).

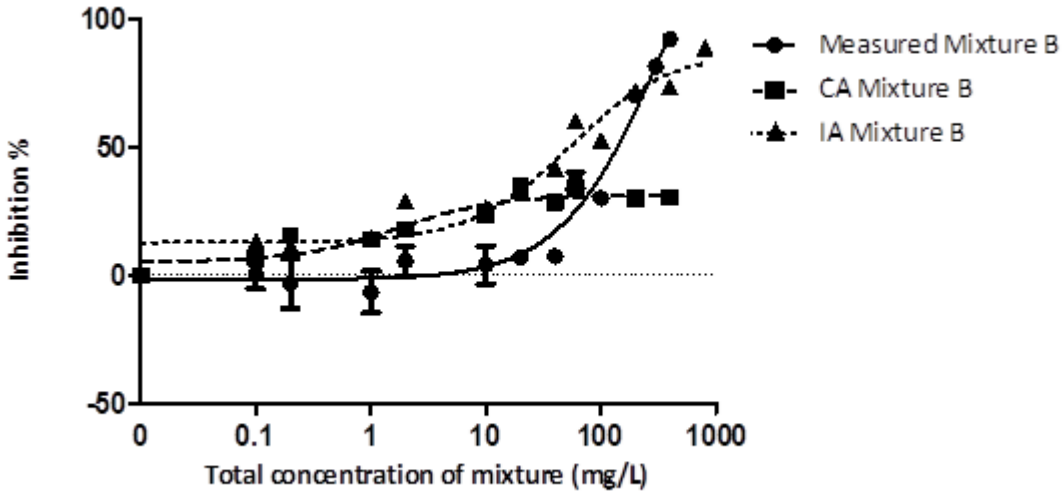


Figure 3.10 Standard curves showing the acute toxicity of mixture B (1:1 ratio of azamethiphos and emamectin benzoate; 0.1- 400 mg/L total concentration) on *A. fischeri,* after 30 minutes incubation (15 °C), compared with the predicted standard curve of mixture B using CA (equation 3.1) and IA (equation 3.2). Cell death was measured by percentage of death when compared to control. Bars show standard error (n = 3) (IA and CA were calculated so error is not shown).

A. fischeri was exposed to mixture C, which was a 1:1 ratio of 3PBA and emamectin benzoate, at total concentrations of 0.1 – 400 mg/L (0.05 – 200 mg/L of each VM). Results from a one-way ANOVA with Tukey's post-hoc test shows that a significant increase in *A. fischeri* cell inhibition from the control started at 200 mg/L (P <0.001) total concentration through to 400 mg/L total concentration of mixture C (P <0.001). Bioluminescence was significantly higher in *A. fischeri* exposed to mixture C beginning at a concentration of 0.1 mg/L (P <0.001) through to 40 mg/L (P <0.001) (figure 3.11).

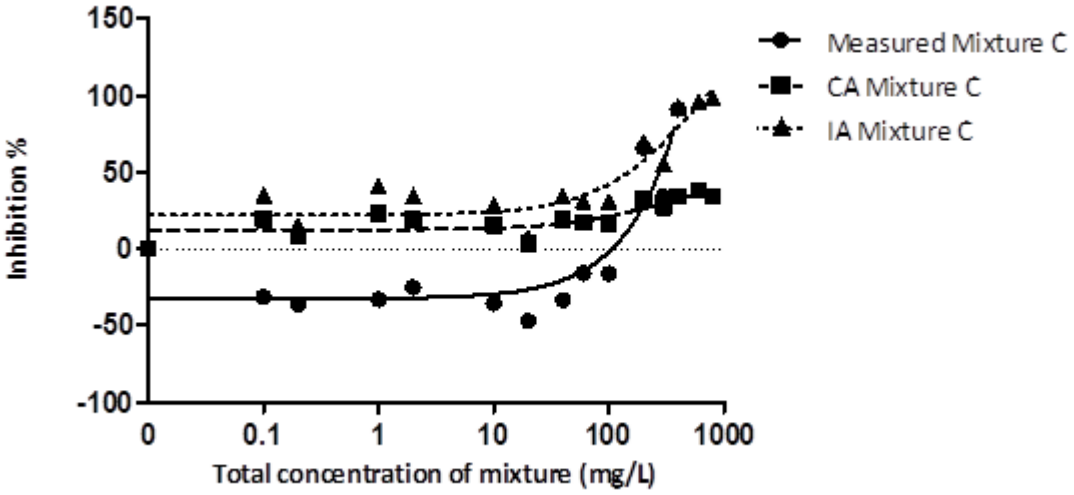


Figure 3.11 Standard curves showing acute toxicity of mixture C (1:1 ratio of 3PBA and emamectin benzoate) on *A. fischeri,* after 30 minutes incubation (15 °C), compared with the standard curve of the predicted mixture C using CA (equation 3.1) and IA (equation 3.2). Bars show standard error (n = 3) (CA and IA were calculated so error is not shown).

A. fischeri was exposed to mixture D, which was a 1:1:1 ratio of azamethiphos, 3PBA and emamectin benzoate, at total concentrations of 0.15 – 300 mg/L (100 –0.05 mg/L of each VM). Results from a one-way ANOVA with Tukey's post-hoc test shows that a significant increase in *A. fischeri* cell inhibition from the control started at 60 mg/L ($P < 0.001$) total concentration through to 300 mg/L total concentration of mixture D ($P < 0.001$). Bioluminescence was significantly higher in *A. fischeri* exposed to mixture D beginning at a concentration of 0.15 mg/L ($P = 0.001$) through to 3 mg/L ($P = 0.008$) (figure 3.12). One way ANOVA of curves shows there is a significant difference between measured curve and the predicted curves calculated using CA (equation 3.1) and IA (equation 3.20) ($P < 0.0001$).

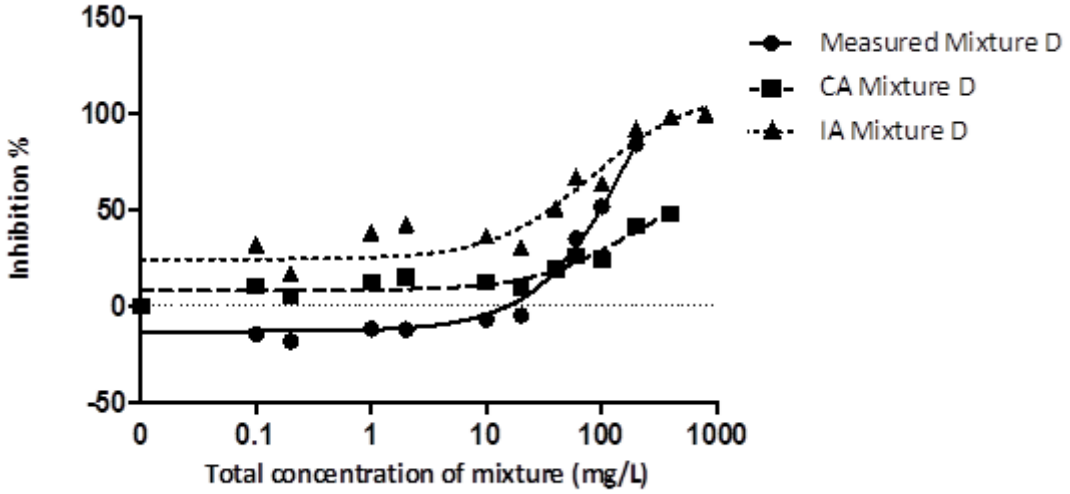


Figure 3.12 Standard curves showing acute toxicity of mixture D (1:1:1 ratio of 3PBA, azamethiphos and emamectin benzoate) on *A. fischeri,* after 30 minutes incubation (15 °C), compared with the standard curve of mixture D prediction using CA (equation 3.1) and IA (equation 3.2). Bars show standard error (n = 3) (IA and CA were calculated so error is not shown).

An EC_{50} value for each of the mixtures was calculated, as were the predicted EC_{50} values using the equations CA (equation 3.1) and IA (equation 3.2) (table 3.2).

Table 3.2 EC_{50} calculations for actual measured mixtures (A-D) and for predictions IA (A-D) and CA (A-D). The value for mixture C CA EC_{50} (mg/L) was unable to be calculated as predicted inhibition using this model did not reach 50%.

Mixture name	**Actual EC_{50} (mg/L)**	**IA EC_{50} (mg/L)**	**CA EC_{50} (mg/L)**
Mixture A	61.82 ± 0.03	22.36	27.88
Mixture B	130.1 ± 1.02	22.22	2736
Mixture C	123.5 ± 1.16	34.93	Not calculated
Mixture D	44.93 ± 0.34	5.345	97.18

Measured inhibition and predictions using CA (equation 3.1) and IA (equation 3.2) of all four mixture combinations were pooled. Statistical analysis using linear regression and Pearson's correlation was performed using GraphPad Prism to determine the reliability of each prediction model. Both prediction models were significantly correlated to actual measured effect, however IA (figure 3.13) had a stronger correlation with actual measured effect than CA (figure 3.14).

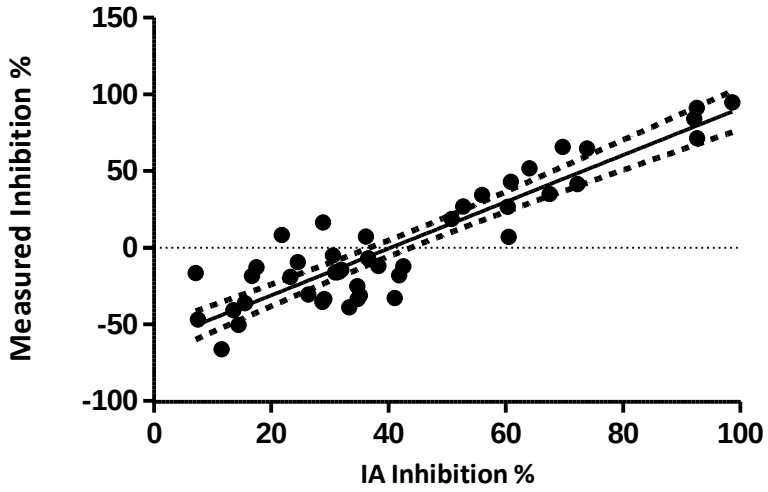


Figure 3.13 Pearson's correlation between measured values of mixtures A-D and predicted values using IA (equation 3.2) Linear regression (continuous line) with 95% confidence intervals (dashed lines) $R^2 = 0.8257$; $P < 0.001$.

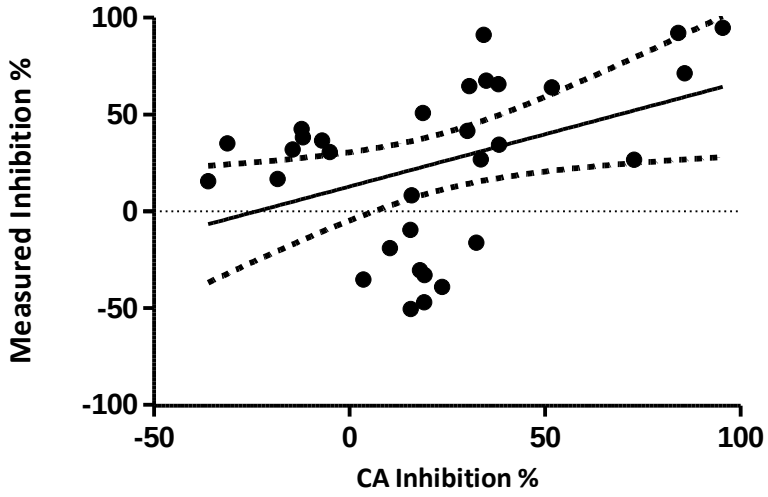


Figure 3.14 Pearson's correlation between measured values of mixtures A-D and predicted values (n = 31) of mixtures using CA. Linear regression (continuous line) with 95% confidence intervals (dashed lines). $R^2 = 0.1691$; $P = 0.0215$.

3.4 Discussion

3.4.1 Single compound toxicity

Of the six compounds tested, azamethiphos was the most potent giving an EC_{50} of 41.25 mg/L, 3PBA had an EC_{50} of 281 mg/L the EC_{50} for emamectin benzoate, deltamethrin, cypermethrin and teflubenzuron could not be calculated as at the highest concentration (400 mg/L, 200 mg/L for deltamethrin) cell inhibition was lower than 50%. Ernst *et al.* (2001) found when using the commercial *A. fischeri* kit Microtox the EC_{50} for azamethiphos was 11 mg/L. When comparing ecotoxicology data of the test compounds, *A. fischeri* appears to be less sensitive than other test species (table 3.3).

Table 3.3 Available EC_{50} or LC_{50} data on *D. magna, O. mykiss* and various algal species on the six test VMs along with the EC_{50} results from this study.

Compound	***D. magna* EC_{50} (mg/L)**	***O. mykiss* LC_{50} (mg/L)**	**Algal species EC_{50} (mg/L)**	***A. fischeri* EC_{50} (mg/L) (This study)**
Azamethiphos	0.00067	>0.115	-	41.25 ± 0.04
Cypermethrin	0.0003	0.0028	>0.01 (*P. subcapita*)	>400
Deltamethrin	0.00056	0.00056	-	>200
Emamectin benzoate	0.001	0.174	0.0072 (*P. subcapita*)	>400
Teflubenzuron	0.0028	0.0186	>0.02 (*S. subipicatus*)	>400
3PBA	0.05	>99	0.10 (*Adetus inaequalis*)	281 ± 0.02

Source: VSDB

Cypermethrin and deltamethrin had no significant effect on *A. fischeri* at the concentrations tested (0.4 – 400 mg/L for cypermethrin and 0.2 - 200 mg/L for deltamethrin). Teflubenzuron caused significant cell inhibition at the lowest concentration, 0.4 mg/L but there was no difference at higher concentrations (see figure 3.5). When testing organic chemicals used in aquaculture on *A. fischeri*, Hernando *et al.* (2007) found that the EC_{50} for deltamethrin was >39.9 mg/L, but there was 30% inhibition at 5 mg/L. This corresponds with other literature as Ernst *et al.* (2001) found the EC_{50} for cypermethrin was >4.95 mg/L. Hernando *et al.* (2007) found that emamectin benzoate had no effect on *A. fischeri* at concentrations up to 6.3 mg/L. Information on the ecotoxicity of 3PBA and teflubenzuron on *A. fischeri* is not available. From the plate counting assay a concentration of 100 mg/L of emamectin benzoate caused a significant decrease in cell viability, however these concentrations are much higher than reported sediment MECs presented in chapter 2 (section 2.3.2; table 2.4 and 2.5) and therefore it is unlikely that emamectin benzoate would cause adverse effects on *A. fischeri* in the environment.

While the acute toxicity for cypermethrin and deltamethrin was too high to obtain an EC_{50} measurement, there was a toxic effect on *A. fischeri* from their degradation product 3PBA (figure 3.3; table 3.1). 3PBA gave an EC_{50} of 281 mg/L, where the EC_{50} for cypermethrin and deltamethrin were >400 mg/L and >200 mg/L respectively. VMs need to pass ecotoxicology testing before they are put on the market. In the EU, VMs are first subject to tier A testing to determine the predicted environmental concentration (PEC) of a parent substance (see chapter 1; section 1.4 for information on testing of VMs). If the resulting risk quotient (RQ) is $\geq$1 then PEC is refined to include information on degradation products. If a degradation product exceeds 10% of the total dose then the degradation product will go through phase II testing alongside the parent compound (Directive 91/414/EEC) (see chapter 1, section 1.4 for more about phase II testing). Cypermethrin and deltamethrin are highly hydrophobic and degradation in water is rapid (Crane, 2007). One of the major degradation products of synthetic pyrethroids, 3PBA, is formed by hydrolysis, photolysis, microbial degradation and animal metabolism which when metabolised forms approximately 15% of cypermethrin (Jones, 1995).

The acute toxicity results obtained from the *A. fischeri* assay show that none of the VMs, and degradation products, tested would cause an acute toxicity effect at environmentally relevant concentrations, as the results shown here are much higher than the sediment MECs presented in the previous chapter (see chapter 2; section 2.3.2; tables 2.4 and 2.5).

3.4.2 Mixture toxicity

IA and CA were calculated for the prediction of mixtures A-D on *A. fischeri*. Dose response curves and EC_{50} were calculated for predictions and for experimental measurements (figures 3.9 – 3.12). Overall IA appeared to be a more effective model for the prediction of effects of VM mixtures (figures 3.12 and 3.13). Villa *et al.* (2012) compared the robustness of both IA and CA against a number of complex mixtures of chemicals to determine which model is a more suitable predictor. They found that both the IA and CA models were effective at predicting acute mixture effects, of compounds with differing mode of action (MOAs), on *A. fischeri.* In their case, the CA model provided a better indicator than IA on the dose response of the tested mixtures.

IA overestimated the toxicity for all four mixtures, by giving a lower EC_{50} value while CA gave more varied results, with underestimation of toxicity for mixtures B and D, and an overestimation of toxicity for mixture A. Using linear regression IA proved to be an effective tool for the prediction of mixture toxicity on *A. fischeri*, giving a prediction accuracy of 82.6% and a significant correlation ($P < 0.001$) (figure 3.13). While linear regression of CA against actual inhibition of mixtures showed a significant positive correlation ($P = 0.0215$), prediction accuracy was substantially lower than that of IA, at 22.6% (figure 3.14). CA is generally considered a model for compounds with a similar MOA, in this case IA proved to be a better indicator for the prediction

of mixture toxicity since the MOA is different for all of the VMs tested (apart from cypermethrin and deltamethrin) (see table 2.1; chapter 2). Cedergreen *et al.* (2008) reviewed the effectiveness of IA and CA. They pooled information on the ability of each model to predict the toxicity of a 98 different mixtures tested against seven test systems from a number of published studies; they found that IA was slightly more effective overall for predicting toxicity than CA (47% and 36% effective prediction respectively).

3.4.3 Exposure to emamectin benzoate and increases in bioluminescence of *A. fischeri*

While there was no effect on *A. fischeri* at concentrations up to 150 mg/L, emamectin benzoate appeared to increase bioluminescence of *A. fischeri* at higher concentrations, with 200 mg/l, 300 mg/L and 400 mg/L having a significantly higher luminescence than the DMSO control (figure 3.7). While emamectin benzoate showed an increase in bioluminescence for the 30 minute *A. fischeri* inhibition assay, plate counts showed an opposite response and inhibited growth at 100 mg/L, 200 mg/L and 400 mg/L (figure 3.8). Some chemicals are known to exhibit chemiluminescent properties, such as luminol a chemical which emits a blue light when it comes into contact with blood (Barni *et al.*, 2007). By testing emamectin benzoate for luminescence without the addition of *A. fischeri,* it was not found to exhibit any chemiluminescence. A potential explanation for the increased luminescence, despite a toxic effect on cells, could be an interaction between emamectin benzoate and the organic solvent all of the substances were dissolved in, DMSO. Organic solvents, such as DMSO, are known to activate luminescence at low concentrations and inhibit luminescence at higher concentrations (Sukovataya and Tyulkova, 2001). To correct for this, DMSO was also added to the control at 2% (the highest concentration). There have been a few other reports of chemical enhancement of bioluminescence on *A. fischeri.* There are reports that interactions between organic solvents and heavy metals, which have been demonstrated to result in an even higher activation of luminescence than an organic solvent alone. When investigating the impact of heavy metals dissolved in organic solvents on the bioluminescence bacteria *Vibrio harveyi* Mariscal *et al.* (2003) found that organic solvents, such as DMSO could increase the bioluminescence of *V. harveyi.* When using organic solvents to dissolve heavy metals, Mariscal *et al.* (2003) found that while the addition of DMSO increased the expected toxicity of cadmium, they also found that DMSO at a concentration of 32000 and 16000 mg/L with mercury at a concentration of 0.5 mg/L, caused a considerable increased luminescence. Concentrations in the Mariscal *et al.* (2003) study were between 8000 and 32000 mg/L, whereas the highest concentration of DMSO in the current study was 2%, or 2000 mg/L this could be a reasonable explanation as to why the addition of emamectin benzoate showed an elevated level of luminescence in this study. There is little evidence for what mechanisms could be involved in this phenomenon as it has scarcely been reported, however it is speculated that changes in cellular permeability may play a role in the activation of luminescence, this may be what is happening

with emamectin benzoate, however there is little evidence to support this in the literature. Dissolving substances in organic solvents that have low solubility in water is common practice, however in light of these results dissolving test substances in DMSO and other organic solvents should be taken with care.

3.4.4 Conclusions

In conclusion while the *A. fischeri* test for bioluminescence is a rapid test to determine the acute toxicity of singular aquatic pollutants and mixtures of aquatic pollutants, it does not appear to be a species with high sensitivity to the specific VMs in this study. Comparing the current concentrations reported in the environment (chapter 2, section 2.3.2, tables 2.4 and 2.5), it is unlikely that the VMs used would cause an adverse effect on *A. fischeri.* Furthermore, dissolving substances in DMSO may increase luminescence especially through interaction with other substances. As discussed in section 3.4.3 of this chapter, the addition of DMSO and other solvents should be carefully taken into consideration when using this assay for the prediction of the toxicity of VMs.

In this study, IA is a more suitable model for the prediction of mixture toxicity on *A. fischeri* than CA, which has been found previously (Cedergreen *et al.*, 2008). This may have occurred as the substances tested did not have similar MOAs and did not produce synergistic effects. In other studies CA has been shown to be a better predictor of mixture toxicity, even for mixtures whose compounds have different MOAs (Villa *et al.* 2012).

Chapter 4

Molecular Docking to Predict the Xenoestrogenic Potential of Veterinary Medicines

4.1. Introduction

There has been a shift in recent years towards the reduction in vertebrate testing in toxicology through the adoption of alternative testing methods such as *in vitro* and *in silico* (Eisenbrand *et al.*, 2002). Despite pressures to reduce the amount of animal testing, and growing evidence that crustaceans amongst other invertebrates may experience pain-like responses, ecotoxicology largely still uses invertebrate and fish models (see chapter 1; section 1.5) (Elwood, 2011; Elwood *et al.*, 2009). The current OECD 'Guidelines for the testing of Chemicals' section 2 'Effects on Biota', (OECD, 2012b) sets out the 40 most common tests for ecotoxicology, of these 19 are acute toxicity tests, 9 are reproduction tests, 7 are tests on microorganisms, bacteria, algae or plants, 3 assess EDCs and one measures growth (OECD, 2012b). All the recommended tests are *in vivo* with 11 of these designed to test the effects on fish. The OECD (2012a) suggests five levels for assessing the endocrine disrupting ability of a chemical. Level one refers to existing data and non-test information, for example physical and chemical properties, any available ecotoxicology or toxicology data, quantitative structure-activity relationship (QSARs), and absorption, distribution, metabolism, and excretion (ADME) predictions. Levels two focuses on specific endocrine pathways using *in vitro* assays whilst levels three, four and five focus on the use of *in vivo* assays to confirm any findings from levels one and two. This chapter will explore the use of *in silico* molecular docking to assist with level one testing of EDCs using the oestrogen receptor alpha (ERα) as the receptor of interest.

One of the primary stages of the environmental risk assessment (ERA) is to assess the QSAR of new substances. QSARs are computational methods which make predictions on a compounds toxicity by comparing the molecular structure and physico-chemical properties, such as the octanol/water partition coefficient (log K_{ow}) to compounds of a known (eco)toxic effect (Schultz *et al.*, 2003) (chapter 1; section 1.4; tables 1.2 and 1.3). QSARs were originally adopted as a high throughput method of drug discovery but are now being applied to other uses such as ecotoxicology (de Roode *et al.*, 2006) (see section 1.6; chapter 1). Another *in silico* method being used for drug discovery is molecular docking (Deng *et al.*, 2014; Gschwend *et al.*, 1996). Increasingly molecular docking is being utilised to predict binding of ligands to protein targets, and is being proposed as an alternative method of *in silico* predictions on protein targets and interactions with various toxic substances, such as VMs. Molecular docking is being suggested as

an additional component in QSARs, to complement the assessment of the structure relationships of ligands.

The regulation of ecotoxic compounds is moving to more intelligent, informed testing and beginning to incorporate new non-destructive techniques. However, there are disadvantages of *in vitro* testing. In particular, the biological and protein targets need to be known before testing can be conducted.

Molecular docking is a computational method to investigate interactions between ligands and receptors and is proposed as a suitable method to incorporate in the QSAR data set (see above). Docking programs can make predictions on binding affinity, hydrogen bonds and Van der Waals interactions (Huey *et al.*, 2007). There are a number of programs that can be used to dock ligands to larger molecules, from open source software such as AutoDock Tools, rDock and SwissDock to commercial programs such as FlexX, hint!® and Discovery Studio. If a mode of action (MOA) is established beforehand then testing can be conducted. However *in silico* has the advantage that multiple receptors from multiple organisms can be tested in a high throughput manner, so that *in silico* methods have the potential to identify targets. This is potentially a powerful adjunct to existing testing methods and deserves further investigation.

The open source docking program AutoDock 4, was used in this study due to its use in a number of studies and open source availability (McCullough *et al.*, 2014; Walker and McEldowney, 2013). AutoDock uses four algorithms to assess and make predictions on the binding efficiency of ligands to receptors. Firstly empirical binding free energy force fields are combined with Lamarckian genetic algorithms using Monte Carlo methods. Genetic algorithms attempt to simulate natural selection using computational techniques. Monte Carlo methods are a set of repeated randomised sampling algorithms. AutoDock 4 also allows for flexible docking of both the ligand and protein. Rotatable torsions allow for the flexibility of rotatable bonds within the ligand while flexibility of certain residues within the binding pocket can also be activated within the receptor (Morris *et al.*, 2009).

Molecular docking provides information on the free energy of binding in kilocalories per mole (kcal/mol). This measurement of energy can be converted into the inhibition constant (Ki). Ki is the concentration of a given substance (in this case a VM) that causes 50% inhibition of the receptor in question. In drug discovery this provides useful information on target receptors and non-target receptors. If the Ki for a target receptor is considerably lower than that of the non-target receptor then the dose of drug needed to elicit a response in the target receptor should not cause serious side effects in the non-target receptor. Therefore, in the context of ecotoxicology regulation if the measured environmental concentration (MEC) and bioavailability

of a VM is a higher concentration than the Ki there could be cause for concern for the exposed species.

Affinity of the ligand-protein complex can be assessed in terms of different intramolecular forces within the complex. AutoDock produces these outputs as free energy binding, measured in kilocalories per molecule (kcal/mol), as well as inhibition constants (Ki) and ligand/receptor interactions such as hydrogen bonds and other non-covalent interactions such as Van der Waals forces (Huey *et al.*, 2007) . Hydrogen bonds are formed in ligand protein complexes when a donor breaks its bond with a water molecule and in exchange forms a new bond with an acceptor which has also broken its bond with water (Zhao and Huang, 2011). Although this type of binding is more unstable than covalent bonding, it is one of the most stable bonds within a ligand-protein complex. A number of studies using AutoDock to investigate ligand-protein interactions have relied not only on free energy of binding, but also on position of binding and possible intermolecular interactions such as hydrogen bonds and Van der Waals interactions (Cui *et al.*, 2013; McCullough *et al.*, 2014). A limitation of using AutoDock to predict ligand/protein interaction is that the software only provides estimates on binding energy and position but does not provide information on whether the ligand could be an agonist or an antagonist. To overcome this limitation it is proposed that hydrogen bond interactions between ligands and residues thought to be involved in activation or deactivation of a receptor will be investigated.

4.1.1 Binding of Oestrogens to the Oestrogen Receptor

The oestrogen receptor (ER) is a ligand dependent nuclear hormone receptor, which primarily binds to the native steroid 17β-oestradiol (E2) (Kumar *et al.*, 2011; Kumar and Chambon, 1988; Webb *et al.*, 1998). While two other oestrogens, oestrone (E1) and oestriol (E3), also bind to the ER, E2 is the most potent and most common. There is evidence that the binding of E1, E2 and E3 is tissue specific (Nelson and Habibi, 2013). The two hydroxyl groups on either end of E2, 11 angstroms (Å) apart (see figure 4.9 [1a]), enable binding positioned between the conserved arginine (Arg), glutamic acid (Glu) and histidine (His) within the ligand binding domain (LBD) (see figure 4.1) (McCullough *et al.*, 2014). The timing and concentration of oestrogens is an important part of sexual and reproductive development in organisms, therefore foreign oestrogenic substances that disrupt the balance of native oestrogens in the body can result in developmental and reproductive abnormalities (Jobling *et al.*, 1998). All vertebrates and some invertebrates have at least one isoform of ER. There are two ER subtypes in humans, α and β (Kumar *et al.*, 2011), while three have been found in Actinopterygii fish (the class under which most bony fish fall), α, β_1 and β_2 (Menuet *et al.*, 2004; Nagler *et al.*, 2007; Nelson and Habibi, 2013).

The ER is a complex receptor which when activated modulates several genes in fish including vitellogenin (*vtg*); growth hormone (*gh*); insulin like growth factor (*igf1*); the thyroid hormone

receptors α and β (*thra* and *thrb*) amongst others (Filby *et al.*, 2006). Gene transcription due to increases in E2 and xenoestrogens has been shown to vary between tissue and sex, for instance in a study by Filby *et al.* (2006) in the presence of 35 ng/L of E2 the fat head minnow showed upregulation of *igf1* in the brain for both sexes but downregulation in the gill in both males and females. VTG is the precursor protein for egg laying vertebrates and has been used as a biomarker in fish for environmental oestrogens, especially as male fish possess the *vtg* gene but it is not normally expressed unless the ER has been activated in the presence of an increase of either natural oestrogens or xenoestrogens (Jobling and Tyler, 2003).

The ER has five distinct domains, these are the N-terminus which includes the activation function 1 (AF-1), the DNA binding domain (DBD), followed by a hinge region, then the ligand binding domain (LBD) which includes the activation function 2 (AF-2) and finally the C-terminal domain (figure 4.1). All subtypes of the ER have the same five domains with varying degrees of similarity between the domains. The DBD and the LBD are well conserved between subtypes and species, whereas the N terminus, hinge region and C terminus are less conserved (Sumida and Saito, 2008).

Figure 4.1 Five domains of the ER . The N terminus (N) containing activation function 1 (AF-1); the DNA binding domain (DBD); the hinge region (H); the ligand binding domain (LBD) containing activation function 2 (AF-2) and the COOH terminus (C). Adapted from Kumar *et al.* (2011).

Prior to ligand binding, the ER is held in the nucleus by a complex of heat shock proteins. Once the ER has bound to a ligand folding at the hinge region (see figure 4.1) takes place, changing the shape of the ER resulting in breaking free of the heat shock protein complex (Webb *et al.*, 1998). The folding of the ER at the hinge region enables the AF-1 within the N-terminus to connect with AF-2 within the LBD. Once the ER is ligand bound and its shape has changed, it can then form a homo or hetero dimer and bind to a section in the DNA called the oestrogen response element (ERE) where downstream transcription can take place to modulate a number of genes with the assistance of co-regulatory proteins (Kumar *et al.*, 2011; Webb *et al.*, 1998). The ERE is a short palindromic sequence (15 basepair) which is activated through binding to the dimerised ER complex (Schwabe *et al.*, 1995).

Antagonists bind to the LBD but block the connection between the LBD and DBD thereby preventing DNA binding (Dayan *et al.*, 2006). The LBD of ER binds to a number of foreign ligands, which can have either an agonistic or antagonistic effect on the ER. The function of ER is essential

to reproductive health, couple this with the promiscuity of the ER, and it is easy to see why it is of such interest in ecotoxicology research.

The appearance of oestrogenic compounds in the environment is of interest to both human and ecosystem health. Prevalent cases of xenoestrogens in the environment include bisphenol A (BPA), a common component of plastics, and the synthetic oestrogen 17α-ethinylestradiol (EE2), a pharmaceutical used in the birth control pill, and their impacts on wild populations of fish downstream from sewage effluent containing these xenoestrogens (Van den Belt *et al.*, 2003). Reported alterations resulting from an increase in oestrogenic substances in the environment include altered sexual development (Jobling *et al.*, 1998); reduced fertility (Jobling *et al.*, 2002); reduction in gonadosomatic growth index (Filby *et al.*, 2007); reduced osmoregulation (Carrera *et al.*, 2007; Lerner *et al.*, 2012); reduced immune functions (Casanova-Nakayama *et al.*, 2011); and altered embryonic development (Jobling *et al.*, 2003). On a wider scale, declines in populations have also been attributed to the increase of xenoestrogens in the environment (Kidd *et al.*, 2007). The reported effects of xenoestrogens on the environment are further described in chapter 1 section 1.2.

4.1.2 Selection of VMs to be included in Study

Five substances were used as controls for this study. Agonist controls were the native steroid E2; the synthetic oestrogen EE2, two known xenoestrogens, dichlorodiphenyltrichloroethane (DDT) and BPA. Tamoxifen was used as an antagonist control (table 4.1).

Table 4.1 Therapeutic effects of a natural oestrogen, synthetic oestrogen, two xenoestrogens and the anti-oestrogen tamoxifen.

Class	Substance	Therapeutic effect/use
Oestrogens	E2	A natural oestrogen that binds to ERα for gene activation. Plays a role in growth, ovulation, pregnancy and lactation in females (Kumar *et al.*, 2011; Webb *et al.*, 1998). The dissociation constant (k_d) value for E2 to the ERα is estimated to be between 0.02 and 2 nM (Barkhem *et al.*, 1998; Gangloff *et al.*, 2001)
	EE2	A synthetic oestrogen used in the contraceptive pill to mimic E2 (Dhont, 2010).
Xenoestrogens	DDT	An organochloride that acts on the voltage-gated sodium channel, primarily used as an insecticide to eradicate malaria. Banned in the 1970s due to high persistence in the environment (Davies *et al.*, 2007).
	BPA	A synthetic bisphenol which is found in plastics. It has been reported as a xenoestrogen in a number of studies (Ben-Jonathan and Steinmetz, 1998; Levy *et al.*, 2004)
Anti-oestrogens	Tamoxifen	An anti-cancer agent that binds to ERα ligand binding domain and prevents binding to ERE due to bulky side-chains blocking DNA binding (Dayan *et al.*, 2006).

Fifteen VMs were used in this study to investigate the effects of VMs on the ERα. The rationale for choosing the VMs in this study differed depending on the VM. All of the sea lice control treatments (azamethiphos, cypermethrin, deltamethrin, emamectin benzoate, teflubenzuron and 3PBA) were used in this chapter for continuity. Cypermethrin, deltamethrin and emamectin benzoate are also included as they appear on the 56 high priority list of VMs likely to enter the environment by Boxall *et al.* (2003). Cypermethrin, deltamethrin and 3PBA have also been previously included in *in vitro* studies suggesting they either have an agonistic or antagonistic effect on the ERα (Chen *et al.*, 2002; Kojima *et al.*, 2004). Fenbendazole, a broad spectrum anthelmintic used as a wormer used in both intensively farmed and companion animals; was included due to evidence of endocrine disruption in invertebrates (Park and Kwak, 2012) and also due to its inclusion on the high priority list by Boxall *et al.* (2003). Diazinon, an organophosphate previously used as a sheep dip, was included due to evidence of its endocrine disrupting potential, and its inclusion on the high priority list (Boxall *et al.*, 2003). Diazinon has reportedly measured in salmon spawning tributaries at concentrations of 18.5 – 35 µg/L, despite the average annual EQS being 0.01 µg/L and MAC being 0.1 µg/L (Moore and Waring, 1996). Diazinon can disrupt the olfactory system of male salmon, inhibiting their ability to detect female pheromones during mating, having a negative impact on reproduction at a concentrations as low as 0.4 µg/L (Moore

and Waring, 1996). Amitraz, an ectoparasiticide, was included due to its inclusion on the high priority list by Boxall *et al.* (2003) and because there is some evidence that it is an anti-oestrogen (Ueng *et al.*, 2004). Sulfadiazine, an antimicrobial used in aquaculture, and ivermectin, an ectoparasiticide, were also included in the high prioritisation list by Boxall *et al.* (2003), however there is no experimental data on their potential to affect the ER.

The other VMs that were chosen were not included on the prioritisation list set out by Boxall *et al.* (2003). Diclofenac, a non-steroidal anti-inflammatory drug (NSAID), has some evidence that it may be an endocrine disruptor in the Mediterranean mussel *Mytilus galloprovincialis* by the increased production of VTG when exposed to concentrations as low as 250 ng/L (Gonzalez-Rey and Bebianno, 2014; Schmidt *et al.*, 2011). More recently, diclofenac has also shown to increase VTG production in the Nile tilapia *Oreochromis niloticus* at concentrations as low as 4 nM (or 1.18 µg/L) (Gröner *et al.*, 2015). Diclofenac has also been recently added to the WFD priority substances watch list (under directive 2008/105/EC) (see chapter 1; section 1). Diflubenzuron, a chitinase inhibitor, was chosen as there is some experimental evidence that it can affect fecundity and development in some invertebrates (Depledge *et al.*, 1999).

Table 4.2 The class and therapeutic effect of the 15 selected VMs.

Class	Substance	Therapeutic effect/use
Synthetic pyrethroids	α-cypermethrin	α-cypermethrin and deltamethrin used as a control for sea lice (see chapter 2). Also used as a sheep dip to control blowflies and tics. They are neurotoxins that act on the voltage-gated sodium channel while modulating the level of gamma-aminobutyric acid (GABA). Was synthesised as an alternative to DDT as it is much less persistent in the environment (Singh *et al.*, 2012).
	Deltamethrin	
	3PBA	Major metabolite of synthetic pyrethroids including deltamethrin and α-cypermethrin. MOA is unknown (Zhao and Huang, 2011).
Antibiotics	Sulfadiazine	Broad spectrum antimicrobials often used in veterinary medicine in combination with other compounds such as trimethoprim or neomycin. Blocks DNA and RNA production in bacteria (Hammesfahr *et al.*, 2008).
	Sulfapyridine	
	Nalidixic acid	Synthetic quinoline used to treat urinary tract infections. Works by inhibiting DNA gyrase in Gram-positive bacteria (Changkwanyeun *et al.*, 2015).
Benzoylurea insecticides	Diflubenzuron	Used as a sea lice control in Norway, not currently registered in the UK. Used as a plant protection product in the UK. Similar to teflubenzuron in that it is a chitinase inhibitor (Selvik *et al.*, 2002).
	Teflubenzuron	Used as a sea lice control (see chapter 2). Chitinase inhibitor (SEPA, 1999).
Benzimidazole anthelmintic	Fenbendazole	Used in livestock and domestic pets to treat gastrointestinal worms. Works by inhibiting the formation of microtubules by binding to β-tubulin, restricting cytoskeleton growth (Martin, 1997).
Formadine pesticide	Amitraz	Primarily used as a topical treatment for dogs in the UK, however it is used in the EU on cattle, sheep and pigs to control ectoparasites. The MOA of amitraz works by binding to the octopamine. Octopamine controls endocrine activity in invertebrates, it has been documented that amitraz is a weak antagonist of ERα in studies performed with MCF-7 cells and immature rats (Ueng *et al.*, 2004).
Avermectins	Emamectin benzoate	Used as a sea lice control (see chapter 2). Agonist of GABA and the glutamate gated chloride channel (Jansson *et al.*, 1997).
	Ivermectin	Used in a variety of livestock including cattle, sheep and pigs. Acts on the GABA receptors in both invertebrates and vertebrates, however it is documented to alter gene mutation in the P-glycoprotein drug pump (*mdr1*) and has been found to cross the blood brain barrier. The unspecific action of azamethiphos means that it has been found to cause lethal and chronic effects to a number of non-target organisms (Geary, 2005).
Non-steroidal anti-inflammatory (NSAIDs)	Diclofenac	Not registered for use in the UK, is registered for use in Spain and Italy to treat cattle. Inhibitor of COX-1 and COX-2. Caused mass mortality of *Gyps* sp. vultures in India and Pakistan after secondary feeding on previously treated carrion (Taggart *et al.*, 2007).
Organophosphate	Azamethiphos	Used as a sea lice control (see chapter 2). Acts by inhibiting acetylcholinesterase (AChE). (Ernst *et al.*, 2001)
	Diazinon	Diazinon was registered for veterinary use as a sheep dip, but it is no longer approved in the EU. It is a cholinesterase inhibitor. It was also used as a plant protection product. There is some evidence for diazinon being a xenoestrogen (Maxwell and Dutta, 2005).

4.1.3 Chapter objectives

In order to address objective 4 (chapter 1; section 1.9.3) the objectives for this chapter are as follows:

- To investigate whether VMs bind differently against ten ERα proteins.
- To determine whether 3 dimensional (3D) structure and binding affinity are correlated for both protein structure and ligand structure.
- To examine evidence for any relationships between binding affinities and experimental chronic data found in the literature.
- To determine whether binding conformation can identify between potential xenoestrogens and potential anti-oestrogens.

4.2 Methods

4.2.1 Obtaining the 3D Structure for ERα

The 622 amino acid (AA) sequence for the rainbow trout *O. mykiss* ERα (accession number P16058) was obtained from UniProt (The UniProt Consortium, 2015). This sequence was then put into SwissModel (Arnold *et al.*, 2006; Kiefer *et al.*, 2009; Peitsch, 1995) 'template mode' to find the pdb file with the highest similarity with *O. mykiss* ERα. This was 3ERT chain A, the ligand binding domain of the *H. sapiens* ERα with a length of 261 AA (Shiau *et al.*, 1998). The accession number for the UniProt reference sequence for the pdb file 3ERT was P03372 with a length of 595 AA. A clustalW (Goujon *et al.*, 2010; Larkin *et al.*, 2007; McWilliam *et al.*, 2013) sequence alignment was performed using the two full-length sequences P16058 and P03372. A clustalW file was made and fed into SwissModel alignment mode, to determine that 3ERT chain A was the template structure. The same method was applied to the other test species using the most suitable crystal structure, of the LBD of the human ERα, pdb entry as a model (table 4.3).

The ERα from nine aquatic species (table 4.3) were used to examine whether there is intra species differentiation in regards to the potency of xenoestrogens, the pacific oyster *Crassostrea gigas* was used as a negative control (see below). With the exception of *H. sapiens* (positive control) and *C. gigas,* which had suitable pdb files thanks to the availability of the crystallography of these structures on the research collaboratory for structural bioinformatics (RCSB)(URL www.rcsb.org; Berman *et al.,* 2000) , ERα structures were modelled using the modelling software SwissModel.

Table 4.3 Details on modelling of the ERα of the ten test species.

Species	**Common Name and habitat(s)**	**Accession number**	**Modelled on (RCSB PDB entry)**	**Number of Amino Acids**	**Similarity with modelled structure %**	**QMEAN4 score**	**RMS from *H. sapiens* (3ERT)**
H. sapiens (positive control)	Human, terrestrial	P03372	3ERT	595	N/A	N/A	N/A
C. gigas (negative control)	Pacific oyster, marine	K1QUU5	4N1Y	485	N/A	N/A	14.411
D. rerio	Zebrafish, freshwater	P57717	3ERT.1.A	569	61.57	-2.08	4.587
G. aculeatus	Three spined stickleback, marine	G3P1N4	1L2I.1.A	623	64.52	-0.62	1.626
O. mykiss	Rainbow trout, freshwater	P16058	3ERT.1.A	622	63.27	-1.79	4.714
P. promelas	Fathead minnow, freshwater	A9XE64	2OCF.1.A	602	59.85	-1.31	4.726
R. rutilus	Common roach, fresh and brackish water	Q5CCT6	3Q97.2.A	588	58.20	-0.46	6.945
Salmo salar	Salmon, marine and freshwater	P50242	3ERT.1.A	535	62.86	-1.88	5.549
Sparus aurata	Gilt-head bream, marine	Q9PVZ9	1L2I.1.A	581	63.67	-0.21	5.588
Xenopus laevis	African clawed frog, freshwater and terrestrial	P81559	3Q97.2.A	586	81.18	-0.23	1.952

Ten ERα homologs were considered during the current study. Aside from *H. sapiens,* the positive control, all other species were a mix of freshwater and marine aquatic organisms (table 4.3). The ERα of *C. gigas* was used as a negative control, as there is evidence that although *C. gigas* possess an ERα there is a semi conserved amino acid mutation from a histidine to a phenylalanine in

position 450, which is conserved in the other species (figure 4.2). This mutation is thought to contribute to the inability of *C. gigas* ERα to bind to oestrogens and therefore has been selected as a suitable negative control (Bannister *et al.*, 2013). The eight test species consisted of seven bony fish from the class Actinopterygii including two salmonids *O. mykiss* and *S. salar;* three cyprinids *D. rerio, R. rutilus* and *P. promelas;* one Sparidae *S. aurata* and one Gasterosteidae *G. aculeatus.* The seven species were chosen as the sequences for their ERα was readily available on UniProt, and because their distribution (with the exception of *D. rerio* and *P. promelas*) is in and around the UK. *D. rerio* and *P. promelas* were also included because of their close relationship with the selected fish and they are often used as test species in ecotoxicology. The ERα of the amphibian *X. laevis* was also included as it is a commonly used test organism in ecotoxicology tests. It is important to note that ecotoxicology tests are only usually carried out on one fish that will act as a model organism for other species within this general class. The reason seven fish species, with such a high similarity, were chosen was therefore to test whether there are any major differences in binding of the test VMs to the different ERα.

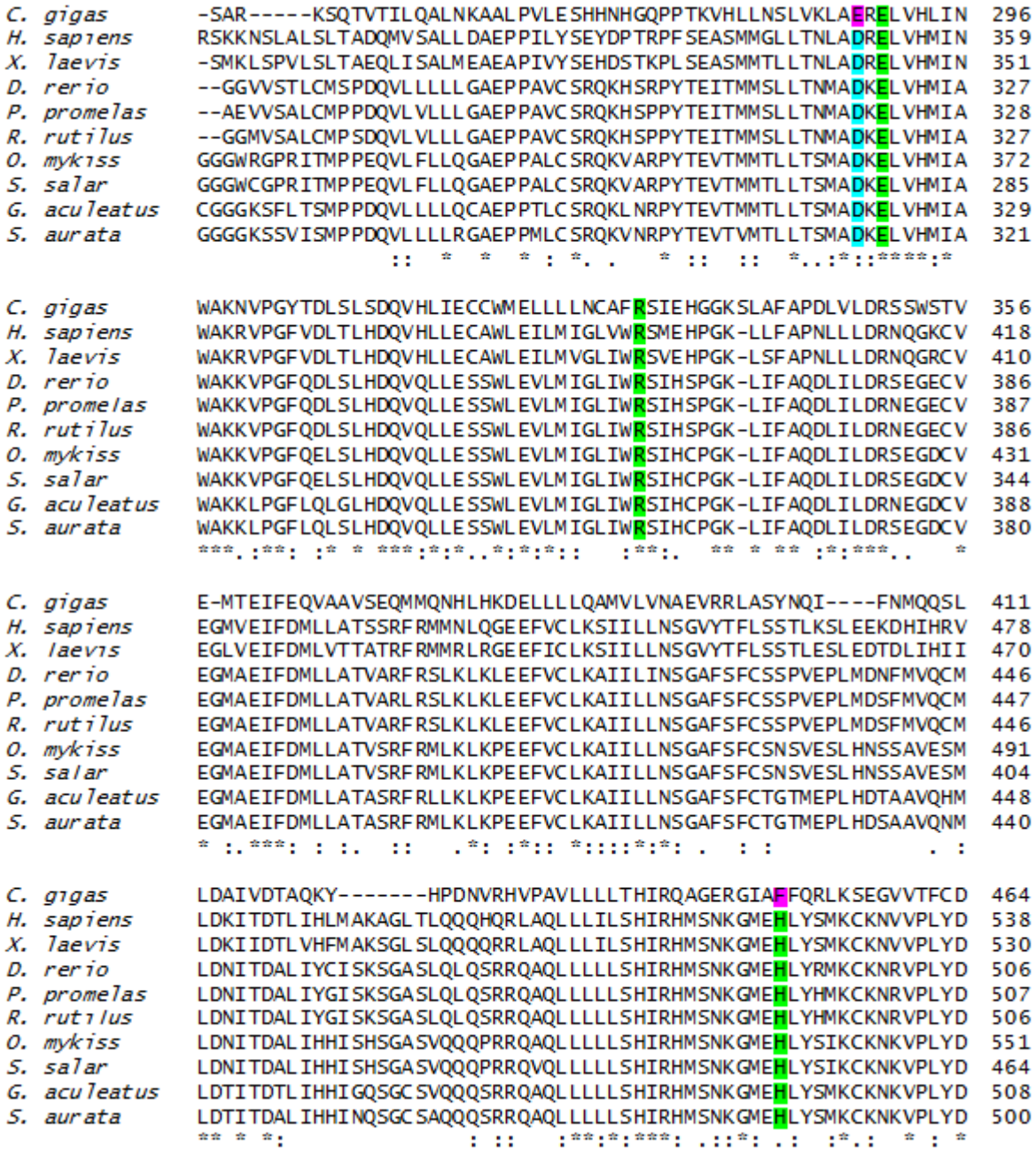


Figure 4.2 Multiple sequence alignment of the ligand binding domain of ERα for the ten test species using clustalW2. Highlighted in green are the residues important for the binding of the native ligand E2, these are glutamic acid (E) (position 354 in *H. sapiens*); arginine (R) (position 394 in *H. sapiens*) and histidine (H) (position 524 in *H. sapiens*). Highlighted in blue is aspartic acid (D) in position 351 (*H. sapiens*), which is important for binding to anti oestrogens such as tamoxifen. These residues are conserved in all ten species apart from in *C. gigas* where the aspartic acid has been replaced by glutamic acid (E) in position 288 and the histidine has been replaced by a phenylalanine (F) in position 450 and (highlighted in pink).

4.2.2 Obtaining the 3D Structure for Ligands

The Simplified Molecular-Input Line-Entry System (SMILES) codes for the ligands used in this chapter (see tables 4.1 and 4.2) were found using chemspider (URL - www.chemspider.com). These SMILES were made into smi files, and converted to pdb files with 3D coordinates using the program open babel (O'Boyle *et al.*, 2011). E2 and EE2 did not convert correctly using open babel, possibly because of their polycyclic structure. As a result E2 and EE2 were downloaded directly from the RCSB website. The pdb file for E2 was made using the pdb file 1QKU and E3 was made using the pdb file for 1X8V.

4.2.3 Docking Ligands to ERα

All pdb files were uploaded to the python molecular viewer program PMV (version 1.5.6) (Sanner, 1999) with the AutoDock tools add-on (Morris *et al.*, 2009). All hydrogens were added to the molecules, polar hydrogens were merged and Gasteiger charges were added. Gasteiger charges the empirical partial charges calculated for atoms within molecules in order to compute the electrostatic interaction energy (Gasteiger and Marsili, 1978; Gasteiger and Marsili, 1980). The literature (Gangloff *et al.*, 2001; Shiau *et al.*, 1998) shows that the binding pocket surrounds the three residues Glu 353, Arg 394 and His 524 in *H. sapiens* ERα, therefore the grid box incorporated these three residues in the *H. sapiens* ERα and homologous residues. The clustalW result (figure 4.2) shows that these residues are highly conserved across all the test species; apart from the negative control *C. gigas* where the histidine was replaced with phenylalanine. The grid box was positioned to incorporate the three identified residues (figure 4.3). The pdb files were then saved as pdbqt files ready to be docked with the control and test ligands. The ligand pdb files were also uploaded to PMV-1.5.6 with the AutoDock Tools add-on, where the adding of Gasteiger charges and the merging of non-polar hydrogens were automatically calculated. The ligands were then saved as pdbqt files.

Fifteen drugs of interest were determined in preliminary studies using AutoDock Vina and through data mining of the literature. The five sea lice treatments (chapter 2) were also included, as well as the synthetic pyrethroid degradation product, 3PBA (also investigated in chapter 3). These were: 3PBA, α-cypermethrin, amitraz, azamethiphos, diazinon, diclofenac, diflubenzuron, emamectin benzoate, fenbendazole, ivermectin, nalidixic acid, sulfadiazine, sulfapyridine and teflubenzuron (table 4.2). Controls for AutoDock 4 were the native ligand E2, the synthetic oestrogen, EE2, and the established xenoestrogens BPA and DDT. Tamoxifen was used as antagonist control (table 4.1).

Each model was generated as a 3D .pdb format. A .pdb file is a universal file in which a number of programs can read in order to generate a 3D model of a structure. This includes the name of each residue in the structure and details of each atom that the structure is built from, such as spatial

coordinates. For AutoDock 4 to run, .pdb files needs to be converted into .pdbqt files. A .pdbqt file is a .pdb file with added partial charges and codes for atom types that AutoDock can read (Huey *et al.*, 2007).

Once .pdbqt files were generated, a grid parameter file (.gpf) was made (see appendix E for example .gpf file). The .gpf contains information on where AutoDock should focus ligand binding. The ten ERα grids were set up to surround the three amino acids that are thought to be crucial to binding (highlighted in table 4.4 and figure 4.2, see above). A grid of 60 $Å^3$ using a spacing of 0.325 Å was created for all receptors (see figure 4.3). Then a docking parameter file (.dpf) was made for each ERα (see appendix F for example .dpf file). The .dpf holds information on how the ligand should move within the grid box, using information set up in the .gpf file previously. The .dpf also determines which search algorithm has been selected, the distances of Van der Waals and hydrogen bonds, how the energy binding (kcal/mol) is calculated and how many energy evaluation runs to complete. Once all of these files were in order AutoDock 4 was run on each receptor for each ligand with 100 energy evaluation runs completed for each experimental scenario. Results were given as docking log output files, or .dlg files. The .dlg output contains information on 3D coordinates and position of the bound ligand; binding in kcal/mol; Ki in nM and the number of conformations within a 2 Å from the reference ligand; these are referred to as binding clusters. Interacting residues with VMs were investigated and compared with the native ligand E2 and the antagonist control tamoxifen (table 4.4). Results were visualised and images were made using PyMOL, Version 1.7.6 (Schrodinger, 2015). The results on binding were expressed as the lowest binding energy (highest affinity) within the biggest binding cluster. For example docking runs produced 100 bound conformations, some ligands (such as E2) had 100 bound conformations that were within 2 Å of one another, in this case the lowest bound ligand was taken (see figure 4.4). However some ligands had a number of clusters, with the biggest cluster not necessarily containing the lowest bound ligand (for example see 3PBA bound to *S. aurata*; figure 4.5). In this case the lowest bound conformation within the biggest cluster was taken. Binding was split into three categories, those with a high likelihood of binding, those with a medium likelihood of binding and those with a low likelihood of binding. When comparing the free binding energy of docked ligands to receptors there is some contention as to what is a 'good hit' (Leach *et al.*, 2006). In reference to others work, correlations between binding and *in vitro* conformation of agonists has been found as low as -7.48 kcal/mol (McCullough *et al.*, 2014; Pavani *et al.*, 2008) therefore ligands that had a binding energy of <-7 kcal/mol were given the status 'high likelihood' of binding. The standard error of docking studies with autodock 4 is given as 2 - 3 kcal/mol (Huey *et al.*, 2007) therefore ligands that bound with a free binding energy of -5 to -7 kcal/mol were put into the medium likelihood of binding category. Finally ligands that

bound with a free binding energy of > -5 kcal/mol were put into the low likelihood of binding category.

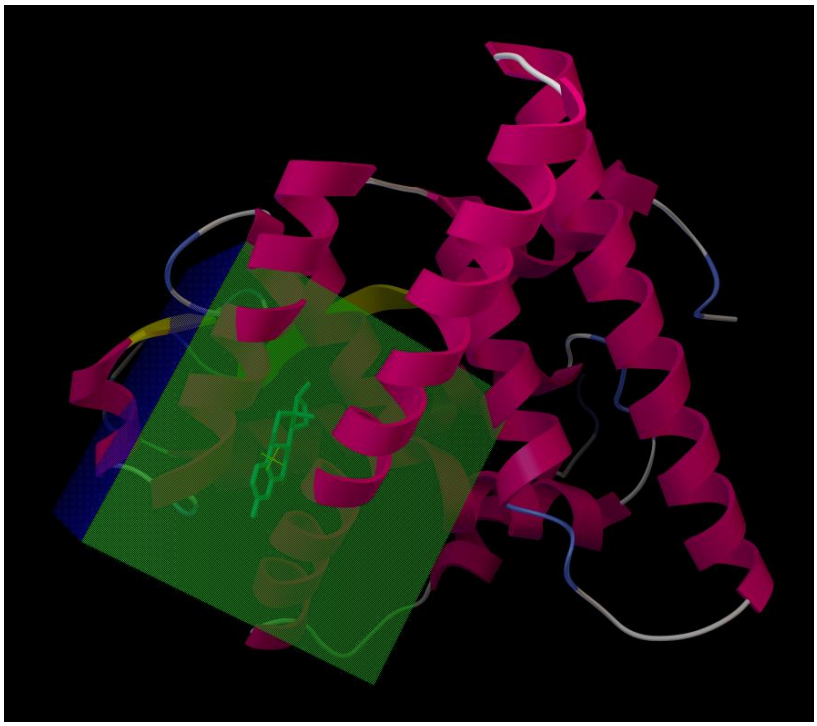


Figure 4.3 Ligand binding domain of the human ERα (ribbon structure, pink α helices, yellow β sheets) bound to E2 (blue ligand within box). Box highlights the grid search space around the active site.

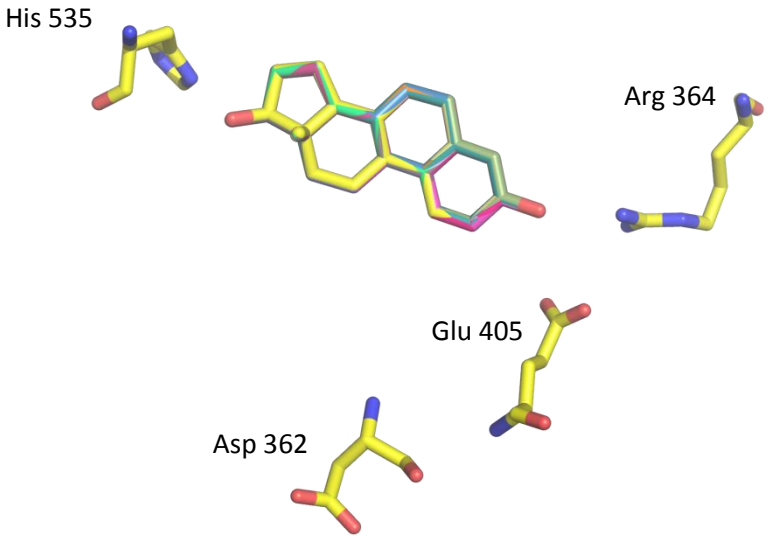


Figure 4.4 E2 bound to All 100 conformations of E2 (multi-coloured ligands) bound to the ERα of *P. promelas* (yellow residues) forming one cluster.

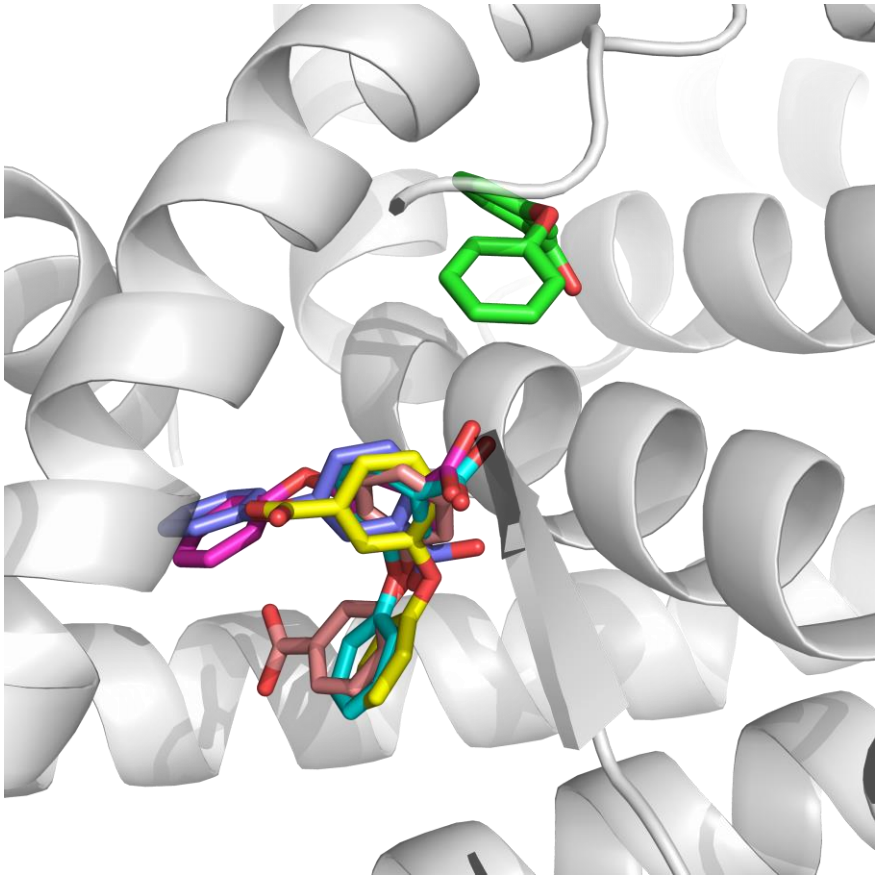


Figure 4.5 3PBA bound to *S. aurata* ERα in six different clusters. Cluster 1 (green ligand) contained one binding conformation and had a binding energy of -6.13 kcal/mol. Cluster 2 (blue ligand) contained 25 binding conformations in the cluster and the lowest binding energy was -5.78 kcal/mol. Cluster 3 (dark pink ligand) contained 19 binding conformations and the lowest binding energy was -5.71 kcal/mol. Cluster 4 (yellow ligand) contained 14 binding conformations and the lowest binding energy was -5.62. Cluster 5 (light pink ligand) had a total of 34 binding conformations and the lowest binding energy was -5.48. Cluster 6 (purple ligand) had a total of 7 binding conformations and the lowest binding energy was -5.32.

Each of the lowest bound conformations within the biggest cluster for each VM and each species ERα was examined for possible hydrogen bonding sites using PyMOL as a visualisation tool. Four residues were the main focus of hydrogen bonding, these were the three residues thought to be important for ligand activation glutamic acid (position 353 in the control *H. sapiens*); the arginine (position 394 in *H. sapiens*); and histidine (position 524 in *H. sapiens*). Binding to aspartic acid (position 351 in *H. sapiens*) was also investigated as this residue is thought to be an important residue for antagonistic binding (see table 4.4).

Table 4.4 Residues important for binding within the LBD of ERα. Glutamic acid (Glu), arginine (Arg) and histidine (His) are important for binding to the native ligand E2, while binding to aspartic acid (Asp) prevents binding to DNA binding domain. In *C. gigas* His was replaced with a phenylalanine (Phe) and Asp was replaced with a Glu, these changes are highlighted with a *.

	Glu	**Arg**	**His**	**Asp**
H. sapiens	353	394	524	351
C. gigas	290	331	*Phe 450	*Glu 288
D. rerio	321	362	492	319
G. aculeatus	350	391	521	348
O. mykiss	366	407	537	364
P. promelas♮	364	405	535	362
R. rutilus	345	386	516	343
S. aurata♮	315	356	486	313
S. salar	279	320	450	277
X. laevis	345	386	516	343

Organisms with ♮ are those shown in figures 4.4 (*P. promelas*) and 4.5 (*S. aurata*).

Table 4.5 Property table for amino acids important in binding in the LBD of the ERα for all ten test species.

Amino acid	**Arg**	**Asp**	**Glu**	**His**	**Phe***
pKa	12.5	3.9	4.2	6	-
Side chain polarity	Positively charged	Negatively charged	Negatively charged	Polar	Hydrophobic
Volume ($A^{\circ 3}$)	173.4	111.1	138.4	153.2	189.9
Hydrogen donor or acceptor	Donor	Acceptor	Acceptor	Donor and acceptor	Neither
Side chain linear formula	$(CH_2)_3NHC(=NH)NH_2$	CH_2CONH_2	$(CH_2)_2CH_2H$	CH_2(4-imidazolyl)	CH_2Ph

pKa = the pH at which half of the side chain is charged. * Phe is present in the LBD of the negative control *C. gigas* only. Information included in property table taken from Jones (2002).

4.2.4 Statistical analysis of results

Statistical analysis was performed using IBM SPSS. Two-way ANOVA was performed to determine significant differences between ligands and between species. Tukey's post hoc was also performed to determine where variance lies. Dendrograms were created using hierarchical

clustering and squared Euclidian distance (see equation 4.1). Tanimoto coefficient (Rogers and Tanimoto, 1960), a measurement of structural distance, was calculated for ligands using open babel software. A dendrogram was built using the information on similarity from the tanimoto coefficient results. Results of dendrogram clusters will be referred to as 'groups' from this point on, to avoid confusion with clusters of binding conformations in docking results.

Equation 4.1 Squared Euclidean Distance

$$d = \sum_{i=1}^{n} (x_i - y_i)^2$$

Where d = distance, x = point x and y = point y

Determining the number of groups within a dendrogram can be interpreted in a number of ways. Therefore a mathematical method (equation 4.2) was employed to find the optimal number of groups within each dendrogram, to avoid bias. Groups were then compared between each dendrogram in order to determine structural and binding differences. The number of groups within a dendrogram was determined by using the variance ratio criterion (VRC) as described by Caliński and Harabasz (1974). VRC was determined by calculating the K-means grouping, with an ANOVA table calculated for each step, for each number of possible groups (for example, if ten groups were possible, the F ratio for 2-10 groups was calculated). The F ratios were summed to determine the VRC_k. The optimum number of groups, ω_k, was calculated as follows:

Equation 4.2 Variance ratio criterion

$$\omega_{\mathrm{k}} = (\mathrm{VRC}_{\mathrm{k+1}} - \mathrm{VRC}_{\mathrm{k}}) - (\mathrm{VRC}_{\mathrm{k}} - \mathrm{VRC}_{\mathrm{k-1}})$$

The ω_{k} with the second lowest value was used to determine the groups while the ω_{k} with the lowest value was used to determine the sub-groups.

Predicted oestrogenic potency (PEP) was calculated as a measure of how potent each VM was in comparison to the native ligand, E2. The calculated PEP was then compared to PEPs found in the literature to determine whether AutoDock 4 can efficiently predict oestrogenic potency of ligands. This equation does not distinguish between agonists and antagonists. The PEP was calculated using equation 4.3.

Equation 4.3 Predicted oestrogenic potential (PEP)

$$\frac{a}{b} \times 100 = PEP$$

Where *a* is the predicted Ki in nM of E2 and *b* is the predicted Ki of the VM in question.

4.3 Results

4.3.1 Structural relationships

Sequence similarity between each of the ten ERα was compared with one another. Sequence similarity tool, ClustalW Emboss water (Goujon *et al.*, 2010; Larkin *et al.*, 2007; McWilliam *et al.*, 2013) was used to determine the sequence similarity between species ERα (table 4.5). This information was then used to create the dendrogram in figure 4.6.

Table 4.5 Sequence similarity of the ten ERα using ClustalW EMBOSS Water. Percentage sequence similarity is shown, with percentage sequence identity shown in brackets.

	C. gigas	***D. rerio***	***G. aculeatus***	***H. sapiens***	***O. mykiss***	***P. promelas***	***R. rutilus***	***S. salar***	***S. aurata***	***X. laevis***
C. gigas	100	53.6 (38.7)	50.6 (37.6)	52.8 (36.8)	52.5 (38.9)	51 (37)	52.9 (36.7)	51.4 (37.5)	51.9 (37.6)	54.7 (37.4)
D. rerio	53.6 (38.7)	100	74 (63.9)	66.3 (53.6)	73.2 (63)	90.8 (86.7)	58 (45.7)	75.6 (65.4)	75.2 (64.2)	65.9 (52)
G. aculeatus	50.6 (37.6)	74 (63.9)	100	65.2 (51.3)	73.4 (65.4)	70.1 (60.4)	56 (43.5)	77.0 (69.8)	79.2 (71.2)	64.4 (49.7)
H. sapiens	52.8 (36.8)	66.3 (53.6)	65.2 (51.3)	100	63.6 (50.3)	63 (51.4)	58.9 (44.1)	66.5 (53.5)	65.8 (50.9)	81.5 (69.9)
O. mykiss	52.5 (38.9)	73.2 (63)	73.4 (65.4)	63.6 (50.3)	100	70.1 (60.4)	56 (43.5)	97.7 (97.2)	79.2 (71.2)	64.4 (49.7)
P. promelas	51 (37)	90.8 (86.7)	70.1 (60.4)	63 (51.4)	70.1 (60.4)	100	58.1 (44.8)	74.6 (64.7)	70.7 (59.9)	64.4 (50.8)
R. rutilus	52.9 (36.7)	58 (45.7)	56 (43.5)	58.9 (44.1)	56 (43.5)	58.1 (44.8)	100	59.3 (46)	61.6 (48.6)	60.9 (46.7)
S. salar	51.4 (37.5)	75.6 (65.4)	77.0 (69.8)	66.5 (53.5)	97.7 (97.2)	74.6 (64.7)	59.3 (46)	100	80.7 (72.8)	68.3 (53.8)
S. aurata	51.9 (37.6)	75.2 (64.2)	79.2 (71.2)	65.8 (50.9)	79.2 (71.2)	70.7 (59.9)	61.6 (48.6)	80.7 (72.8)	100	67 (52.3)
X. laevis	54.7 (37.4)	65.9 (52)	64.4 (49.7)	81.5 (69.9)	64.4 (49.7)	64.4 (50.8)	60.9 (46.7)	68.3 (53.8)	67 (52.3)	100

A dendrogram was developed using SPSS and the information on sequence similarity (table 4.5) to determine groups of related receptors. In theory those receptors which have sequence and structure similarity should bind with a similar affinity to tested drugs (figure 4.6). Four distinct groups could be determined from this dendrogram. Group 1, which had three sub-groups, consisted of six species in total. These were *O. mykiss* and *S. salar* (figure 4.6 [1a]) *G. aculeatus* and *S. aurata* (figure 4.6 [1b]); and *D. rerio* and *P. promelas* (figure 4.6 [1c]). Group 2 contained two species; *H. sapiens* and *X. laevis* (figure 4.6 [2]); Group 3 contained one species, *R. rutilus* (figure 4.6 [3]). Group 4 had one species the negative control *C. gigas* (figure 4.6 [4]).

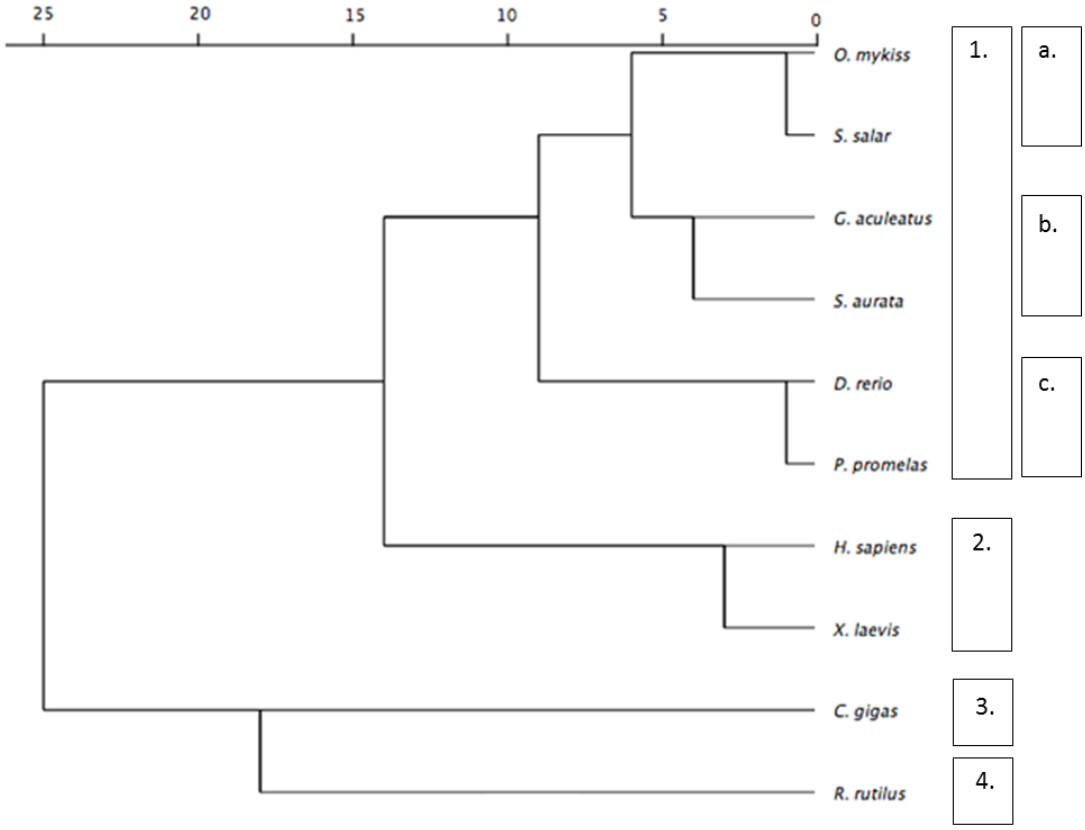


Figure 4.6 Dendrogram showing the evolutionary distance between ERα receptors of ten selected aquatic species.

When lowest binding energy within the biggest cluster was used to find relationships between the species tested six distinct groups appeared. The avermectins ivermectin and emamectin benzoate, were excluded due to their high binding affinity. Group 1 contained four species *O. mykiss , S. salar* and *D. rerio* and *S. aurata* (figure 4.7 [1]). Group 2 contained the two species *R. rutilus* and *X. laevis* (figure 4.7 [2]). Group 3, which had two sub-groups, contained the positive control *H. sapiens, G. aculeatus* (figure 4.7 [3a]) and *P. promelas* (figure 4.7 [3b]). Group 4 contained the negative control *C. gigas*. Group 4 was not shown in figure 4.7 as the distance of the relationship for the negative control, *C. gigas,* would have skewed results.

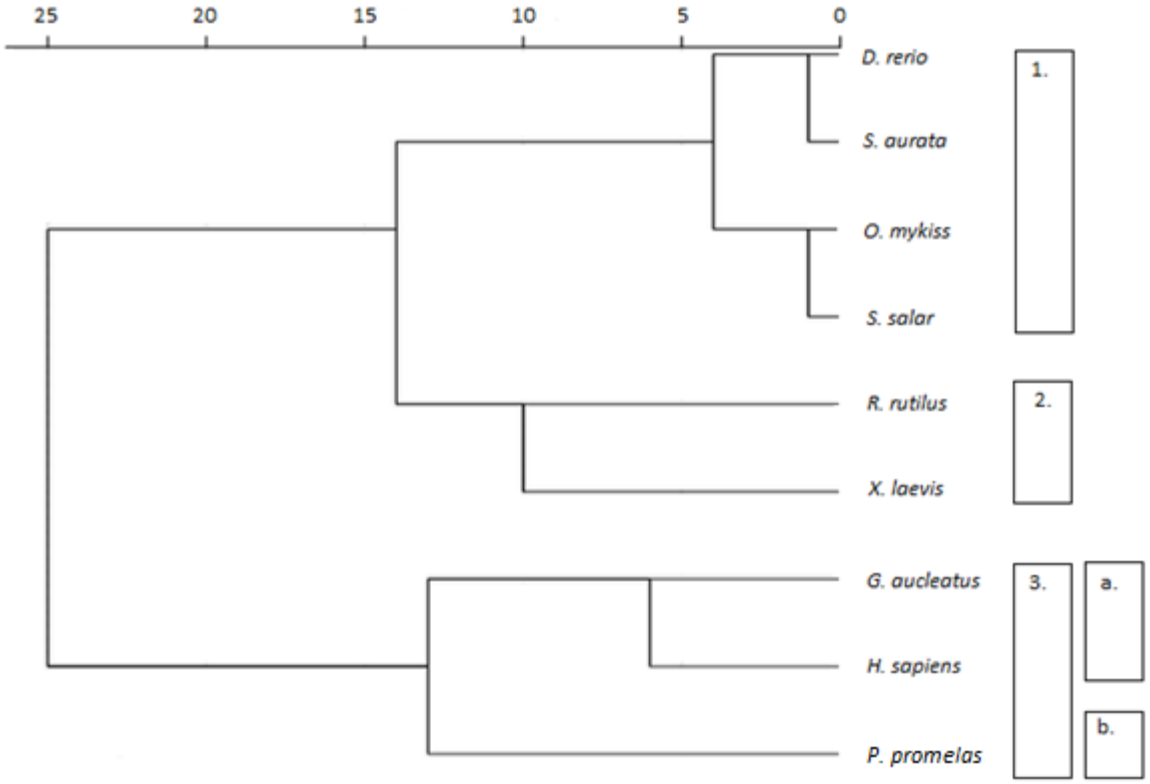


Figure 4.7 Dendrogram representing groups of species with the largest relationship in terms of lowest binding energy (highest affinity) in the biggest cluster for all test substances, excluding emamectin benzoate and ivermectin. The negative control *C. gigas* was excluded from this dendrogram.

Tanimoto coefficient was determined to show percentage structural similarity of the test ligands. Hierarchical clustering with a squared Euclidean distance was performed to produce a dendrogram (figure 4.8) based on the hypothesis that structurally similar drugs will bind with a similar affinity to receptors. Five main groups of structures were determined. Group 1, which had two sub-groups, consisted of the oestrogens E2 and EE2; the known xenoestrogens BPA and DDT and tamoxifen (figure 4.8 [1a]); and the pyrethroids α-cypermethrin, deltamethrin and their metabolite 3PBA (figure 4.8 [1b]). Group 2, which consisted of two sub-group, contained the benzoylurea insecticides, teflubenzuron and diflubenzuron (figure 4.8 [2a]); the formadine pesticide, amitraz and the NSAID, diclofenac (figure 4.8 [2b]). Group 3 consisted of the antibiotics, sulfadiazine and sulfapyridine and the benzimidazole anthelmintic, fenbendazole (figure 4.8 [3]). Group 4, which was split into two sub-groups, contained the organophosphates, azamethiphos and nalidixic acid (figure 4.8 [4a]) and the antibiotic, diazinon (figure 4.8 [3b]). Group 5 contained the two avermectins, emamectin benzoate and ivermectin (figure 4.8 [4]).

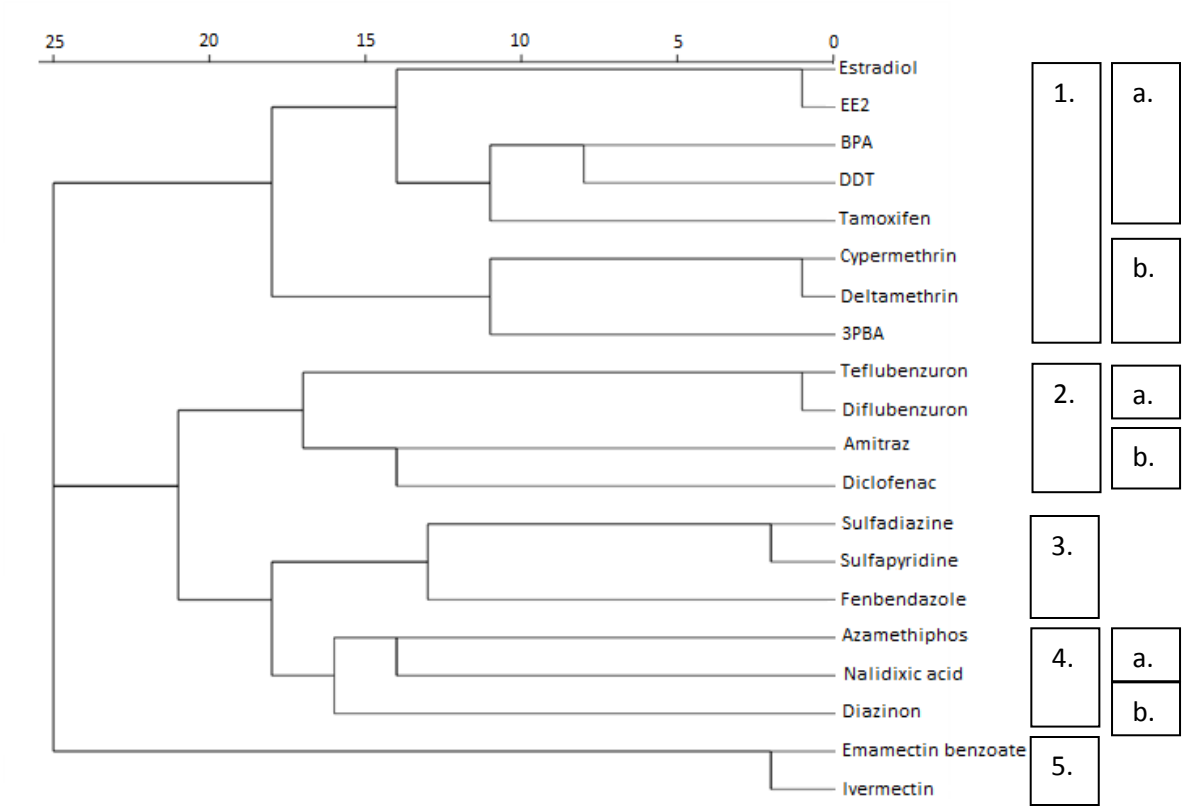
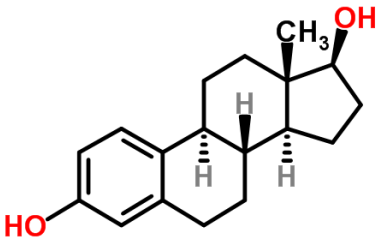


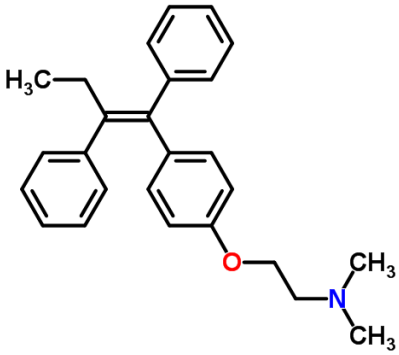
Figure 4.8 Dendrogram based on structural relationships of test VMs, E2 and EE2; known xenoestrogens DDT and BPA; and the anti-oestrogen tamoxifen using the tanimoto coefficient to determine structural similarity.

Figures 4.8 – 4.12 show the 2D structures of the 20 ligands used in the present study, sorted into the groups given by the dendrogram based on tanimoto coefficient for structural similarity (figure 4.9). Group 1a (figure 4.9 [1a]) contains the five control substances. All of these structures have a phenolic ring (except DDT which has a chlorobenzene ring instead) which is thought to contribute to strong binding within the binding pocket (McCullough *et al.*, 2014). Group 1b contains the synthetic pyrethroids deltamethrin, α-cypermethrin and their metabolite 3PBA. These three structures all have diphenyl ether in common (figure 4.9 [1b]).

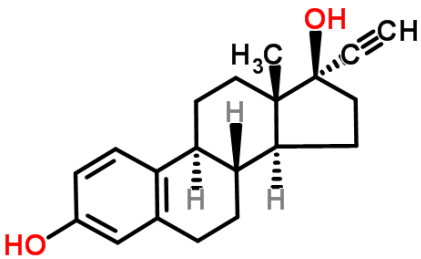
Group 1a



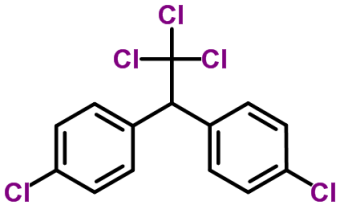
E2



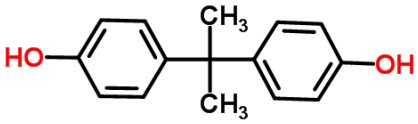
Tamoxifen



EE2

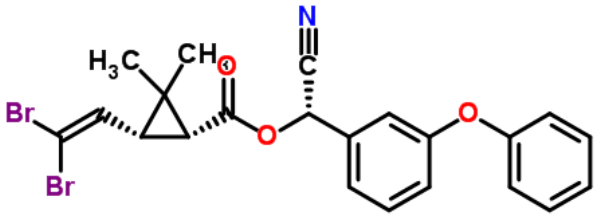


DDT

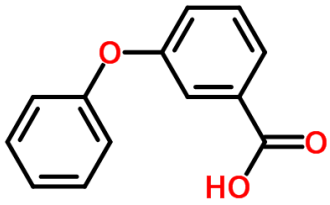


BPA

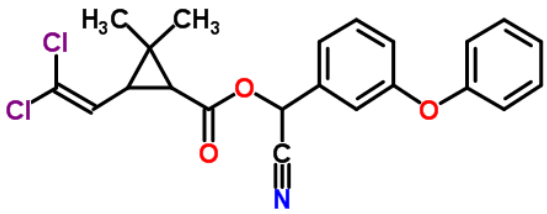
Group 1b



Deltamethrin



3PBA

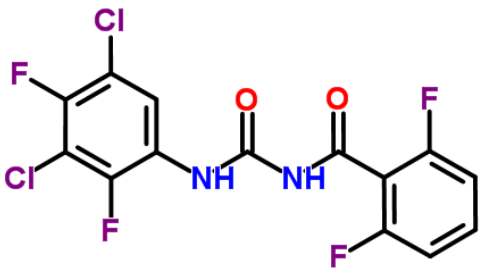


Cypermethrin

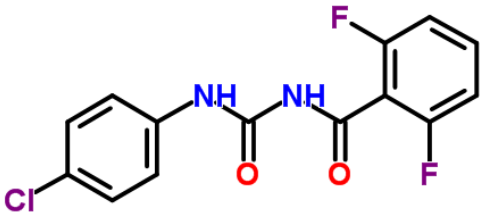
Figure 4.9 2D structures of VMs and controls in group 1a and group 1b according to dendrogram based on structural similarity (figure 4.8). Source: ChemSpider.

Group 2a contains the two benzoylurea VMs, teflubenzuron and diflubenzuron which both share a 2,6-difluorobenzamide (figure 4.9 [2a]). Group 2b contained amitraz and diclofenac, which were not closely related (figure 4.8), they share two benzene rings and a nitrogen (figure 4.10 [2b]).

Group 2a

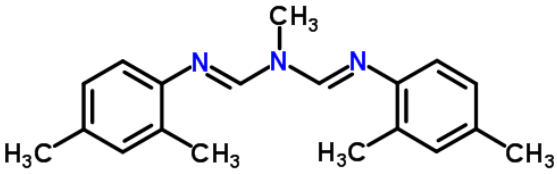


Teflubenzuron

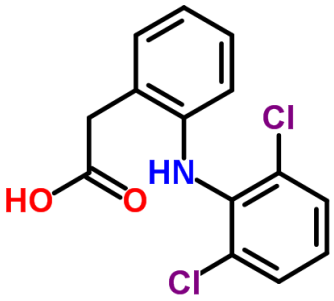


Diflubenzuron

Group 2b



Amitraz

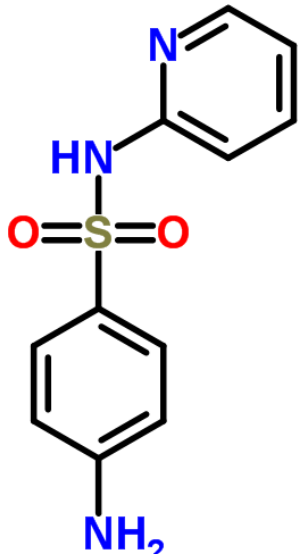


Diclofenac

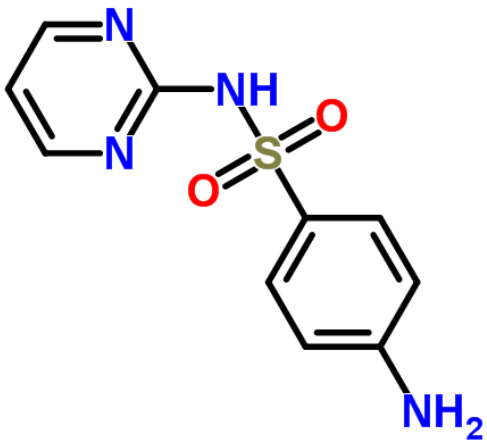
Figure 4.10 Structures of VMs in group 2a and group 2b according to dendrogram based on structural similarities (figure 4.8). Source: ChemSpider.

Group 3 contained the antibiotics sulfadiazine and sulfapyridine as well as benzimidazole anthelmintic, fenbendazole. These three VMs share a phenylsulfanyl (figure 4.10).

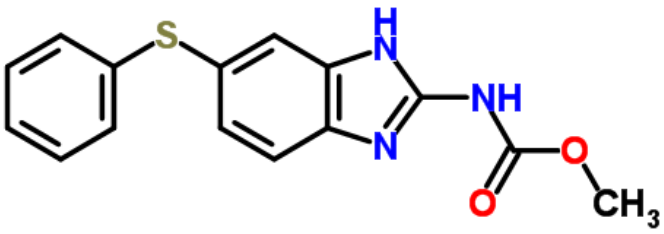
Group 3



Sulfapyridine



Sulfadiazine

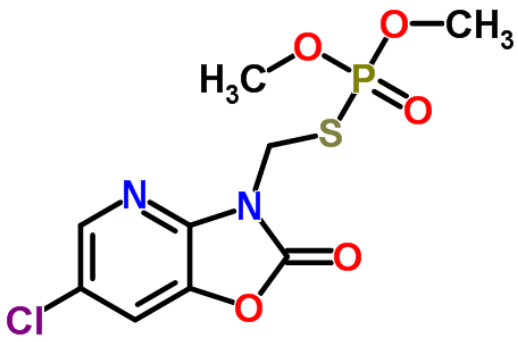


Fenbendazole

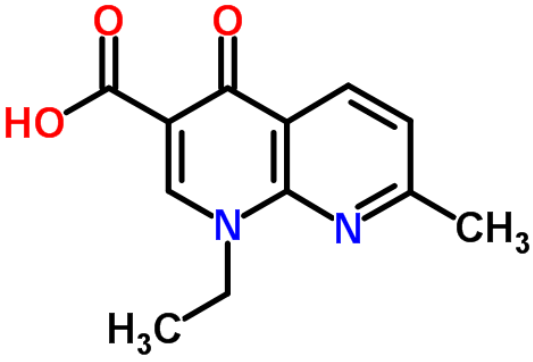
Figure 4.11 Structures of VMs in group 3 according to dendrogram based on 2D structural similarities (figure 4.8). Source: ChemSpider.

Group 4 (figure 4.12) contained three VMs that were not that closely related (figure 4.8). Azamethiphos and diazinon share a phosphorothioate group. While azamethiphos and nalidixic acid share a nitrite group.

Group 4a

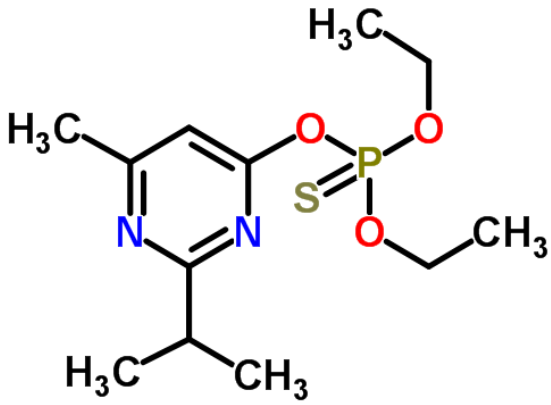


Azamethiphos



Nalidixic acid

Group 4b

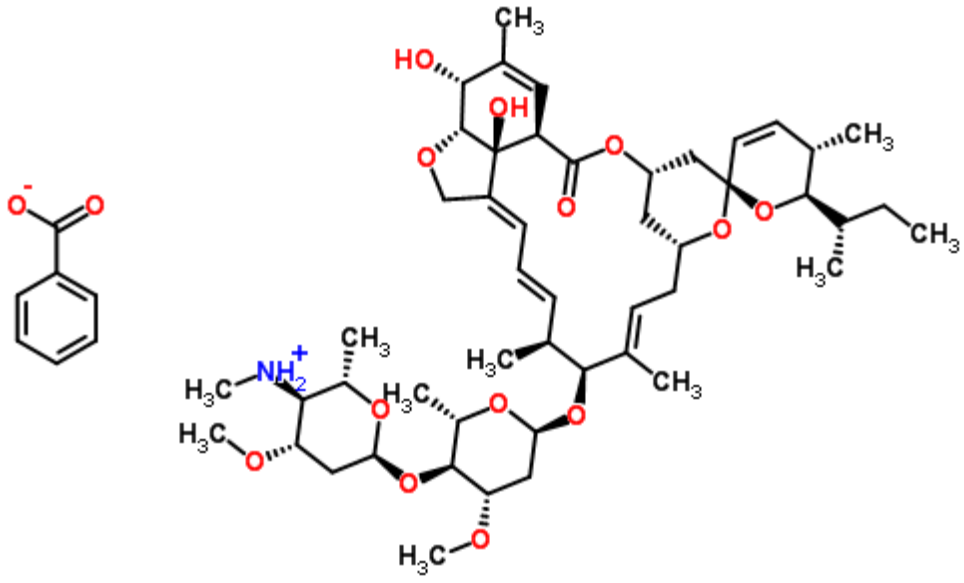


Diazinon

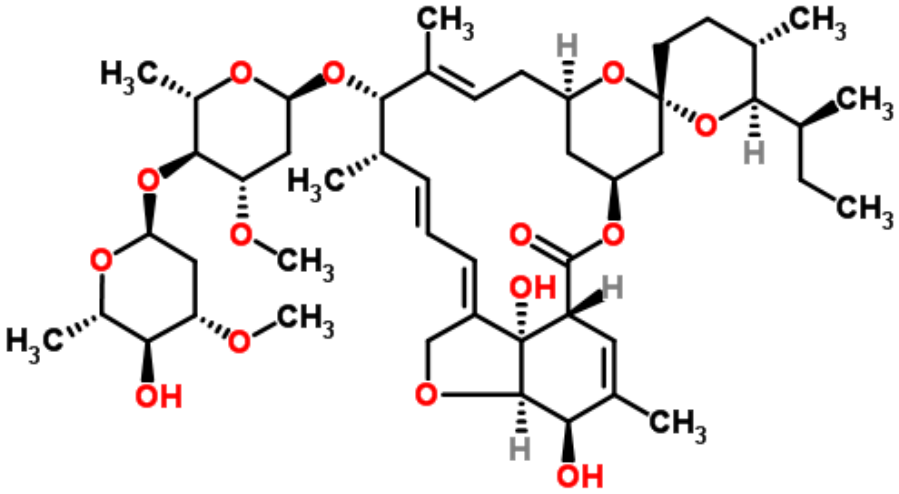
Figure 4.12 Structures of VMs in group 4a and group 4b according to dendrogram based on 2D structural similarity (figure 4.8). Source: ChemSpider.

Group 5 contained the avermectins which both have a (16) annulene ($C_{16}N_{16}$) at their core (figure 4.13).

Group 5



Emamectin benzoate



Ivermectin

Figure 4.13 Structure of the VMs in group 5 according to dendrogram based on 2D structural similarity (figure 4.8). Source: ChemSpider.

A further dendrogram was constructed to examine whether a structural relationship was related to lowest binding energy (highest affinity) within the biggest cluster. This was determined by taking the average of the lowest binding energy (highest affinity) in the biggest cluster from all test species (excluding the negative control *C. gigas*). Five distinct groups were observed from this dendrogram (figure 4.14). Group 1, which had two sub-groups, contained the oestrogen E2, synthetic oestrogen EE2, the anti-oestrogen tamoxifen (figure 4.14 [1a]) and the synthetic pyrethroids α-cypermethrin and deltamethrin (figure 4.14 [1b]). Group 2 contained the known

xenoestrogens DDT and BPA and the benzimidazole anthelmintic, fenbendazole (figure 4.14 [2]). Group 3, which had two sub-groups, contained the organophosphates azamethiphos and diazinon, the NSAID diclofenac, the antibiotics sulfadiazine and sulfapyridine (figure 4.14 [3a]); the formadine pesticide, amitraz and benzoylurea insecticide diflubenzuron (figure 4.14 [3b]). Group 4, which had two sub-groups, contained the synthetic pyrethroid degradation product, 3PBA; the antibiotic nalidixic acid (figure 4.14 [4a]); and the benzoylurea insecticide, teflubenzuron (figure 4.14 [4b]). Group 5, which is not included in figure 4.6, contained the avermectins, emamectin benzoate and ivermectin. These compounds were not included in figure 4.6 as their binding was < -5, which would have significantly skewed the dendrogram results.

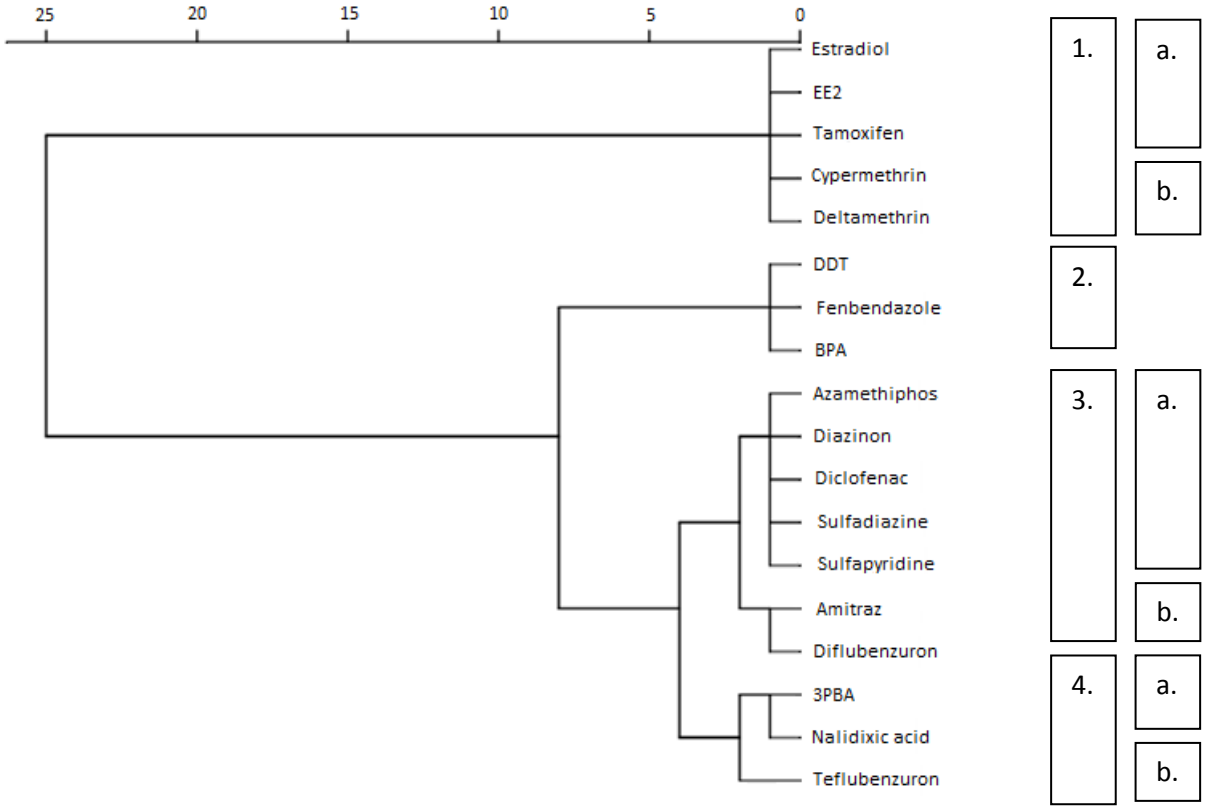


Figure 4.14 Dendrogram representing the relationship between substances between the eight test species and the positive control *H. sapiens* in terms of lowest binding energy (highest affinity) in the biggest cluster. Emamectin benzoate and ivermectin have been excluded due to them skewing the result.

4.3.2 Binding affinity

The VM with the lowest average binding energy (highest affinity) to the eight test receptors was deltamethrin with an average binding energy of -9.55 (±0.71) kcal/mol; this was closely followed by fellow pyrethroid α-cypermethrin which had an average binding energy of -9.42 (±0.65) kcal/mol (see tables 4.6a and 4.6b). Two VMs had an average binding affinity that was higher than

the cut off energy for a potential binder (tables 4.6a and 4.6b). These two VMs were emamectin benzoate which had an average binding energy of 338.38 (± 147.18) kcal/mol and ivermectin which had an average binding energy of 477.9 (± 203.93) kcal/mol. Since binding of emamectin benzoate and ivermectin is unlikely further investigation using these two VMs was discontinued and they were not included in statistical analysis.

The test VMs were sorted by lowest binding energy (highest affinity) in the biggest cluster. Although binding energy in deltamethrin and α-cypermethrin was lowest for all test receptors, docking produced a number of clusters with the largest cluster for α-cypermethrin being 35% of all binding conformations of α-cypermethrin to the *P. promelas* ERα. For deltamethrin the species which had the biggest cluster in terms of binding energy was the control *H. sapiens* with the biggest cluster having 26% of conformations followed by *G. aculeatus* which had a cluster of 24% of conformations.

4.3.2.1 Predicted oestrogenic potency (PEP)

The PEP of each drug to the ten ERα was calculated using equation 4.2. α-cypermethrin, deltamethrin and tamoxifen were predicted to be more potent than the native ligand E2 to the ERα of all ten species. EE2 was predicted to be more potent than E2 in *C. gigas, D. rerio, X. laevis, R. rutilus* and *G. aculeatus.* Fenbendazole was shown to have a more than 10% PEP for five of the ten species, *O. mykiss, S. salar, D. rerio, X. laevis* and *S. aurata* which was higher than the PEP for the known xenoestrogens DDT and BPA in these species (figure 4.15).

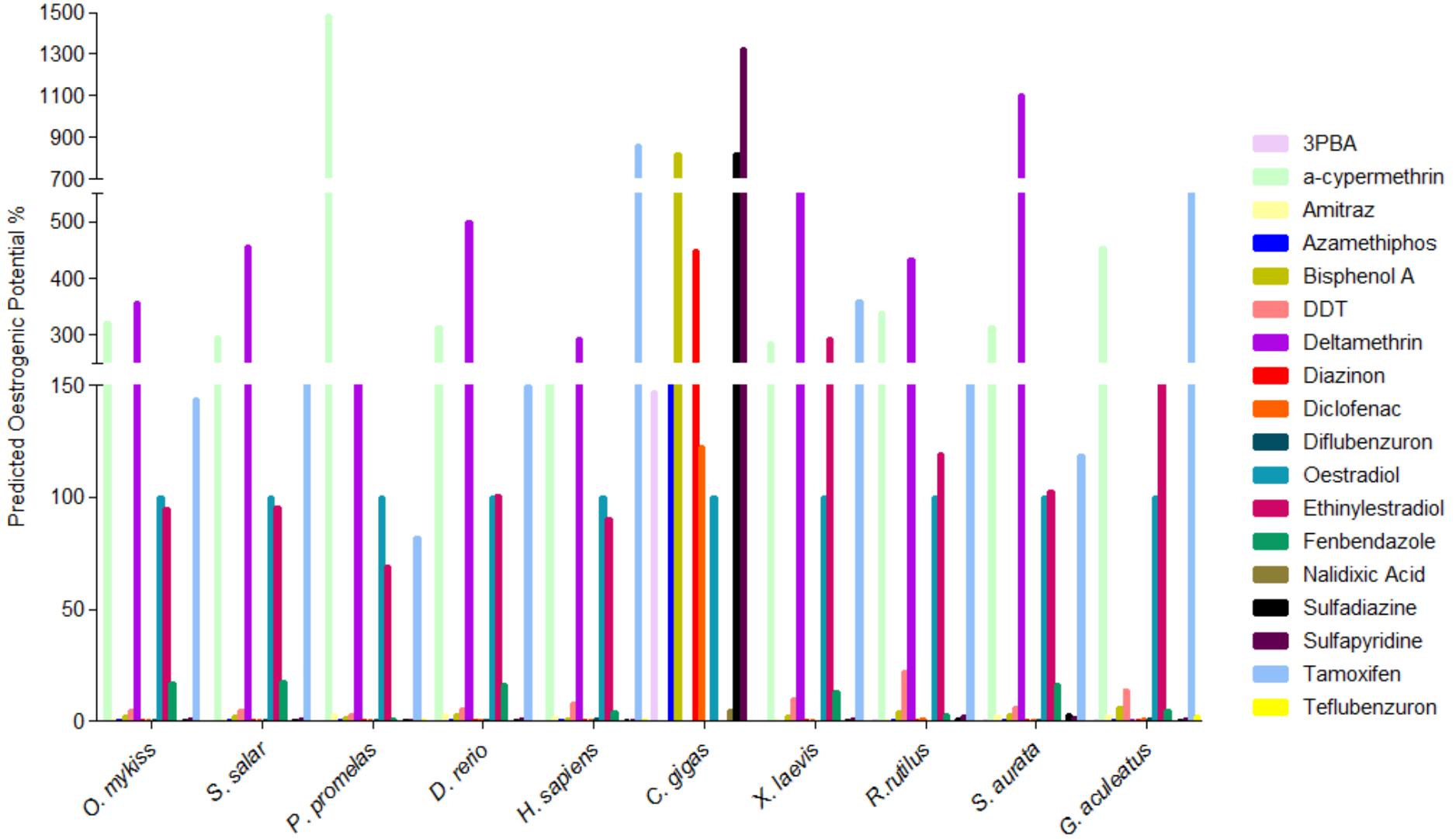


Figure 4.15 Predicted oestrogenic potency (PEP) of 18 of the test compounds. Emamectin benzoate and ivermectin were excluded due high binding and a lack of information on Ki values. PEPs were estimated using equation 4.2 using the lowest binding energy in the biggest cluster for E2 as the baseline for each species.

4.3.3 Binding affinities of selected VMs to ERα

Binding affinity of the 15 test VMs and the 5 control compounds varied between species ERα. Results on the lowest binding energy (kcal/mol) were taken from the lowest bound conformation within the biggest cluster (see figures 4.4 and 4.5). VMs and controls were categorised into compounds with a high likelihood of binding to the ERα (those with a binding energy of <-7 kcal/mol), those with a medium likelihood of binding to the ERα (those with a binding energy between -5 and -7 kcal/mol) and those with a low likelihood of binding to the ERα (those with a binding energy of > -5 kcal/mol) (table 4.6 a and 4.6b).

Emamectin benzoate and ivermectin were the only VMs that were in the low likelihood category for all ten of the tested ERα. Teflubenzuron was the only VM that appeared in all three of the likelihood categories. Teflubenzuron was in the low likelihood category for *D. rerio, S. aurata, O. mykiss S. salar, R. rutilus* and the negative control *C. gigas.* Teflubenzuron had a high likelihood of binding in the ERα of *G. aculeatus* and *H. sapiens* and a medium likelihood of binding for *P. promelas* and *X. laevis.* Three VMs were in the high likelihood of binding category for all species (excluding the negative control *C. gigas*) these were deltamethrin, α-cypermethrin and fenbendazole. All of the control substances, E2, EE2, BPA, DDT and tamoxifen were in the high likelihood of binding category for all ERα (excluding the negative control *C. gigas*). Sulfapyridine was in the high likelihood of binding category for all species ERα excluding *G. aculeatus.* Amitraz had a high likelihood of binding to five of ERα, *D. rerio, S. aurata G. aculeatus, H. sapiens,* and *P. promelas* (see tables 4.6a and 4.6b). Sulfadiazine was in the high likelihood of binding category for three species ERα, these were *R. rutilus, P. promelas* and the negative control *C. gigas.* Diclofenac was in the high likelihood of binding category for the ERα of *R. rutilus* and diflubenzuron appeared in the high likelihood of binding category for the ERα of *P. promelas.*

Table 4.6a Lowest binding energy (highest affinity) in the biggest cluster for the ERα for *D. rerio, S. aurata, O. mykiss* and *R. rutilus* . Highlighted in red are the VMs and controls that pose a high liklihood of binding to the ERα; highlighted in orange are the VMs and controls that pose a medium liklihood of binding to the ERα and highlighted in green are the VMs and controls that pose a low likelihood of binding to the ERα.

D. Rerio		***S. aurata***		***O. mykiss***		***S. salar***		***R. rutilus***	
Deltamethrin	-10.58	Deltamethrin	-10.4	Deltamethrin	-10.5	Deltamethrin	-10.64	Deltamethrin	-10.54
α-cypermethrin	-10.3	α-cypermethrin	-10.32	α-cypermethrin	-10.44	α-cypermethrin	-10.39	α-cypermethrin	-10.39
Tamoxifen	-9.86	Tamoxifen	-9.74	Tamoxifen	-9.89	Tamoxifen	-10.02	Tamoxifen	-10.2
EE2	-9.63	EE2	-9.65	E2	-9.74	E2	-9.74	EE2	-9.77
E2	-9.62	E2	-9.64	EE2	-9.71	EE2	-9.72	E2	-9.67
Fenbendazole	-8.56	Fenbendazole	-8.57	Fenbendazole	-8.27	Fenbendazole	-8.71	DDT	-8.77
DDT	-7.92	DDT	-7.96	DDT	-7.93	DDT	-7.92	BPA	-7.77
Amitraz	-7.51	BPA	-7.5	BPA	-7.53	BPA	-7.53	Fenbendazole	-7.64
BPA	-7.49	Amitraz	-7.47	Sulfapyridine	-7.01	Sulfapyridine	-7.02	Sulfapyridine	-7.37
Sulfapyridine	-7.03	Sulfapyridine	-7.07	Sulfadiazine	-6.75	Sulfadiazine	-6.75	Diclofenac	-7.1
Diflubenzuron	-6.59	Sulfadiazine	-6.75	Diflubenzuron	-6.65	Diflubenzuron	-6.65	Sulfadiazine	-7.06
Sulfadiazine	-6.55	Diflubenzuron	-6.58	Diclofenac	-6.34	Diclofenac	-6.35	Diazinon	-6.54
Diclofenac	-6.34	Diclofenac	-6.33	Amitraz	-6.31	Amitraz	-6.31	Amitraz	-6.16
Diazinon	-6.28	Diazinon	-6.24	Diazinon	-6.21	Diazinon	-6.28	Azamethiphos	-6.01
Azamethiphos	-5.85	Azamethiphos	-5.85	Azamethiphos	-5.91	Azamethiphos	-5.94	3PBA	-5.92
3PBA	-5.49	3PBA	-5.48	3PBA	-5.43	3PBA	-5.43	Nalidixic acid	-5.28
Nalidixic Acid	-4.97	Nalidixic acid	-4.96	Nalidixic Acid	-4.99	Nalidixic Acid	-4.99	Diflubenzuron	-4.92
Teflubenzuron	-4.84	Teflubenzuron	-4.95	Teflubenzuron	-4.55	Teflubenzuron	-4.78	Teflubenzuron	-4.77
Emamectin Benzoate	79.02	Emamectin Benzoate	269.09	Emamectin Benzoate	87.57	Emamectin Benzoate	87.87	Ivermectin	153.72
Ivermectin	231.51	Ivermectin	278.53	Ivermectin	238.85	Ivermectin	246.74	Emamectin Benzoate	394.06

Table 4.6b Lowest binding energy (highest affinity) in the biggest cluster for the ERα for X. laevis, G. aculeatus, H. sapiens, P. promelas and C. gigas. Highlighted in red are the VMs and controls that pose a high likelihood of binding to the ERα; highlighted in orange are the VMs and controls that pose a medium likelihood of binding to the ERα and highlighted in green are the VMs and controls that pose a low likelihood of binding to the ERα.

X. laevis		***G. aculeatus***		***H. sapiens***		***P. promelas***		***C. gigas***	
Deltamethrin	-11.14	Tamoxifen	-10.63	Tamoxifen	-11.29	α-cypermethrin	-11.95	Sulfapyridine	-8.04
Tamoxifen	-10.81	Deltamethrin	-10.44	Deltamethrin	-10.66	Deltamethrin	-10.69	BPA	-7.76
EE2	-10.69	α-cypermethrin	-10.41	α-cypermethrin	-10.28	E2	-10.35	Sulfadiazine	-7.76
α-cypermethrin	-10.68	EE2	-9.84	E2	-10.02	Tamoxifen	-10.23	Diazinon	-7.4
E2	-10.05	E2	-9.51	EE2	-9.96	EE2	-10.13	Azamethiphos	-6.85
Fenbendazole	-8.87	DDT	-8.35	DDT	-8.55	Amitraz	-8.22	3PBA	-6.74
DDT	-8.7	BPA	-7.87	Fenbendazole	-8.14	DDT	-8.22	Diclofenac	-6.63
BPA	-7.83	Fenbendazole	-7.72	Amitraz	-7.69	BPA	-7.84	E2	-6.51
Sulfapyridine	-7.33	Teflubenzuron	-7.33	BPA	-7.44	Fenbendazole	-7.79	Nalidixic Acid	-4.73
Sulfadiazine	-6.99	Amitraz	-7.29	Sulfapyridine	-7.3	Diflubenzuron	-7.32	DDT	-1.82
Diclofenac	-6.95	Diclofenac	-6.84	Diflubenzuron	-7.26	Sulfapyridine	-7.27	Deltamethrin	-1.55
Diazinon	-6.43	Sulfapyridine	-6.73	Teflubenzuron	-7.01	Sulfadiazine	-7	Fenbendazole	-1.09
Amitraz	-6.35	Diflubenzuron	-6.66	Sulfadiazine	-6.94	Diclofenac	-6.91	α-cypermethrin	0.31
3PBA	-6	Sulfadiazine	-6.5	Diclofenac	-6.56	Teflubenzuron	-6.81	EE2	1.89
Azamethiphos	-6	Diazinon	-6.38	Diazinon	-6.37	Diazinon	-6.62	Tamoxifen	10.61
Diflubenzuron	-5.77	Azamethiphos	-6.1	Azamethiphos	-6.25	Azamethiphos	-6.37	Amitraz	12.65
Nalidixic Acid	-5.49	3PBA	-5.88	3PBA	-5.53	3PBA	-6	Diflubenzuron	16.99
Teflubenzuron	-5.03	Nalidixic acid	-5.32	Nalidixic Acid	-5.27	Nalidixic Acid	-5.76	Teflubenzuron	21.15
Emamectin Benzoate	77.03	Ivermectin	197.5	Ivermectin	78.97	Emamectin Benzoate	244.89	Ivermectin	964.53
Ivermectin	368.1	Emamectin Benzoate	434.03	Emamectin Benzoate	125.93	Ivermectin	368.4	Emamectin Benzoate	1772

In order to determine whether binding of selected VMs showed intra-species variation to the ten ERα statistical analysis was performed. Using SPSS a one-way ANOVA was completed to determine any statistical differences in energy binding (kcal/mol) between VMs and between species ERα. A two-way ANOVA was performed to determine any differences between the two variables VM and species ERα. Tukey's post hoc was performed to determine if there were statistical differences in binding of each VM to each ERα.

Statistical analysis was performed on the ligands that had a sufficiently low binding energy (< -5 kcal/mol) to the ten ERα receptors. Ivermectin with an average binding affinity of 477.9 (± 203.93) kcal/mol, and emamectin benzoate with an average binding affinity of 338.38 (± 147.18) kcal/mol were excluded from statistical analysis since their binding was far higher than the cut off of -5 kcal/mol and therefore, as outliers, the addition of these two VMs in statistical analysis would have substantially skewed results.

Two-way ANOVA was performed with binding affinity as a dependent variable of species and drug. There was a statistical difference between binding affinity and species ($P < 0.001$), binding affinity and VM ($P < 0.001$) and binding affinity when compared with species by VM ($P < 0.001$).

Tukey's post hoc test was run to establish which of the species had statistically different binding affinity to individual drugs. Of the drugs tested (excluding emamectin benzoate and ivermectin) 11 had statistically different binding depending on which species ERα was targeted (table 4.7) (appendix G for full results).

Eleven ligands showed statistically different binding between at least two species (excluding the negative control *C. gigas* and the positive control *H. sapiens*). These were 3PBA, amitraz, DDT, diazinon, diclofenac, diflubenzuron, E2, EE2, fenbendazole, tamoxifen and teflubenzuron. The results of the highest and lowest binding energy of each VM along with the species ERα is presented in table 4.7.

Table 4.7 Highest average binding energy and lowest average binding energy (kcal/mol) for each VM and control ligands that had statistical difference in average binding energy between at least two species using Tukey's post hoc test (excluding the negative control *C. gigas* and the positive control *H. sapiens*).

Test VM	**Highest average binding energy (kcal/mol); species ERα**	**Lowest average binding energy (kcal/mol); species ERα**	**Number of species with a statistical difference in average binding energy (n = 8)**	**P value**
3PBA	-5.5; *S. salar*	-5.99; *P. promelas*	2	0.048
Amitraz	-5.10; *R. rutilus*	-7.36; *S. aurata*	8	< 0.001
DDT	-7.49; *O. mykiss*	-8.74; *R. rutilus*	8	< 0.001
Diazinon	-5.74; *S. aurata*	-6.41; *P. promelas*	5	0.007
Diclofenac	-6.29; *S. aurata*	-6.94; *R. rutilus*	7	0.008
Diflubenzuron	-4.77; *R. rutilus*	-6.62; *G. aculeatus*	8	< 0.001
E2	-9.51; *G. aculeatus*	-10.35; *P. promelas*	8	0.001
EE2	-9.63; *D. rerio*	-10.69; *X. laevis*	8	< 0.001
Fenbendazole	-7.6; *G. aculeatus*	-8.23; *X. laevis*	5	0.01
Tamoxifen	-8.25; *P. promelas*	-10.10; *G. aculeatus*	8	< 0.001
Teflubenzuron	-4.14; *R. rutilus*	-7.09; *G. aculeatus*	8	< 0.001

4.3.3.1 E2 and Tamoxifen

E2, the native ligand to ERα acted as one of the controls. Binding of E2 to seven of the eight test ERα were in a similar conformation and affinity to the control *H. sapiens* which had the lowest binding in the biggest cluster of -10.02 kcal/mol (figure 4.16). Binding energy of E2 to *G. aculeatus* ERα was significantly higher than the binding energy of *H. sapiens* ERα (-9.51 kcal/mol; P = 0.039). Binding energy of E2 was significantly higher in the negative control *C. gigas* than all test ERα and *H. sapiens* (-6.47 kcal/mol; P <0.001) (figure 4.17).

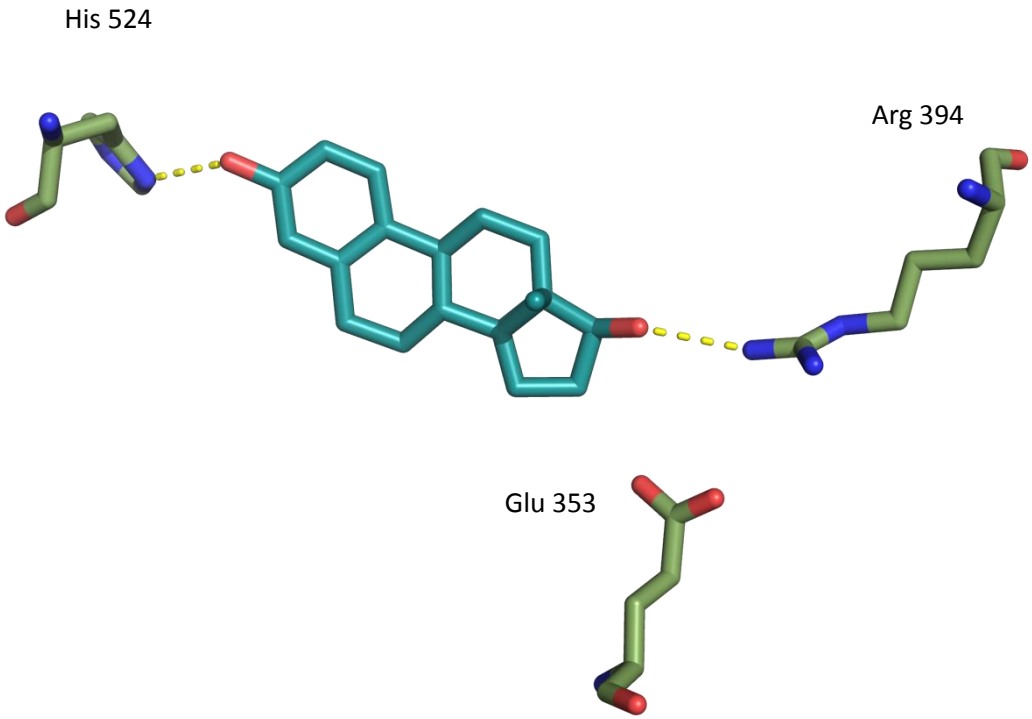


Figure 4.16 The native ligand E2 (blue ligand) bound to the control *H. sapiens* ERα (khaki green receptors). The binding energy was -10.02 kcal/mol for all 100 conformations. Yellow dashed line shows hydrogen bonds.

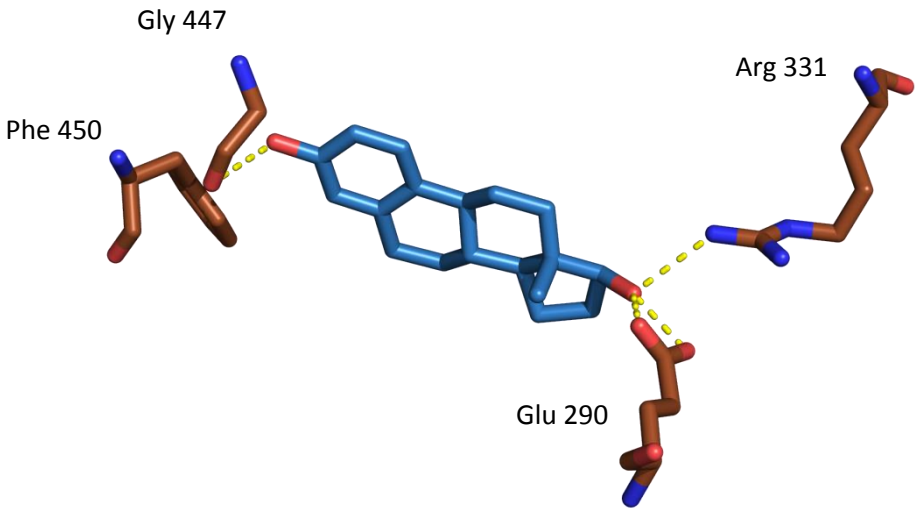


Figure 4.17 The native ligand E2 (blue ligand) bound to the negative control *C. gigas* ERα (brown receptors). All 100 conformations bound forming one cluster, lowest binding (highest affinity) was -6.51. Yellow dashed lines represent hydrogen bonds.

Tamoxifen was used as an antagonist, or anti-oestrogen control. Tamoxifen bound to the *H. sapiens* ERα with a significantly lower binding energy than all other test ERα (-11.29 kcal/mol) (figure 4.18).

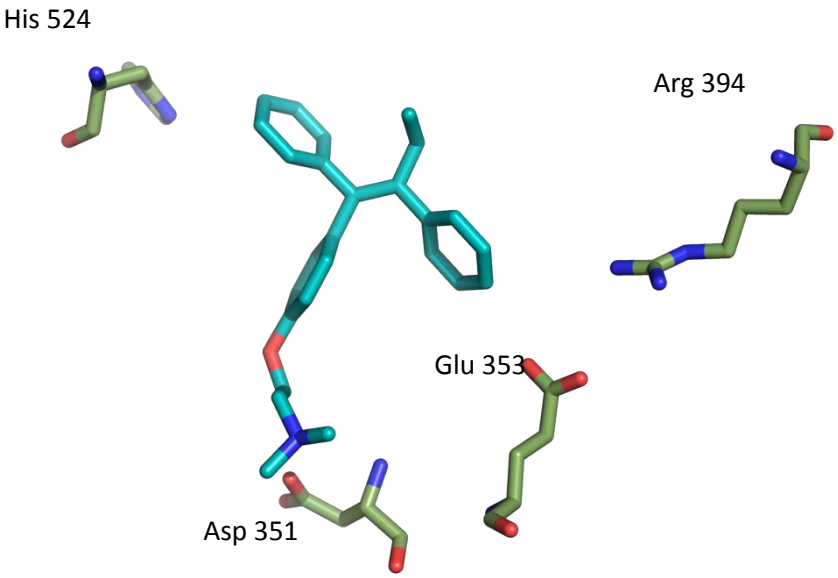


Figure 4.18 Lowest binding in the biggest cluster of tamoxifen (blue ligand) bound to *H. sapiens* ERα (khaki green resides). Binding energy, -11.29 kcal/mol.

4.3.3.1 Deltamethrin

Deltamethrin bound with a 'high likelihood' to all ERα (excluding *C. gigas*) (tables 4.6a and 4.6b). Deltamethrin was the VM that had the lowest binding energy to all of the test receptors. Despite the low binding energy, the biggest cluster of deltamethrin within one docking run was 26% of all binding conformations in *H. sapiens* and 24% in *G. aculeatus* which could suggest unspecific binding, which could suggest that deltamethrin has both agonistic and antagonistic properties. Lowest binding in the biggest cluster showed conformations between receptors that were variable, leading to the possibility of unspecific binding (figure 4.19).

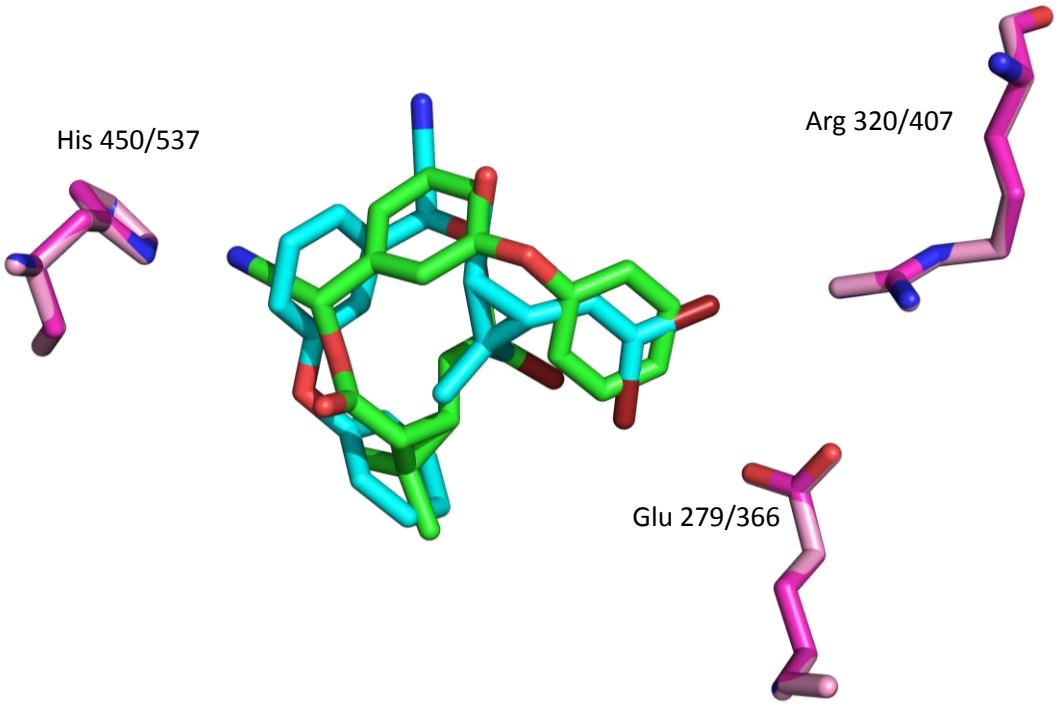


Figure 4.19 Lowest binding (highest affinity) within the biggest cluster for deltamethrin in ERα for *S. salar* (light pink residues, blue ligand) and *O. mykiss* (dark pink residues, green ligand). The binding energy for deltamethrin to *S. salar* ERα was -10.64 kcal/mol and for *O. mykiss* ERα was -10.5 kcal/mol.

4.3.3.2 α-cypermethrin

α-cypermethrin bound with a 'high likelihood' to all ERα (excluding *C. gigas*) (tables 4.6a and 4.6b). The VM with the second lowest binding energy to ERα for all species, apart from the negative control *C. gigas* was α-cypermethrin (figure 4.20). The largest binding cluster for cypermethrin was 35% of binding conformations; this was in the *P. promelas* ERα. As with deltamethrin (figure 4.19), the unspecific nature of α – cypermethrin binding could suggest that the VM has both agonistic and antagonistic properties.

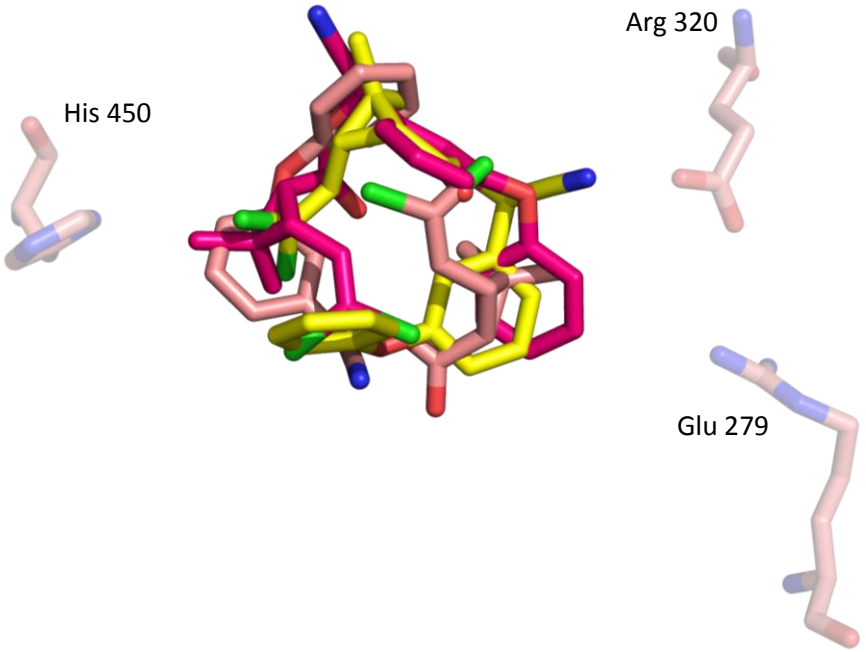


Figure 4.20 Lowest binding energy (highest affinity) within the biggest cluster for α-cypermethrin in ERα for *P. promelas* (yellow ligand), *S. salar* (light pink ligand) and *R. rutilus* (dark pink ligand). Glu 279, Arg 320 and His 450 are included to show context and are *S. salar* residues (faded pink). The binding energy for α-cypermethrin to *P. promelas* ERα was -11.95, to *S. salar* ERα was -10.39 and to *R. rutilus* ERα was -10.39

4.3.3.3 Teflubenzuron

Teflubenzuron had the biggest range of binding between receptors and was the only VM to be included in all three likelihood categories (tables 4.6a and 4.6b). The highest average binding affinity was for *R. rutilus* at -4.14 (± 0.43) kcal/mol which did not show hydrogen bonds with amino acid side chains (see table 4.14). The lowest average binding affinity was for *G. aculeatus* at -7.09 (± 0.16) kcal/mol, which showed hydrogen bonding with the polar amino acid threonine in position 334 (table 4.11). Despite the differences in binding energy, teflubenzuron bound to the ERα of both *G. aculeatus* and *R. rutilus* in a similar conformation (figure 4.21).

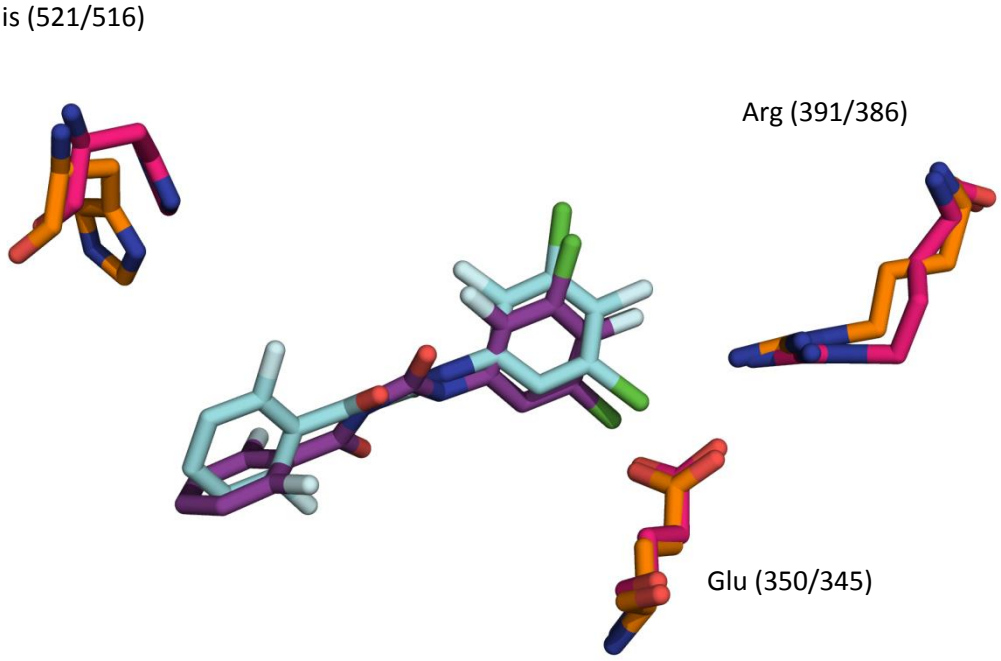


Figure 4.21 Teflubenzuron bound to *G. aculeatus* (purple ligand, orange residues) and *R. rutilus* (blue ligand, dark pink residues). Conformations shown are the lowest energy within the biggest cluster.

4.3.3.4 Fenbendazole

The receptor with the highest average binding to fenbendazole was that of *G. aculeatus* -7.6 (± 0.13) kcal/mol. There was no evidence of hydrogen bonds between fenbendazole and any of the residues within the binding pocket of *G. aculeatus* ERα (table 4.11). The binding of fenbendazole to *G. aculeatus* was significantly higher than the binding energy in *O. mykiss, S. salar* and *X. laevis* ($P < 0.05$). The receptor with the lowest binding energy (highest affinity) to fenbendazole was *X. laevis* at -8.23 (± 0.44) kcal/mol. There was evidence for hydrogen bonding between fenbendazole and Arg 386 and Glu 345 in the *X. laevis* ERα (figure 4.22; table 4.17).

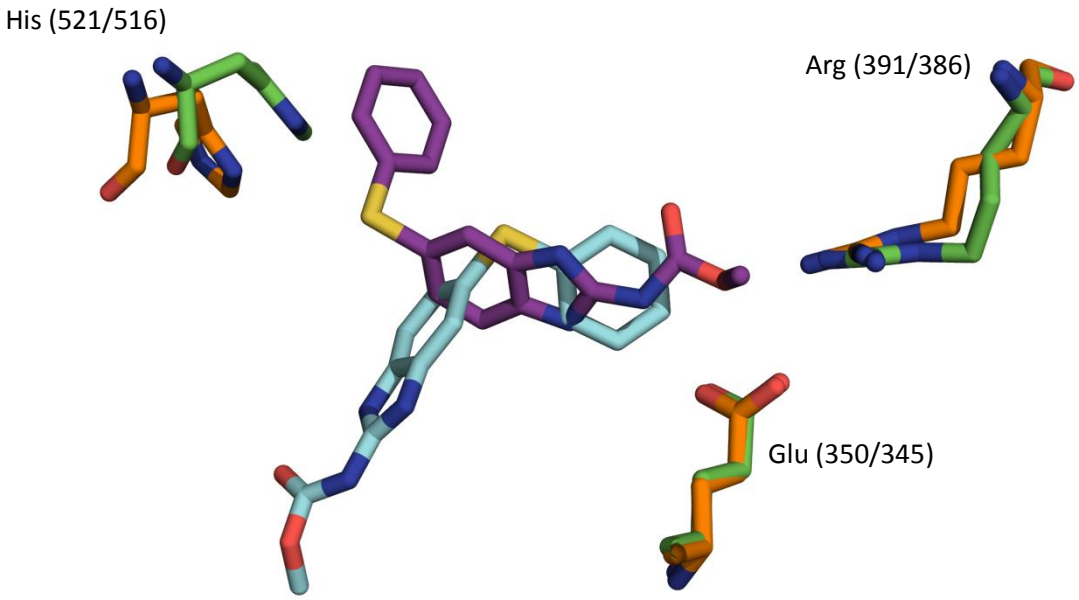


Figure 4.22 Fenbendazole bound to *G. aculeatus* (purple ligand, orange residues) and *X. laevis* (blue ligand, green residues). Conformations shown are the lowest energy within the biggest cluster.

4.3.3.5 Amitraz

Amitraz bound with a 'high likelihood' affinity to ERα LBD of *P. promelas, G. aculeatus, D. rerio* and *H. sapiens* (see table 4.6a and 4.6b). There were significant differences in the energy binding of amitraz to a number of the ERα (see table 4.6a, 4.6b and 4.7), including a significant difference in binding between two species with a highly conserved ERα (P <0.001), these were *S. salar* and *D. rerio* which had a whole sequence similarity of 75.6% (identity of 65.4%) (see table 4.5) and a LBD sequence similarity of 90.6% (identity of 82.8%) (figure 4.2). Amitraz bound to the *D. rerio* ERα with an average energy of -7.35 kcal/mol (± 0.11). There was no evidence of hydrogen bonds between amitraz and any of the residues within the binding pocket of *D. rerio* (table 4.10). Amitraz bound to the *S. salar* ERα with an average energy of -6.03 kcal/mol (± 0.54) (figure 4.23). There was no evidence of hydrogen bonding between amitraz and any residues within the binding pocket for *S. salar* ERα (table 4.15).

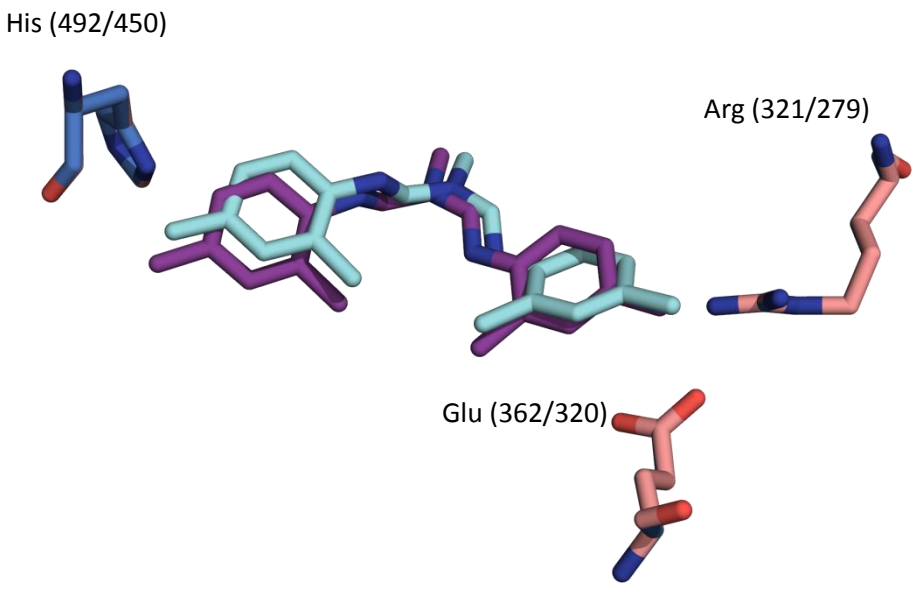


Figure 4.23 Amitraz bound to *D. rerio* (purple ligand; overlapping dark blue residues) and *S. salar* (light blue residue; overlapping pink residues). The position of the residues in the LBD of *D. rerio* and *S. salar* are so similar that the residues overlap making it difficult to distinguish between the two.

4.3.4 Hydrogen bonding

Nine of the twenty tested compounds (7 of 15 VMs; 2 of 5 controls) showed hydrogen bonding capabilities with the negative control *C. gigas*. Of these, azamethiphos, E2 and EE2 bound to both Glu 290 and Arg 331. α-cypermethrin, sulfadiazine and sulfapyridine bound to Glu 290. Emamectin benzoate, ivermectin and 3PBA bound to other residues within the binding pocket. None of the tested VMs had hydrogen bonding with Phe 450 (table 4.8). The VMs that did not have any evidence of hydrogen bonding to *C. gigas* ERα were: amitraz, BPA, DDT, deltamethrin, diazinon, diclofenac, diflubenzuron, fenbendazole, nalidixic acid, tamoxifen and teflubenzuron.

Table 4.8 Residues with hydrogen bonding for VMs and controls to *C. gigas* ERα (negative control). All results are from the lowest bound conformation in the biggest cluster.

VM	**Glu 290**	**Arg 331**	**Phe 450**	**Glu 348**	**Others**	**Binding energy (kcal/Mol)**
3PBA	-	-	-	-	Gly 447	-6.74
Azamethiphos	+	+	-	-	Leu 283, 324	-6.85
α-cypermethrin	+	-	-	-	Phe 342	0.31
E2	+	+	-	-	Gly 447	-6.51
EE2	+	+	-	-	Phe 342	1.89
Emamectin benzoate	-	-	-	-	Ala 287	1772
Ivermectin	-	-	-	-	Leu 324, 325	964.53
Sulfadiazine	+	-	-	-	Phe 342	-7.76
Sulfapyridine	+	-	-	-	Phe 342	-8.04

Thirteen of the twenty tested compounds (15 VMs and 5 controls) showed hydrogen bonding with residues within the control ERα of *H. sapiens.* Of these, fenbendazole showed hydrogen bonding with Asp 351, a residue thought to be important for antagonistic effects (Heldring *et al.*, 2007). Azamethiphos, 3PBA and ivermectin bound to Arg 394; EE2 bound to Glu 353; emamectin benzoate, sulfadiazine and sulfapyridine bound to His 524; E2 bound to both Arg 394 and His 524; nalidixic acid bound to both Glu 353 and Arg 394. BPA, deltamethrin, and teflubenzuron bound to other residues within the binding pocket (table 4.9). The VMs that did not have any evidence of hydrogen bonding to *H. sapiens* ERα were: amitraz, α-cypermethrin, DDT, diazinon, diclofenac, diflubenzuron, and tamoxifen.

Table 4.9 Residues with hydrogen bonding for VMs and controls to *H. sapiens* ERα (positive control). All results are from the lowest bound conformation in the biggest cluster.

VM	**Glu 353**	**Arg 394**	**His 524**	**Asp 351**	**Others**	**Binding energy (kcal/Mol)**
3PBA	-	+	-	-	Phe 404	-5.53
Azamethiphos	-	+	-	-	-	-6.25
BPA	-	-	-	-	Leu 346	-7.44
Deltamethrin	-	-	-	-	Gly 420	-10.66
E2	-	+	+	-	-	-10.02
EE2	+	-	-	-	Thr 347	-9.96
Emamectin benzoate	-	-	+	-	Ala 350, Gly 420, Met 421	125.93
Fenbendazole	-	-	-	+	Thr 347	-8.14
Ivermectin	-	+	-	-	Met 388	78.97
Nalidixic acid	+	+	-	-	-	-5.27
Sulfadiazine	-	-	+	-	Gly 521	-6.94
Sulfapyridine	-	-	+	-	Gly 521	-7.3
Teflubenzuron	-	-	-	-	Thr 347	-7.01

D. rerio ERα showed hydrogen bonding with eleven of the tested VMs. None of the tested VMs bound to *D. rerio* ERα Asp 319. Emamectin benzoate and ivermectin bound to Glu 321; 3PBA, BPA and EE2 bound to Arg 362; deltamethrin, sulfadiazine and sulfapyridine bound to His 492; fenbendazole bound to both Glu 321 and Arg 362. E2 did not bind to any of the residues thought to be important within the LBD of ERα, it did however, form a hydrogen bind with the residue Leu 353 (table 4.10). The VMs that did not have any evidence of hydrogen bonding to *D. rerio* ERα were: amitraz, azamethiphos α-cypermethrin, DDT, diazinon, diclofenac, diflubenzuron, tamoxifen and teflubenzuron.

Table 4.10 Residues with hydrogen bonding for VMs and controls in *D. rerio* ERα. All results are from the lowest bound conformation in the biggest cluster.

VM	**Glu 321**	**Arg 362**	**His 492**	**Asp 319**	**Others**	**Binding energy (kcal/Mol)**
3PBA	-	+	-	-	Glu 345	-5.49
BPA	-	+	-	-	Leu 355	-7.49
Deltamethrin	-	-	+	-	-	-10.58
E2	-	-	-	-	Leu 353	-9.62
EE2	-	+	-	-	Leu 353	-9.63
Emamectin benzoate	+	-	-	-	Leu 352, 355	79.02
Fenbendazole	+	+	-	-	Leu 355	-8.56
Ivermectin	+	-	-	-	-	231.51
Nalidixic acid	-	-	-	-	Leu 314	-4.97
Sulfadiazine	-	-	+	-	Gly 489	-6.55
Sulfapyridine	-	-	+	-	Gly 489	-7.03

Fourteen test VMs showed hydrogen bonding to residues within the *G. aculeatus* ERα. Fenbendazole and ivermectin formed hydrogen bonds with Asp 348, the residue thought to be important for antagonistic binding to the ERα (Heldring *et al.*, 2007). E2 bound to Glu 350; 3PBA, BPA and diazinon bound to Arg 391; EE2, nalidixic acid, sulfadiazine and sulfapyridine formed hydrogen bonds with Glu 350 and Arg 391. Deltamethrin, diclofenac, and teflubenzuron did not form hydrogen bonds with any of the residues important for binding with the *G. aculeatus* ERα. None of the tested VMs bound to His 521 (table 4.11). The VMs that did not have any evidence of hydrogen bonding to *G. aculeatus* ERα were: amitraz, BPA, α-cypermethrin, DDT, diflubenzuron and tamoxifen.

Table 4.11 Residues with hydrogen bonding for VMs and controls in *G. aculeatus* ERα. All results are from the lowest bound conformation in the biggest cluster.

VM	**Glu 350**	**Arg 391**	**His 521**	**Asp 348**	**Others**	**Binding energy (kcal/Mol)**
3PBA	-	+	-	-	Leu 384	-5.88
Azamethiphos	-	+	-	-	Leu 384	-6.1
Deltamethrin	-	-	-	-	Gly 417	-10.44
Diazinon	-	+	-	-	-	-6.38
Diclofenac	-	-	-	-	Leu 343	-6.84
E2	+	-	-	-	Glu 350, Phe 401	-9.51
EE2	+	+	-	-	Phe 401	-9.84
Emamectin benzoate	-	-	-	-	Met 339, 340; Phe 401	434.03
Fenbendazole	-	-	-	+	Leu 343	-7.72
Ivermectin	-	-	-	+	Thr 344, Leu 343, 384; Ala 347, Met 385	197.5
Nalidixic acid	+	+	-	-	-	-5.32
Sulfadiazine	+	+	-	-	Leu 384	-6.5
Sulfapyridine	+	+	-	-	Leu 384	-6.73
Teflubenzuron	-	-	-	-	Leu 343, Thr 344	-7.33

Eleven VMs showed hydrogen bonding with *O. mykiss* ERα. EE2 bound to Glu 366; fenbendazole, ivermectin and tamoxifen bound to Arg 407; sulfadiazine and sulfapyridine bound to His 537; emamectin benzoate bound to Glu 366 and Arg 407; 3PBA, E2 and nalidixic acid did not bind to any of the residues thought to be important for binding. None of the VMs formed a hydrogen bond with Asp 364 (table 4.12). The VMs that did not have any evidence of hydrogen bonding to *O. mykiss* ERα were: amitraz, azamethiphos, α-cypermethrin, BPA, DDT, diazinon, diclofenac, diflubenzuron and teflubenzuron.

Table 4.12 Residues with hydrogen bonding to VMs and controls in the *O. mykiss* ERα. All results are from the lowest bound conformation in the biggest cluster.

VM	**Glu 366**	**Arg 407**	**His 537**	**Asp 364**	**Others**	**Binding energy (kcal/Mol)**
3PBA	-	-	-	-	Gly 534	-5.43
Deltamethrin	-	-	+	-	-	-10.5
E2	-	-	-	-	Leu 400, Gly 534	-9.74
EE2	+	-	-	-	Leu 400	-9.71
Emamectin benzoate	+	+	-	-	Met 401	87.57
Fenbendazole	-	+	-	-	-	-8.27
Ivermectin	-	+	-	-	Met 401	238.85
Nalidixic acid	-	-	-	-	Leu 359	-4.99
Sulfadiazine	-	-	+	-	Leu 359, Gly 534	-6.75
Sulfapyridine	-	-	+	-	Leu 359, Gly 534	-7.01
Tamoxifen	-	+	-	-	-	-9.89

Fifteen of the tested VMs bound to *P. promelas* ERα. Amitraz, EE2, emamectin benzoate, nalidixic acid, tamoxifen and teflubenzuron bound to Arg 405; E2, sulfadiazine and sulfapyridine bound to His 535; BPA, diflubenzuron and fenbendazole bound to both Glu 364 and Arg 405; 3PBA, azamethiphos and ivermectin formed hydrogen bonds with other residues surrounding the binding pocket. None of the VMs bound the Asp 362 (table 4.13). The VMs that did not have any evidence of hydrogen bonding to *P. promelas* ERα were: α-cypermethrin, DDT, deltamethrin, diazinon and diclofenac.

Table 4.13 Residues with hydrogen bonding to VMs and controls in the *P. promelas* ERα. All results are from the lowest bound conformation in the biggest cluster.

VM	**Glu 364**	**Arg 405**	**His 535**	**Asp 362**	**Others**	**Binding energy (kcal/Mol)**
3PBA	-	-	-	-	Thr 358	-6
Amitraz	-	+	-	-	Leu 398	-8.22
Azamethiphos	-	-	-	-	Gly 532	-6.37
BPA	+	+	-	-	Leu 398	-7.84
Diflubenzuron	+	+	-	-	Pro 336	-7.32
E2	-	-	+	-	Leu 398, Gly 532	-10.35
EE2	-	+	-	-	Leu 398	-10.13
Emamectin benzoate	-	+	-	-	Pro 336	244.89
Fenbendazole	+	+	-	-	-	-7.79
Ivermectin	-	-	-	-	Ile 435	368.4
Nalidixic acid	-	+	-	-	Leu 398	-5.76
Sulfadiazine	-	-	+	-	Gly 532, Leu 357	-7
Sulfapyridine	-	-	+	-	Gly 532, Leu 357	-7.27
Tamoxifen	-	+	-	-	-	-10.23
Teflubenzuron	-	+	-	-	-	-6.81

Fourteen of the tested VMs and control substances bound to *R. rutilus* ERα. Amitraz, cypermethrin, deltamethrin, sulfadiazine and sulfapyridine formed a hydrogen bond with Glu 345; azamethiphos bound to Arg 386; 3PBA, BPA, E2, EE2, ivermectin and nalidixic acid formed a hydrogen bond with both Glu 345 and Arg 386; diclofenac, diflubenzuron and emamectin benzoate formed hydrogen bonds with other residues within the binding pocket. None of the VMs or controls formed hydrogen bonds with His 516 or Asp 343 (table 4.14). The VMs that did not have any evidence of hydrogen bonding to *R. rutilus* ERα were: amitraz, DDT, diazinon, fenbendazole, tamoxifen and teflubenzuron.

Table 4.14 Residues with hydrogen bonding to VMs and controls in the *R. rutilus* ERα. All results are from the lowest bound conformation in the biggest cluster.

VM	**Glu 345**	**Arg 386**	**His 516**	**Asp 343**	**Others**	**Binding energy (kcal/Mol)**
3PBA	+	+	-	-	-	-5.92
Azamethiphos	-	+	-	-	-	-6.01
BPA	+	+	-	-	Phe 396	-7.77
α-cypermethrin	+	-	-	-	-	-11.95
Deltamethrin	+	-	-	-	-	-10.54
Diclofenac	-	-	-	-	Leu 338	-7.1
Diflubenzuron	-	-	-	-	Leu 338	-4.92
E2	+	+	-	-	Gly 513	-9.67
EE2	+	+	-	-	-	-9.77
Emamectin benzoate	-	-	-	-	Ala 342	394.06
Ivermectin	+	+	-	-	Met 380	153.72
Nalidixic acid	+	+	-	-	Leu 379	-5.28
Sulfadiazine	+	-	-	-	Leu 338	-7.06
Sulfapyridine	+	-	-	-	Leu 338	-7.37

Twelve VMs and control substances formed hydrogen bonds to *S. salar* ERα. Emamectin benzoate and ivermectin bound to Glu 279; EE2 and tamoxifen bound to Arg 320; azamethiphos, sulfadiazine and sulfapyridine bound to His 450; fenbendazole bound to both Glu 279 and Arg 320; 3PBA, BPA and nalidixic acid did not bind to any of the key residues, but formed hydrogen bonds with other residues within the binding pocket. None of the VMs or controls bound to Asp 277 (table 4.15). The VMs that did not have any evidence of hydrogen bonding to *S. salar* ERα were: Amitraz, BPA, α-cypermethrin, DDT, deltamethrin, diazinon, diclofenac, diflubenzuron and teflubenzuron.

Table 4.15 Residues with hydrogen bonding to VMs and controls in the *S. salar* ERα. All results are from the lowest bound conformation in the biggest cluster.

VM	**Glu 279**	**Arg 320**	**His 450**	**Asp 277**	**Others**	**Binding energy (kcal/Mol)**
3PBA	-	-	-	-	Gly 447	-5.43
Azamethiphos	-	-	+	-	Gly 447, Leu 451	-5.94
E2	-	-	-	-	Leu 313, Gly 447	-9.74
EE2	-	+	-	-	Leu 313	-9.72
Emamectin benzoate	+	-	-	-	Leu 313	87.87
Fenbendazole	+	+	-	-	Leu 313	-8.71
Ivermectin	+	-	-	-	Leu 313	246.74
Nalidixic acid	-	-	-	-	Leu 272	-4.99
Sulfadiazine	-	-	+	-	Leu 272, Gly 447	-6.75
Sulfapyridine	-	-	+	-	Leu 272, Gly 447	-7.02
Tamoxifen	-	+	-	-	-	-10.02

Ten VMs and control substances formed hydrogen bonds with *S. aurata* ERα. EE2 and tamoxifen bound to Arg 356; sulfadiazine and sulfapyridine bound to His 486; fenbendazole bound to both Glu 315 and Arg 356; 3PBA, E2, Emamectin benzoate and nalidixic acid bound to residues within the LBD but not those that are thought to be important for ligand binding. None of the VMs or controls bound to Asp 313 (table 4.16). The VMs that did not have any evidence of hydrogen bonding to *S. aurata* ERα were: amitraz, azamethiphos, α-cypermethrin, BPA, DDT, deltamethrin, diazinon, diclofenac, diflubenzuron and teflubenzuron.

Table 4.16 Residues with hydrogen bonding to VMs and controls in the *S. aurata* ERα. All results are from the lowest bound conformation in the biggest cluster.

VM	**Glu 315**	**Arg 356**	**His 486**	**Asp 313**	**Others**	**Binding energy (kcal/Mol)**
3PBA	-	-	-	-	Gly 483	-5.48
E2	-	-	-	-	Leu 349 Gly 483	-9.64
EE2	-	+	-	-	Leu 349	-9.65
Emamectin benzoate	-	-	-	-	Gly 483	269.09
Fenbendazole	+	+	-	-	Leu 349,	-8.57
Ivermectin	-	+	-	-	Leu 349, Met 350	278.53
Nalidixic acid	-	-	-	-	Leu 308	-4.96
Sulfadiazine	-	-	+	-	Gly 483	-6.75
Sulfapyridine	-	-	+	-	Gly 483	-7.07
Tamoxifen	-	+	-	-	-	-9.74

Thirteen VMs and control substances bound with hydrogen bonds to at least one residue within the *X. laevis* ERα. Ivermectin bound to Glu 345; 3PBA and tamoxifen bound to Arg 386; azamethiphos, α-cypermethrin, sulfadiazine and sulfapyridine bound to His 516; BPA, diflubenzuron, E2, EE2, fenbendazole and nalidixic acid bound to both Glu 345 and Arg 386 (table 4.17). The VMs that did not have any evidence of hydrogen bonding to *X. laevis* ERα were: amitraz, DDT, deltamethrin, diazinon, diclofenac, emamectin benzoate and teflubenzuron.

Table 4.17 Residues with hydrogen bonding to VMs and controls in the *X. laevis* ERα. All results are from the lowest bound conformation in the biggest cluster.

VM	**Glu 345**	**Arg 386**	**His 516**	**Asp 343**	**Others**	**Binding energy (kcal/Mol)**
3PBA	-	+	-	-	Leu 379	-6
Azamethiphos	-	-	+	-	-	-6
BPA	+	+	-	-	Phe 396	-7.83
α-cypermethrin	-	-	+	-	Gly 513	-10.68
Diflubenzuron	+	+	-	-	-	-5.77
E2	+	+	-	-	-	-10.05
EE2	+	+	-	-	-	-10.69
Fenbendazole	+	+	-	-	Leu 379	-8.87
Ivermectin	+	-	-	-	Leu 379	368.1
Nalidixic acid	+	+	-	-	Leu 338	-5.49
Sulfadiazine	-	-	+	-	Leu 338, Gly 513	-6.99
Sulfapyridine	-	-	+	-	Leu 338, Gly 513	-7.33
Tamoxifen	-	+	-	-	-	-10.81

4.4 Discussion

4.4.1 Structural relationships

4.4.1.1 ERα relationships

A dendrogram illustrating the evolutionary distance between the ten ERα receptors shows that six of the seven bony fish ERα are closely related and highly conserved (figure 4.6 [1] page 105). The seventh bony fish, *R. rutilus*, appears to have a less conserved ERα when compared to the other bony fish (figure 4.6 [4]), and is more distant to these than both *H. sapiens* and the African clawed frog *X. laevis* (figure 4.6 [2]; table 4.5 page 104). Published data on the evolutionary distance between Actinopterygii, or bony fish, by Li *et al.* (2007) found that the Salmonid *O. mykiss* and the Gaterostedae *G. aculeatus* were more closely related than the Cyprinid *D. rerio.* The results from Li *et al.* (2007) are comparable to the relationship dendrogram in this study where the Salmonids *O. mykiss* and *S. salar* were more closely related to the Gasterostedae *G. aculeatus* than the Cyprinids *D. rerio, R. rutilus* and *P. promelas* (figure 4.6). Ecotoxicology testing focuses on model species or the most 'sensitive' species and makes the presumption that closely related species will interact in the same way to substances. Traditionally only one species from each group of animals is used in regulatory ecotoxicology testing. It is speculated that highly conserved receptors will bind to ligands in a similar manner to one another (Wass and Sternberg, 2009). To check this theory, ligands bound to each ERα and their binding affinities were compared with the species phylogeny. Comparing the sequence identity dendrogram (or phylogenetic tree) (figure 4.6) to a dendrogram built using lowest binding energy (highest affinity) within the biggest cluster of ligands to each ERα (figure 4.7), showed that groups do not match as expected. Four of the six bony fish, which were found in the same group as one another when checking for phylogeny (figure 4.6 [1]), were within the same group in the dendrogram; these are *O. mykiss, D. rerio, S. aurata* and *S. salar* (figure 4.7 [1a]). However the other two bony fish that are closely related in terms of phylogeny, *G. aculeatus* and *P. promelas* are much more distant than other species in terms of ligand binding affinity. The ERα of *P. promelas,* which shares 90.8% sequence similarity with the ERα of *D. rerio* (table 4.5), was the most distant of the ERα in terms of lowest binding energy (highest affinity) in the biggest cluster (excluding the negative control *C. gigas*). There has been some evidence to support these results. Well known xenoestrogens such as the alkylphenols 4-*tert*-octylphenol and nonylphenol have displayed significant differences in their oestrogenic potency between *D. rerio* and *O. mykiss,* which share 73.4% similarity (table 4.5), with *O. mykiss* being 5 times more sensitive to the oestrogenic effects of nonylphenol than *D. rerio* when VTG production was used as a biomarker (Van den Belt *et al.*, 2003).

This finding would suggest that although the two receptors are highly conserved, even a small percentage of different amino acids can cause significant changes in terms of binding affinity. This

is especially concerning as ecotoxicology testing, based on the assumption that related species will respond similarly, may miss impacts. This finding further confirms that testing should focus on native species rather than model species and that molecular docking could serve as a useful tool for screening and selection of sensitive species.

4.4.1.2 Ligand structure relationships

When the VM ligands were ordered in a dendrogram based on their structural relationships using the tanimoto coefficient, they appeared to be grouped in a logical order depending on their chemical properties (figure 4.8). Group 1 containing E2 and EE2 (figure 4.8 [1a]) also contained the known xenoestrogens BPA and DDT as well as tamoxifen (figure 4.7 [1b]). This was expected, as it is known that all of these compounds bind to the ERα, and also have a phenolic ring (apart from DDT which has a chlorobenzene). Group 1 also contained α-cypermethrin, deltamethrin and 3PBA (figure 4.8 [1c). Comparatively, the dendrogram which was made of the lowest binding energy (highest affinity) in the biggest cluster for each ligand, shows that group 1 contains E2, EE2, tamoxifen, deltamethrin and α-cypermethrin (figure 4.14 [1]). This suggests whilst structure is not directly linked to binding affinity, it does play a part and that structural similarity between ligands is more likely to be a factor in binding affinity than sequence similarities between receptor. The structure of the native ligand E2 is comprised of two hydroxyl groups on either end of the ligand that are 11 Å apart, a lot of xenoestrogens share a hydroxyl group (Baker, 2014; McCullough *et al.*, 2014). However not all xenoestrogens have this hydroxyl group, DDT is a known xenoestrogen (Fu *et al.*, 2007; Oien *et al.*, 1997) and it does not have this quality (figure 4.9). Group 2 contained the known xenoestrogens BPA and DDT as well as fenbendazole (figure 4.7 [2]). Although there are no current reports on fenbendazole being a xenoestrogen in vertebrates, it has been reported as being an EDC in the invertebrate *Chironomus riparius* (Park and Kwak, 2012). Structurally fenbendazole does not possess a hydroxyl group (figure 4.11) unlike many xenoestrogens, the molecular docking results indicate that fenbendazole is worthy of significantly more investigation to determine its xenoestrogenic impacts.

4.4.2 Ligand binding affinity

The two ligands that had the lowest binding energy (highest affinity) for all ERα molecules, excluding the negative control *C. gigas*, were deltamethrin and α-cypermethrin. The lowest binding energy (highest affinity) within the biggest cluster of these two VMs did not significantly differ between any of the species. There have been mixed reports on whether deltamethrin is a xenoestrogen or an anti-oestrogen.

McCullough *et al.* (2014) used AutoDock 4 alongside a commercial *in vitro* cell culture assay and found that there was a correlation between binding energy and oestrogenic potential. Compounds with a binding energy of ≤ -8.22 (kcal/mol) in the LBD of the human ERα were

inhibitors in the *in vitro* assay whereas those compounds with a binding energy of ≥ -7.37 possessed no oestrogenic activity *in vitro.* McCullough *et al.* (2014) chose to select the lowest binding energy (highest affinity) within the biggest cluster, which gave them the estimates on binding. The results from the McCullough *et al.* (2014) study suggest using results on binding energy from the biggest cluster is a good indicator of oestrogenic compounds within the ERα. In Chinese hamster CHO cells, Kojima *et al.* (2004) reported deltamethrin as being an anti-oestrogen. In contrast other studies have shown deltamethrin to be a xenoestrogen in human MCF-7 cells (Andersen *et al.*, 2002). In studies on pregnant rats Presibella *et al.* (2005) showed that deltamethrin, in combination with the xenoestrogen endosulfan did not have an endocrine disrupting effect at oral concentrations up to 4 mg/kg body weight per day. It would appear that deltamethrin is both an ERα agonist and antagonist, this could explain why deltamethrin had such a high affinity to the test ERα in this study, but binding was fairly unspecific with various possible binding positions (see figure 4.19).

4.4.3 Hydrogen bonding

Interactions between VMs and the possible binding site for agonistic and antagonistic substances within the ERα were investigated by looking at residues that formed hydrogen bonds. Amitraz has been reported as being an EDC and appears to be a weak agonist on the α_2-adrenergic receptor in mammals. It reduces gonadotropin-releasing hormone (GnRH) and noradrenalin secretion altering growth and reproduction (Altobelli *et al.*, 2001). Amitraz has also been reported as being a weak antagonist of the ERα in human MCF-7 cells (Ueng *et al.*, 2004). The findings from the present study do not suggest that amitraz is an antagonist, as there is no evidence of hydrogen bonding to aspartic acid (position 351 in *H. sapiens*) in any of the binding conformations. As a consequence, at this stage, it is difficult to say whether amitraz is an agonist or antagonist. Only two VMs showed hydrogen bonding to aspartic acid, these were fenbendazole in *H. sapiens* (table 4.7) and *G. aculeatus* (table 4.8). The binding of fenbendazole to aspartic acid in this position suggests that this VM could have an antagonistic effect on the *H. sapiens* and *G. aculeatus* ERα. If this is the case then this finding further highlights the complexity of the affinity of certain VMs to different species. Ivermectin also had hydrogen bonding with aspartic acid in *G. aculeatus* (table 4.11). As ivermectin bound with such a high binding energy (197.5 kcal/mol), it is unlikely that it would have an antagonistic effect on the ERα of *G. aculeatus.* There is no evidence that it is a xenoestrogen and the high binding energy shown in this study also strongly indicates this (see tables 4.6b and 4.11). Fenbendazole had a strong affinity to both *H. sapiens* (-8.14 kcal/mol) and *G. aculeatus* (-7.72 kcal/mol). Therefore it is possible that fenbendazole could have mixed agonistic and antagonistic effects on the ERα.This suggests once again (see section 4.4.1.2) that the lack of evidence in the literature about the endocrine disrupting effects of fenbendazole does not necessarily imply it is not an EDC but rather that there is a substantial knowledge gap for this VM that should be remedied.

4.4.4 Sea lice treatments

Of the five sea lice treatments, emamectin benzoate did not demonstrate affinity to any of the ten ERα receptors (see tables 4.6a and 4.6b). Deltamethrin and α-cypermethrin showed high affinity to all the ERαs investigated, apart from the negative control *C. gigas* (see table 4.6b) and were categorised as 'high likelihood' VMs (see tables 4.6 a and 4.6b). The use of cypermethrin is decreasing in Scottish salmon farms while the use of deltamethrin is increasing (chapter 2; section 2.3.1; table 2.3). However, data on the sediment MEC of cypermethrin and deltamethrin from 2008 – 2012 show that both VMs were under the level of detection. This does not mean that they were not present in concentrations that could cause harm, especially as it has been documented that mixtures of xenoestrogens in concentrations below the LOEC have elicited an oestrogenic response (Silva *et al.*, 2002). Teflubenzuron showed a high likelihood of binding to the three-spined stickleback *G. aculeatus* ERα. In chapter 2 (section 2.3; table 2.4) it was shown that use of teflubenzuron dramatically increased between 2008 – 2012 going from 72.73 kg total use in 2002 to 261.96 kg total use in 2013. *G. aculeatus* is an anadromous (lives in fresh, marine and brackish water) fish and is distributed in and around UK coastal waters, as this is where Scottish salmon farms are based then it is likely that *G. aculeatus* would be exposed to concentrations of teflubenzuron that could cause an endocrine disrupting effect.

Azamethiphos showed a medium likelihood of binding to all ten of the tested ERα. Since azamethiphos is the most hydrophilic of the sea lice treatments and is more likely to remain in the water column rather than adsorb to sediment (see chapter 2; section 2.1; table 2.1) then exposure to fish living in the water column is high. Since the likelihood of binding is medium, but the exposure likelihood is high then it is recommended that azamethiphos also be tested for oestrogenic activity using an *in vitro* assay.

4.4.5 Binding affinity

Nine of the fifteen VMs appeared in the high likelihood of binding category for binding to the ERα for at least one species (see tables 4.6a and 4.6b). Fenbendazole was in the high likelihood of binding category for all of the ERα (excluding the negative control *C. gigas*) and was chosen to be investigated because there is evidence of its endocrine disrupting properties. For example, *C. riparius* ribosomal gene RpS3 when exposed to fenbendazole showed significant upregulation of the ribosomal protein gene RpS3 (Park and Kwak, 2012). Fenbendazole has been included on several lists for the prioritisation of veterinary pollutants in the aquatic environment in the UK (Boxall *et al.*, 2003; Boxall *et al.*, 2002), Korea (Kim *et al.*, 2008) and Spain (Guillén *et al.*, 2012). Fenbendazole is on the priority list for these countries because use is high but information on effects on non-target organisms is lacking (Boxall *et al.*, 2003). From the results of this study fenbendazole is likely to have an effect on the ERα on all of the test species, suggesting that the

oestrogenic potential of this VM is investigated further with an *in vitro* reporter assay such as a modified YES.

Teflubenzuron had a wide binding range between the species, with the lowest binding energy (highest affinity) in the biggest cluster for *R. rutilus* at -4.77 (average binding was -4.14 ± 0.43) kcal/mol (figure 4.21; table 4.6b) and the lowest binding energy (highest affinity) in the biggest cluster for *G. aculeatus* at -7.33 (average binding was -7.09 ± 0.16) kcal/mol (figure 4.21; table 4.7b). This difference in binding energy could indicate that teflubenzuron is a mild xenoestrogen in *G. aculeatus* but does not act on the ERα of *R. rutilus.* Deciding on what is a good binding 'hit', is something of a grey area. However, from other studies and from the AutoDock user guidelines, it is generally accepted that anything below -7 kcal/mol is worthy of further investigation (McCullough *et al.*, 2014; Pavani *et al.*, 2008). In this study the results for teflubenzuron indicate that even in conserved proteins binding can differ vastly, resulting in the need for more species specific testing in both chronic and lethal effects of substances.

4.4.6 Predicted oestrogenic potency (PEP)

A number of *in vitro* assays, when testing for oestrogenicity, compare the compounds being tested with oestrogens to determine the compounds potency related to native ligands (Nagel *et al.*, 1999; Segner *et al.*, 2003). In the present study, deltamethrin, α-cypermethrin and tamoxifen gave PEPs that were orders of magnitude higher than E2. For example deltamethrin was 110x more potent than E2 in the *S. aurata* ERα (see figure 4.19 and tables 4.6a and 4.6b). Whilst α-cypermethrin has been shown to possess some xenoestrogenic properties, deltamethrin has been reported to have both mild oestrogenic and anti-oestrogenic properties (Kojima *et al.*, 2004). In this study, α-cypermethrin bound with a high affinity to all ERα receptors (excluding the negative control *C. gigas*) however binding was not consistently in the same position within the receptors. This unspecific binding could be an indicator of how α-cypermethrin and deltamethrin can be both agonistic and antagonistic to the ERα as shown in *in vitro* studies (Chen *et al.*, 2002; Kojima *et al.*, 2004). Although the PEPs calculated in the present study have often been in agreement with which ligands have been found to cause an effect in other studies, it wildly overestimates the oestrogenic potential for some. This suggests that PEP calculations based on molecular docking cannot be used as a reliable measure of potency; at best it may provide an indication of substances that might have a potential oestrogenic effect. In the present study BPA had a PEP ranging between 1.28% in *H. sapiens* and 6.28% in *G. aculeatus* whereas in other studies the PEP of BPA varies between 0.00006 – 0.01% depending on the assay (Nagel *et al.*, 1999; Segner *et al.*, 2003).

When investigating 200 pesticides for their xenoestrogenic potential, Kojima *et al.* (2004) found that deltamethrin was a possible anti-oestrogen and had a 20% relative inhibitory concentration (RIC_{20}) of 8.1 x 10^{-6} (equivalent to 4.09 mg/L) compared with the RIC_{20} for tamoxifen with a

reported RIC_{20} of 3.2 x 10^{-9} (equivalent to 1.19 µg/L). Cypermethrin was reported as being a mild xenoestrogen, with a 28% PEP being measured as 8.1 x 10^{-6} (equivalent 3.37 mg/L). According to this study, α-cypermethrin had a stronger affinity to the ERα of all tested species (apart from the negative control *C. gigas*). It would seem that α-cypermethrin does pose a risk to aquatic wildlife. Cypermethrin has been detected in surface freshwater at concentrations up to 85.1 µg/L (Boxall *et al.*, 2002). When comparing the reported concentrations that would cause a response in Chinese hamster ovary (CHO) cells with MECs it seems that α-cypermethrin and deltamethrin would not reach the effect threshold. From the Kojima *et al.* (2004) study it shows that deltamethrin and α-cypermethrin would cause little effect at environmentally relevant concentrations, however it does not mean that they are unable to cause an effect within the aquatic environment. As these compounds generally occur in mixtures and may be present in the environment along with other xenoestrogens their incidence in the aquatic environment should be closely monitored.

4.4.7 Predictions on mixtures of VMs in the environment

In a study using the YES as a reporter assay Silva *et al.* (2002) showed that a mixture of eight mild xenoestrogens at concentrations below their NOEC or EC_{01} caused an oestrogenic response. When using the prediction equations CA (see chapter 3, equation 3.1) and IA (see chapter 3, equation 3.1) they found that the equation IA gave EC_{50} results that underestimated the effects of the mixture, however CA results were in line with their findings. The reliability of CA in this experiment was to be expected. CA is an equation which favours similar MOA effects, and since in this case only one pathway was available for measurement CA would inevitably be the equation that fits the best. In chapter 3 (equation 3.2; section 3.1) IA gave the best prediction in terms of mixture effects, however the MOA of the VMs tested in chapter 3 were different to one another. This further confirms that in order to effectively predict the effects of mixtures on non-target organisms the equation used should be carefully selected with consideration of MOA similarity or dissimilarity in order to maximise the potential of the predictive equations. If molecular docking were to be used as a tool to aid the predictions of mixture effects on a specific target, in this case the ERα, then CA would be the equation to best fit this task. It should be noted, however, that this equation does not take into account synergistic effects.

4.5 Conclusion

It appears that molecular docking can serve as a useful pre-screening tool in order to distinguish which species may be more sensitive than others to different compounds. Binding affinity of ligands between the ERα of the ten species in this study has shown profound differences, even between species with very high ERα similarity (up to 90.8% sequence identity) (see figure 4.23). Previously it was assumed that species with high phylogenetic similarities would interact with compounds in a similar enough way that ecotoxicology testing was only needed on a model

species (Brooks *et al.*, 2008). These results highlight the need for more intelligent ecotoxicology testing.

This study has also highlighted two VMs that may hold xenoestrogenic or anti-oestrogenic properties to certain species of bony fish that have not been tested for their xenoestrogenic properties before. These are fenbendazole, which has previously been described as having an endocrine disrupting effect on the invertebrate *C. riparius* and teflubenzuron, which currently has not been tested for xenoestrogenic properties. The uncertainty surrounding binding and whether a substance is an antagonist or agonist is a significant problem for molecular docking, therefore it is proposed that substances with low binding affinity should be further tested using *in vitro* reporter assays such as YES, ER-Calux ®, or VTG induction assays (these *in vitro* assays are discussed in full in chapter 5, section 5.1). *In vitro* reporter assays are preferable at the stage to *in vivo* assays to reduce the number of animals used and because *in vitro* reporter assays can give information on specific targets. However it has been documented that *in vitro* assays are not as sensitive at detecting the toxicity of compounds as *in vivo* assays (Segner *et al.*, 2003). It appears however that molecular docking cannot offer any information at this stage as to whether a VM is an antagonist or agonist. In order to provide a more comprehensive assessment it is recommended that *in vitro* assays are performed afterwards to identify whether a VM is an antagonist or agonist.

One problem with molecular docking is the analysis of results. It is not obvious which docking conformation is the best to choose in an experiment. If out of 100 docking conformations there is only one cluster of results (see figure 4.4) then the conformation with the lowest binding energy and highest affinity can be chosen. However, there is often more than one cluster of binding conformation (see figure 4.5) and sometimes the lowest bound conformation does not appear in the biggest cluster. This is where analysis becomes difficult. There is not much information on analysing results in peer-reviewed papers, however according to the AutoDock website FAQ section inspection by eye may be the most suitable form of identification for the 'right' conformation (The Scripps Research Institute, 2007). Ideally, for this technology to be of use in ecotoxicology testing, docking should be usable as an automated process to optimise screening potential. It is not only binding energy (kcal/mol) that should be considered when analysing docking results, but also clusters and the orientation of bound molecules, hydrogen bonding and Van der Waals interactions. While the orientation and type of bonds are important in binding (Cui *et al.*, 2013), inspecting each conformation is a time consuming process and not a practical proposition if this method is to be employed as a high throughput technique as part of ecotoxicology screening. Although molecular docking can be useful for identifying ligands that may be problematic, a lot more research is needed to fully understand whether it can be a viable option for structure based estimates on ligand binding.

Pharmaceuticals are designed to have a biological target, or MOA. Some VMs and pesticides have targets that are specific to invertebrates, making them relatively safe for non-target vertebrates. With the advance of molecular docking, however, it may be possible to identify alternative targets. The results from this study show that no matter what the intended MOA may be, a VM can still be a target for the ERα. This technology can therefore serve to identify alternative biological targets, which may have an adverse chronic effect on a number of species. With the increase in sequencing data and as 3D models improve molecular docking can become a stronger tool in the identification of sensitive species across a greater diversity of organisms.

Molecular docking may provide important insights into chronic effects of VMs on a variety of species. It also has the ability to make predictions on compounds with relatively little background information. Although there are limitations to the capabilities of molecular docking as a prediction method, it may be able to assist in ecotoxicology testing as a precursor for the identification of VMs capable of binding to the ERα causing a chronic effect on the reproductive system in a variety of aquatic organisms without the need for using living organisms. With the increase in sequencing data, molecular docking may prove to be a useful tool for focusing on more sensitive species as well as identifying possible molecular targets that were previously unknown.

Chapter 5

In vitro Yeast Oestrogen Screen

5.1 Introduction

In order to confirm the results from the molecular docking in chapter 4 an *in vitro* assay was proposed. *In vitro* assays may not be as accurate as *in vivo* assays but they do have some advantages over their whole body counterpart. While *in vitro* assays do not mimic adsorption, distribution, metabolism and excretion (ADME) in the way that *in vivo* assays do their advantages include rapid high-throughput results, reproducibility, insights in to specific mechanism of action and a lack of animal testing (Segner *et al.*, 2003). There are numerous *in vitro* reporter assays that can be used to examine and measure the impacts of xenoestrogens. These include biomarker studies using liver cells measuring hepatic vitellogenin (VTG) induction, where hepatic cells are exposed to media containing the test chemical for 72 hours and then samples are analysed for VTG using enzyme-linked immunosorbent assay (ELISA) (Hultman *et al.*, 2015). Cell line fluorescent labelled reporter gene assays (Cosnefroy *et al.*, 2009; Cosnefroy *et al.*, 2011) also can be used in which a plasmid containing the ER gene co-transfected with a plasmid is containing a gene for a florescent protein such as green florescent protein (GFP). Recombinant yeast assays such as the yeast oestrogen screen (YES) (described in more detail below) have been used as well. There are also commercial kits that can measure the oestrogenic potential of compounds or field samples such as ER-calux® which is a dual reporter luciferase similar to cell line fluorescent labelled reporter gene assays, except with luciferase as the reporter.

A recombinant YES was selected to validate the results from the molecular docking in this study in part due to its reproducibility but also because of results from a study by Segner *et al.* (2003). This study compared four *in vitro* xenoestrogen reporter assays, YES was the assay that gave results that had the greatest similarity to *in vivo* experiments. YES was developed by Routledge and Sumpter (1996) and has been widely used to test for xenoestrogenic substances (Arnold *et al.*, 1996; Beck *et al.*, 2006; Fu *et al.*, 2007; Le Grand *et al.*, 2015; Rehmann *et al.*, 1999). Recombinant DNA from the human oestrogen receptor alpha (ERα) is transformed into an auxotrophic strain of *Saccharomyces cerevisiae* along with the oestrogen response element (ERE) and the *lacZ* gene. Once the transformed yeast comes into contact with an oestrogenic compound (either synthetic or natural) the ERα will be activated thereby binding to the ERE which acts a promotor to transcribe the *lacZ* gene which in turn releases the enzyme β-galactosidase, which catalyses the hydrolysis of the chlorophenol red-ß-D-galactopyranoside (CPRG) substrate, turning the yellow media red (Routledge and Sumpter, 1996). The colour change can then be measured using a spectrophotometer, and oestrogenic activity can be determined by comparing the

compound to a control oestrogen such as E2 (see figure 5.1). By inserting the ERα cDNA of *D. rerio* and *O. mykiss* species variation can be measured, as well as direct comparisons with the molecular docking work, as described in chapter 4.

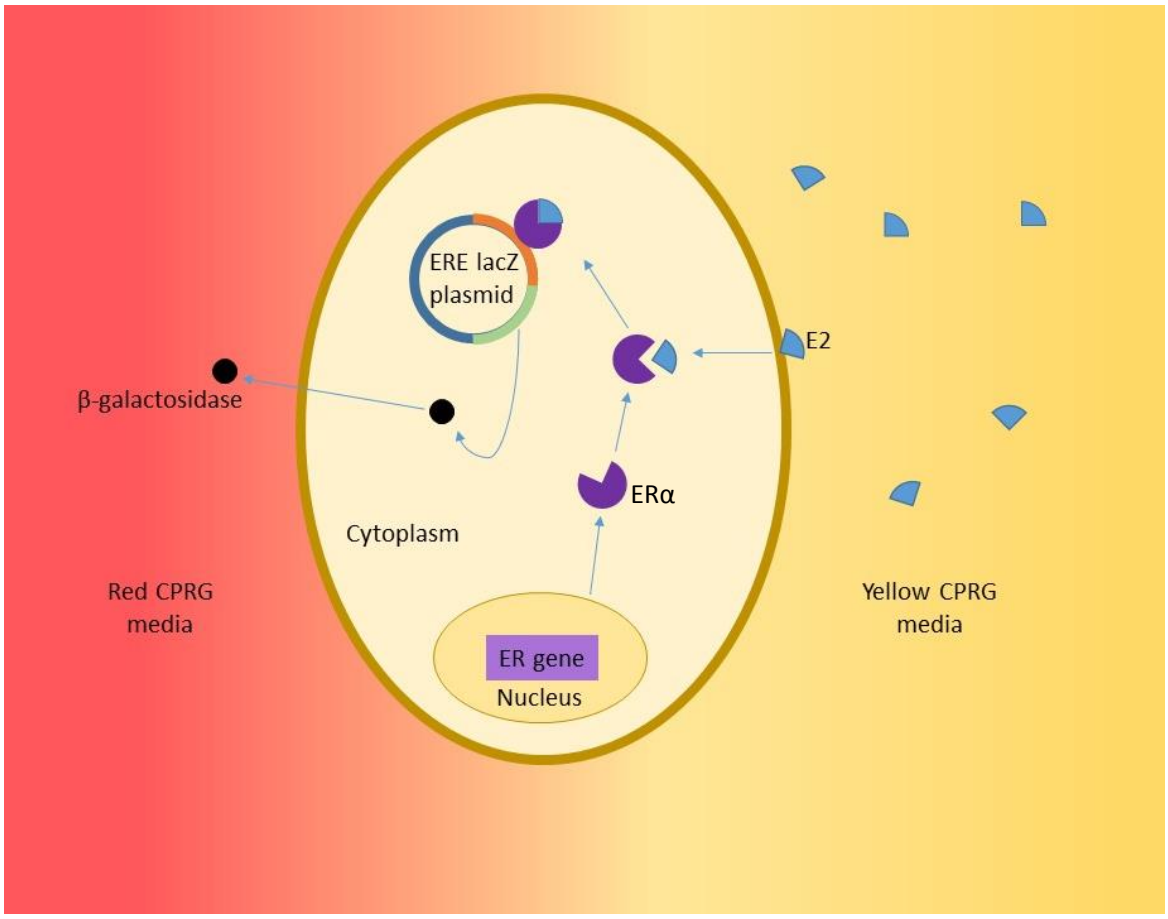


Figure 5.1 *S. cerevisiae* cell transfected with ER gene (purple box) and reporter plasmid containing ERE and lacZ gene (blue, orange and green circle). ER gene transcribes ERα (purple ¾ circle) which is released into the cytoplasm. Upon binding to an oestrogenic substance (in this case E2, blue ¼ circle) the ERα binds to the ERE (orange section within the ERE-lacZ plasmid). Activation of the ERE causes downstream transcription of the lacZ gene (green section within the ERE-lacZ plasmid) which produces the β-galactosidase enzyme (black circle). *S. cerevisiae* excretes β-galactosidase into the media containing CPRG, where β-galactosidase converts the yellow substrate in the CPRG to red. *Adapted from Rutledge and Sumpter (1996).*

5.1.1 Chapter objectives

In order to address objective 4 (chapter 1; section 1.9.3) the aims of this chapter were:

- To compare the oestrogenic potential of VMs investigated in chapter 4.
- To identify species variation between the ERα of *D. rerio* and the ERα of *O. mykiss.*
- To apply CA and IA (previously used in chapter 3; equations 3.1 and 3.2) to determine which equation, if any is supported by this assay.

- Determine which VMs are ERα agonists, which are ERα antagonists and which VMs have no effect on the ERα.

5. 2 Methods

5.2.1 Cloning of plasmids

Three plasmids were constructed, pESC-TRP-rtERα (using *O. mykiss* ERα); pESC-TRP-zfERα (using *D. rerio* ERα) and pYES2-lacZ-ERE (see figures 5.1a and b; 5.2) using the commercial vector backbones pESC-TRP (Agilent Technologies, Stockport, UK) and pYES2 (ThermoScientific, Loughborough, UK). Mammalian compatible plasmids (pCMV5) containing cDNA rtERα (*O. mykiss*) and zfERα (*D. rerio*) were kindly donated by Dr. F Pakdel at Rennes University. In order to transform into the genetically modified *Saccharomyces cerevisiae* yeast strain INV*Sc*1 (Invitrogen, Loughborough, UK), cDNA had to be digested out of mammalian vectors and inserted into yeast compatible vectors (see below).

5.2.1.1 Polymerase Chain Reaction (PCR)

As the mammalian plasmids containing zfERα and rtERα did not have complementary restriction sites to pESC-TRP, primers (Eurofins, Abington, UK) were designed, using mRNA sequences from GenBank, online software to convert mRNA to cDNA sequence, and the online software primer designer program primer3 (Koressaar and Remm, 2007; Untergasser *et al.*, 2012). In order to ensure successful ligation into yeast vectors, the restriction sites EcoRI and PacI which are complementary to the yeast plasmid pESC-TRP were added on either end of primers. An overhang of 2 base pair (bp) was added to the end of restriction sites to decrease the chance of asymmetrical cleavage. cDNA was then amplified using polymerase chain reaction (PCR). Amplified DNA was then digested and inserted into relevant backbones (see table 5.1).

Table 5.1 PCR primers for rtERα and zfERα. Restriction sites are bold and underlined. Overhang is to the 5' end of restriction sites and cDNA specific primer directly after the restriction sites.

PCR Primers for rtERα	
Direction	Primer Sequence
Forward 5'	AG**GAATTC**ATGCTGGTCAGACAGTCCCA
Reverse 5'	CA**TTAATTAA**TCACGGAATGGGCATCTG
PCR Primers for zfERα	
Direction	Primer Sequence
Forward 5'	AG**GAATTC**ATGTACCCTAAGGAGGAGCACA
Reverse 5'	CA**TTAATTAA**TCAGGGGTCAGGGCTATG

PCR reaction contained in (0.2 ml) tubes (Eppendorf, Stevenage, UK): 1 µg of plasmid DNA, 10 µl of KAPA HiFi HotStart ReadyMix DNA polymerase master mix (containing DNA polymerase, buffer and dNTPs) (Kapa Biosystems, London, UK), 5 µM of forward primer, 5 µM of reverse primer and RNAase free water (Qiagen, Manchester, UK) made up to 20 µl. PCR reaction tubes (5 tubes in total, two samples, two controls containing primers and one blank) were placed in a PCR machine (BioRad). The PCR cycle was as follows: initial denaturation was at 95°C for 3 minutes, followed by 20 cycles of denaturation of 98°C for 20 seconds, annealing at 67°C for 15 seconds and extension of 72°C for 30 seconds. After 20 cycles of denaturation, annealing and extension, there was a final extension of 72°C for 1 minute. Following these steps the reaction was held at 4°C until analysis by gel electrophoresis (see below) was carried out (no longer than 16 hours).

5.2.1.2 Construction of pESC-TRP-zfERα

The 3 kb PCR product of zfERα insert (as described in section 5.2.1.1) was digested using the restriction enzymes EcoRI and PacI (New England Biosciences, Hitchin, UK). The empty plasmid vector pESC-TRP was also digested using the restriction enzymes PacI and EcoRI. Approximately 300 ng of cDNA or empty vector DNA was added to separate 1.5 ml Eppendorf tubes (Eppendorf, Stevenage, UK), 0.5 µl of each enzyme was added along with 2 µl of 10X cutsmart buffer to each tube (New England Biosciences, Hitchin, UK) and left to digest for 2 hours at 37°C. The reaction was then heat inactivated at 65°C for 10 minutes. Once digested both insert and vector were run on a 0.4% agarose gel for 90 minutes at 120V. The digested insert (~3 kb) and the digested vector (~6.5 kb) were cut out of the gel and a gel DNA extraction was performed using the GeneJET gel extraction kit (ThermoFisher Scientific, Loughborough, UK). Insert and vector were ligated at a molar ratio of approximately 3:1, 36 ng of digested rtERα, 36 ng of digested pESC-TRP, 2 µl of 10X ligase buffer (New England Biosciences, Hitchin, UK), 1 µl of T4 DNA ligase (New England Biosciences, Hitchin, UK) and 10 µl of sterile dH_2O was added to an Eppendorf tube and left at 4°C

to ligate overnight (see figure 5.2a for construct map of pESC-TRP-zfERα). The ligation, now referred to as pESC-TRP-zfERα was immediately transformed into TOP10 *Escherichia coli* cells as described below (section 5.2.2).

5.2.1.3 Construction of pESC-TRP-rtERα

The 2 kilobase (kb) PCR product of rtERα (as described in section 5.2.1.1) insert was digested using the restriction enzymes EcoRI and PacI (New England Biosciences, Hitchin, UK). The same method as in the construction of pESC-TRP-zfERα (section 5.2.1.2) was applied. The ligation, from now on referred to as pESC-TRP-rtERα, was transformed into TOP10 *Escherichia coli* cells as described in section 5.2.2 (see figure 5.2b for construct map of pESC-TRP-rtERα).

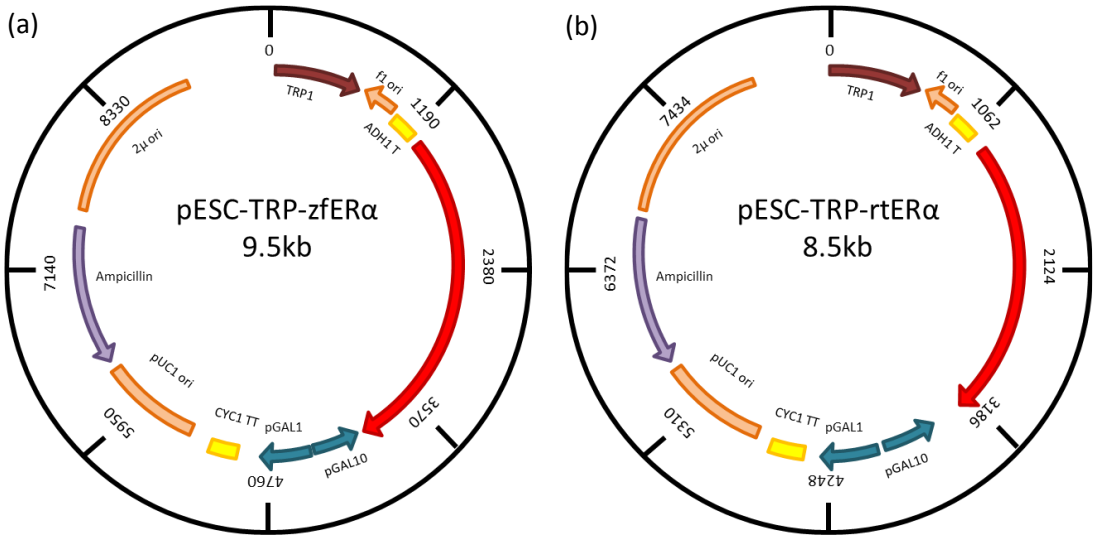


Figure 5.2 Construct maps of pESC-TRP containing either (a) the zfERα (creating pESC-TRP-zfERα) or (b) rtERα (creating pESC-TRP-rtERα).

5.2.1.4 Construction of pYES2-LacZ-ERE

LacZ was digested out of the plasmid PCSLZW using the restriction enzymes BamHI and NotI (New England Biosciences, Hitchin, UK). Each reaction was made up to 20 µl using sterile dH_2O. To remove *LacZ* from PCSLZW the following reaction was prepared: 0.5 µl BamHI, 0.5 µl NotI, 2 µl cutsmart buffer and 269 ng of PCSLZW were added to a sterile Eppendorf tube To digest pYES2 273 ng of the plasmid was digested using 0.5 µl of BamHI, 0.5 µl of NotI, 2 µl cutsmart buffer and made up to 20 µl using sterile dH_2O. Both reactions were left to incubate at 37°C for 2 hours and heat inactivated at 65°C for ten minutes. To isolate DNA to be ligated the reaction was run on a 0.4% agarose gel stained with GelRed (10 µl of reaction mixture per well) for 90 minutes at 120V. *LacZ* was identified as a ~3 kb band whereas pYES2 was identified as a ~5.9 kb band. Bands were cut out of the gel and a gel extraction was performed using a gel extraction kit according to the kit

protocol (ThermoScientific, Loughborough, UK). Following digestion and gel extraction ligation was performed at a 3:1 inset to vector molar ratio. Insert *LacZ* (76ng) was added to an Eppendorf tube with 52.8 ng of digested pYES2, 1 µl T4 DNA ligase, 2 µl ligase buffer and brought up to 20 µl using sterile dH_2O. The reaction was left at 4°C overnight and heat inactivated at 70°C for 20 minutes. The ligation, from now on referred to as pYES2-*LacZ,* was transformed into TOP10 *E. coli* cells as described below (section 5.2.2).

Following the construction of pYES2-*LacZ* the addition of the ERE was needed to make pYES2-*LacZ*-ERE. ERE was made by annealing two oligonucleotides that incorporated the 16 base pair (bp) palindromic ERE and the restriction sites HindIII and BamHI were added to the end in order to create sticky ends when digested.

ERE Forward 5' **AAGCTT**AGGTCACAGTGACCT**GGATCC**

ERE Reverse 5' **GGATCC**AGGTCACTGTGACCT**AAGCTT**

The two ERE strands were annealed by adding 9 µl of each synthetic oligonucleotide (at a concentration of 2 µg/µl) and 2 µl of 10 X ligase buffer to total volume of 20 µl. The reaction was heated to 95°C for 5 minutes and left to cool to room temperature. This reaction was then run on a 2% agarose gel (containing 0.001% GelRed Nucleic acid stain [Biotium, Hayward, USA]) at 120V for 30 minutes. The 27 bp band was cut out and a gel extraction was performed as described previously. Approximately 200 ng of the now double stranded synthetic ERE was digested using 0.5 µl of the restriction enzymes HindIII and BamHI. pYES2-*lacZ* (~200 ng) was also digested using HindIII and BamHI at 37°C for 2 hours; the reaction was inactivated at 65°C for ten minutes. Digested ERE was run on a 2% agarose gel using electrophoresis at 120V for 30 minutes while digested pYES2-*lacZ* was run on a 0.8% agarose gel containing GelRed at 120V for 90 minutes. Both bands were cut out and a gel extraction was performed on each as described before (see figure 5.3 for construct map of pYES2-*LacZ*-ERE).

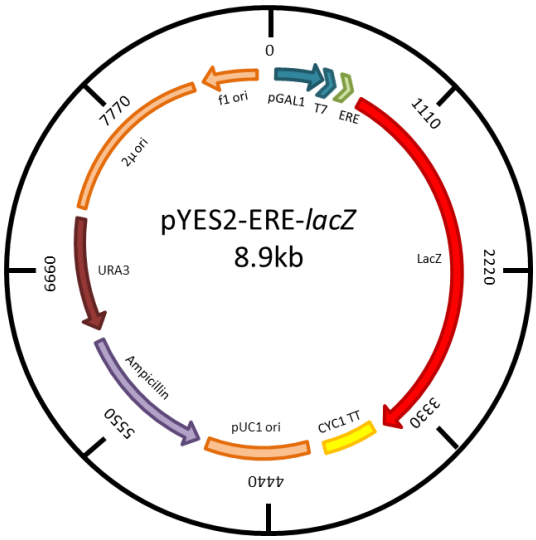


Figure 5.3 Construct map of pYES2 with inserts ERE and *LacZ* to create pYES2-ERE-*lacZ.*

5.2.2 Transformation of Plasmids into *E. coli*

For each transformation, 50 µl of competent Top10 *E. coli* cells were used (ThermoFisher. Scientific, Loughborough, UK). Cells were taken out of the -80°C freezer and left on ice for 5 minutes to thaw. Ligated plasmid (5 µl) was added to 50 µl of cells. Tubes were left on ice for a further 15 minutes. Cells were then heat shocked at 42°C for 45 seconds before being placed on ice for a further 5 minutes. SOC broth (Sigma, Poole, UK) (250 µl) was added to the cells, which were then incubated at 37°C at 250 rpm for 60 minutes. Selective ampicillin Lysogeny broth (LB) agar plates (see appendix D for constituents) were spread with 100 µl of transformed *E. coli* and left to incubate at 37°C overnight (approximately 16 hours).

Colonies were picked from plates the following day. Five colonies in total were added to separate falcon tubes containing 2ml of ampicillin LB broth (see appendix D for constituents) and grown at 37°C at 150 rpm for 16 hours. Once grown each tube was centrifuged at 3000 rpm for 5 minutes and a plasmid miniprep was completed using the GeneJET^{TM} Plasmid Miniprep Kit as instructed (ThermoFisher Scientific, Loughborough, UK).

Once the mini prep was completed, an aliquot of each sample, was re-digested and run on an agarose gel. Samples were then sent for sequencing (GATC Biotech, Konstanz, Germany) to confirm the digestion and ligation had worked.

Samples (n = 5) were digested to test whether the rtERα had successfully ligated into the pESC-TRP plasmid showed evidence of ligation (figure 5.4). All five samples showed signs of digestions, samples in lanes 5 and 7 were sent for sequencing.

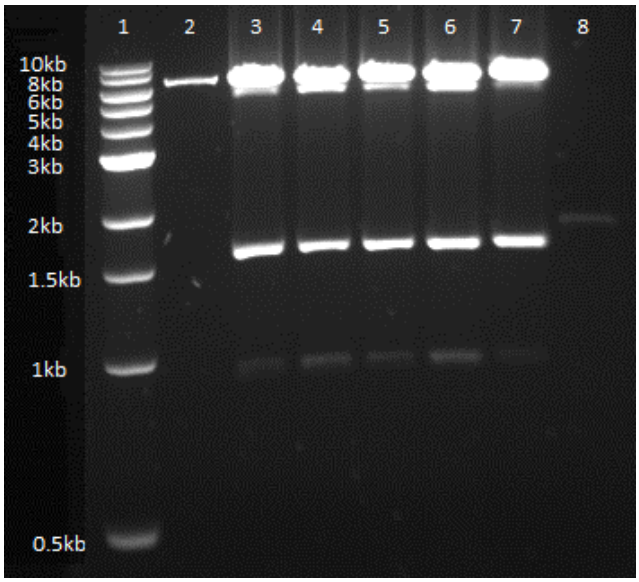


Figure 5.4 Electrophoresis gel of uncut pESC-TRP-rtERα (lane 2); and samples of plasmid DNA containing pESC-TRP-rtERα digested with the restriction enzymes EcoRI and PacI (lanes 3-7) and uncut rtERα insert (lane 8). Using a 1kb molecular weight DNA ladder (lane 1) (New England Biosciences, Hitchin, UK). All digested samples showed signs of successful transformation and the samples in lanes 5 and 7 were sent for sequencing. Samples in lanes 3-7 come from different bacterial colonies transformed with pESC-TRP-rtERα.

Samples (n = 5) were digested to test whether the zfERα had successfully ligated into the pESC-TRP plasmid (figure 5.5). Three out of five samples showed signs of digestions, samples in lanes 6, 7 and 8 were sent for sequencing.

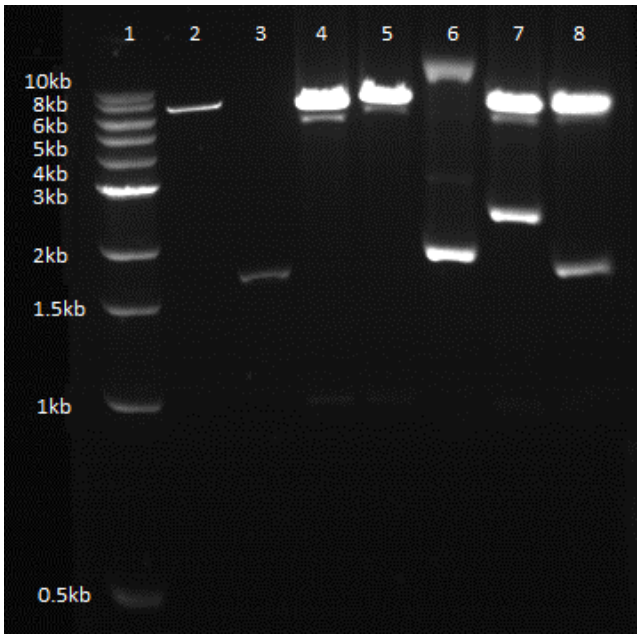


Figure 5.5 Electrophoresis gel of uncut pESC-TRP-zfERα (lane 2); uncut zfERα insert (lane 3) and samples of plasmid DNA containing pESC-TRP-zfERα digested with the restriction enzymes EcoRI and PacI (lanes 4-8). Lane 1, 1kb molecular weight DNA ladder Digested plasmids in lanes 6, 7 and 8 indicate successful transformation. Samples in lanes 4-8 come from different bacterial colonies transformed with pESC-TRP-zfERα.

Samples (n = 5) were digested to test whether the *lacZ* gene had successfully ligated into the pYES2 plasmid. Four out of five showed evidence of ligation (figure 5.6). Samples 2 and 4 were sent for sequencing.

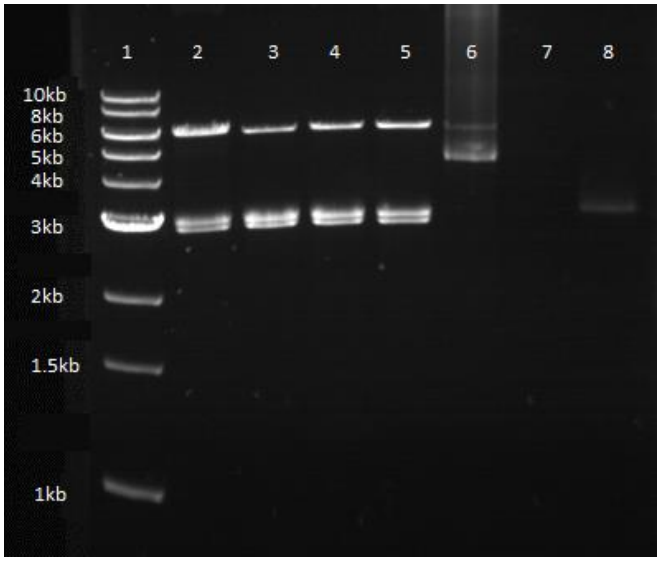


Figure 5.6 Electrophoresis gel of digested pYES2-*lacZ* (lanes 2-6) using the restriction enzymes BamHI and NotI; and uncut *lacZ* (lane 8). Uncut plasmid was not included in this picture; lane 1, 1kb molecular weight DNA ladder (lane 1). Samples in lanes 2-6 come from different bacterial colonies transformed with pYES2-*lacZ*.

5.2.3 Transformation into INV*Sc*1 using Lithium Acetate (LiAc) method

5.2.3.1 INVSc1 competency

Plasmids were transformed into INV*Sc*1 (Invitrogen, Loughborough, UK), an auxotrophic strain of *S. cerevisiae*. A sample of INV*Sc*1 was spread onto yeast peptone dextrose (YPD) (see appendix D for constituents) agar plates and incubated at 30°C for 72 hours. A colony of INV*Sc*1 was added to 1ml YPD broth (see appendix D for the constituents) and vortexed; this volume was added to 50ml YPD broth and grown on a shaking incubator (250rpm) at 30^{o} C for 16 hours. The OD_{600} after 16 hours was ~4; 15ml of this culture was added to 300ml YPD to dilute to an OD_{600} of 0.2 and grown on a shaking incubator at 250 rpm at 30^{o}C for 3 hours until the OD_{600} was 0.4. Cells were centrifuged at 1000 x*g* for 5 minutes at room temperature. The supernatant was removed and cells were pooled and re-suspended in 10ml sterile dH_2O and centrifuged at 1000 x*g* for 5 minutes at room temperature. The supernatant was removed and the cells were re-suspended in 1.5ml 1X T.E/LiAc solution (Sigma-Aldrich, Poole, UK) at room temperature. Competent cells were used immediately.

5.2.3.2 Transformation

To each 1.5 ml Eppendorf tube, 10 µl (or 0.1 mg) of carrier DNA, ~100 ng of each plasmid DNA (see table 5.2 for concentrations and plasmid details) and 100 µl of competent INV*Sc*1 was added and vortexed. To each Eppendorf tube, 600 µl of plate buffer was added and vortexed again. Tubes were incubated at 30°C shaking at 150 rpm for 30 minutes. DMSO (70 µl) was added to each tube and inverted 6-8 times. Cells were then heat shocked on a heat block for 15 minutes at

42°C. Tubes were then spun at 3000 rpm for 5 seconds and re-suspended in 500 µl of sterile dH_2O. Synthetic dextrose (SD) plates (see appendix D for constituents) (see table 5.2 below for appropriate SD plates) were spread with 100 µl of each transformation and incubated at 30°C for 4 days until colonies appeared (see table 5.2 for colony numbers).

Table 5.2 Information on the plasmids added to each transformation tube, which selective media was used and the number successful of colonies grown.

Plasmids added	Amount in µl to make ~100 ng	SD media	Number of colonies
pYES2-*lacZ*-ERE and pESC-TRP-rtERα	1 µl of pYES2-*lacZ*-ERE 0.45 µl of pESC-TRP-rtERα	Yeast nitrogen base (YNB) – Uracil (URA) – tryptophan (TRP)	7
pESC-TRP-rtERα	0.45 µl of pESC-TRP-rtERα	YNB -TRP	147
pYES2-*lacZ*-ERE	1 µl of pYES2-*lacZ*-ERE	YNB - URA	214
Control plasmid – pRS316	0.4 µl of pRS316	YNB - URA	236
pESC-TRP-zfERα	1.5 µl of pESC-TRP-zfERα	YNB - TRP	0
pYES2-*lacZ*-ERE and pESC-TRP-zfERα	1 µl of pYES2-*lacZ*-ERE 1.5 µl of pESC-TRP-zfERα	YNB - URA - TRP	0
Negative control – no plasmid	N/A	N/A	0

INV*Sc*1 that was successfully transformed with pYES2-*lacZ*-ERE and pESC-TRP-zfERα was used in the assay to determine the oestrogenicity of VMs and will from now on be referred to as INV*Sc*1-rtERα.

5.2.4 Assay for determination of oestrogenicity of VMs

Due to complications with cloning the YES could not be completed. The methods below are how the YES assay should be carried out. Test VMs are dissolved in either absolute EtOH or DMSO. Solutions are to be made up on the day of use. Each assay should include an E2 standard curve from 0.25 nM – 50 nM. Chemicals dissolved in absolute EtOH should be serially diluted and a volume of 10 µl should be added to each well. EtOH should be allowed to evaporate to dryness before cells are added. VMs diluted in DMSO should be added at a volume of 4 µl before cells are added. A row of blanks and a row of negative controls (cells with EtOH or DMSO and no test substance) are to be added to each 96 well plate. Glassware should be scrupulously scrubbed with

laboratory grade washing up liquid, rinsed with EtOH twice and then autoclaved to ensure there was is contamination between experiments.

Add a colony of INV*Sc*1-rtERα to 50ml of YNB-URA-TRP and shake at 30°C at 200 rpm for 24 hours, until the OD_{600} reaches 5. Add 500 µl of CPRG, at a concentration of 10 mg/ml, to 50 ml of YNB-URA-TRP. CPRG is the substrate for β-galactosidase and can therefore be used to measure the expression of β-galactosidase from the reporter gene *lacZ*. Inoculate 50 ml of fresh YNB-URA-TRP with CPRG with INV*Sc*1- zfERα to reach an OD_{600} of ~1 (or 3 x 10^7 cells per ml). Seed cells at a concentration of 6 x 10^6 cells per well or a volume of 200 µl per well. Once cells are added to the 96 well plates, put on a plate shaker for two minutes to ensure thorough mixing and incubate at 32°C. Shake once a day for three days, return plates to the incubator after shaking. On the fourth day left for one hour for cells to settle after shaking. Read the plate at 540 nm for CPRG and 620 nm for turbidity. The following equation should be used to correct for turbidity.

Equation 5.1

$$R = A_{540nm} - (A_{620nm} - B_{620nm})$$

Where *A* is the absorbance of the test VM and *B* is the average absorbance of the blank wells and *R* is the result.

5.3 Results and Discussion

Due to difficulties in cloning the ERE insert into pYES2-*lacZ* (see appendix H for sequence), the two hybrid recombinant YES was in this instance, not successful. Although co-transformation of pYES2-TRP-zfERα and pYES2-*lacZ* into INV*Sc*1 was successful, without the ERE to act as a promotor to the *lacZ* gene, expression in the YES assay failed. There are a number of solutions to rectify this problem however due to time constraints this was not possible. There are companies that offer the commercial construction of synthetic plasmids such as ThermoFisher's GeneArt™. Synthetically constructed plasmids are offered in a generic backbone, digestion and ligation is still needed and it is still not guaranteed to work.

A number of reasons could explain why the cloning did not work. When performing a gel extraction, DNA is exposed to UV light, which can cause mutations. If mutations were present in one of the restriction sites then the restriction enzyme would not have digested the site properly, resulting in the plasmid self-ligating. If this is the reason why the cloning did not work then it should have been repeated from scratch. The sequencing data shows that there are several mutations within the sequence, this could be due to UV light or alternatively it could be due to the small size of the ERE insert. The ERE insert is relatively small (15 bp), cloning small pieces of DNA can be difficult due to the ratios of DNA to plasmid and ligation also can be a problem. A possible solution to this could have been to repeat the sequence up to four times. In some two-

hybrid systems used by other researchers this has been done to minimise this problem (Ackermann *et al.*, 2002; Katsu *et al.*, 2006). In the study described above the ERE insert was made from two synthesised oligonucleotide sequences, in order to create a bigger product of around 60 bp, double stranded DNA would have to have been synthesised which would have been more costly. As the ERE sequence is palindromic it is likely that it would self-anneal (Cheskis and Freedman, 1998), therefore the product that was inserted into the pYES2 backbone may not have been purely double stranded ERE, although a gel extraction was performed after annealing the two strands to prevent this possibility.

5.3.1 Conclusion

Due to technical difficulties in cloning the YES did not work, and therefore the aims of this chapter could not be achieved (section 5.1). However, this does not mean it is not a reasonable method for validating molecular docking of VMs to the ERα. In other studies (Arnold *et al.*, 1996; Beck *et al.*, 2006; Fu *et al.*, 2007; Rehmann *et al.*, 1999; Routledge and Sumpter, 1996) it has been a successful *in vitro* method for determining xenoestrogens and its application to validate molecular docking is an area to be considered in future work.

Chapter 6

General Discussion

6.1 Hypothesis Testing

Hypothesis 1 'Veterinary medicines used in intensive farming enter the environment as mixtures with the potential to have an additive effect, impacting negatively on non-target organisms' (chapter 1; section 1.9.1[1]) was addressed in chapter 2 and chapter 3. Chapter 2 showed that veterinary medicines were being released directly into the environment in mixtures (see chapter 2; section 2.3; table 2.4; figures 2.6-2.8). Chapter 3 addressed the additive effect these VMs had on the bioluminescent bacteria *A. fischeri* and found that there was an additive effect, rather than a synergistic or antagonistic effect (see chapter 3; section 3.3.3; figure 3.12; $R^2 = 0.8257$; $P < 0.001$). Therefore hypothesis 1 (chapter 1; section 1.9.1) can be accepted.

Hypothesis 2 'Molecular docking is a viable tool for determining the environmental impacts of veterinary medicines on non-target organisms' (chapter 1; section 1.9.1[2]) was addressed in chapter 4 and chapter 5. There was evidence that molecular docking could be used to predict binding of VMs to specified proteins in non-target organisms and is, therefore, potentially a viable tool for determining the impacts of pollutants on non-target organisms (Baker, 2014; Li *et al.*, 2015; McCullough *et al.*, 2014; Selvam *et al.*, 2015). The *in vitro* YES assay, however, was unsuccessful (chapter 5) and, therefore, could not provide supportive evidence for this hypothesis. As a consequence hypothesis 2 cannot fully be answered. This is an area for future work.

6.2 Mixtures

6.2.1 Cypermethrin and diazinon

Cypermethrin and diazinon were both used as sheep dip and are commonly found together in contaminated water and sediment. They are both reported to affect the olfactory system in male salmon, in low concentrations (<0.001 µg/L for cypermethrin and 0.4 µg/L for diazinon) (Moore and Waring, 1996; Moore and Waring, 2001). They have routinely been measured in the rivers at concentrations exceeding 0.85 µg/L and 35 µg/L respectively (Potter and Dare, 2003), with reports of cypermethrin occurring at concentrations as high as 85.1 µg/L (Boxall *et al.*, 2002) which is higher than the effect concentration described above. Out of 573 freshwater sites sampled for cypermethrin contamination in England and Wales, 20% of sites had concentrations of cypermethrin higher than the PNEC of 0.1 ng/L (UK Technical Advisory Group, 2008). Molecular docking results gathered in chapter 4 show that α-cypermethrin is showed a high likelihood of binding to the ERα of all of the aquatic species tested (apart from *C. gigas*) (chapter 4; section 4.3;

table 4.6a and 4.6b). Diazinon showed a medium likelihood of binding to all of the ERα (apart from *C. gigas* where it showed a high likelihood of binding) (chapter 4; section 4.3; table 4.6a and 4.6b). Suggesting that in waters containing diazinon and cypermethrin, an additive effect on the ERα of non-target organisms is possible.

6.2.2 Cypermethrin, deltamethrin and teflubenzuron

Three VMs that are often used together in aquaculture are cypermethrin, deltamethrin and teflubenzuron. In chapter 2, where the use of sea lice treatments in Scotland in the years 2007-2011 was mapped and compared to MECs for the years 2008-2012, it was found that teflubenzuron was used as a treatment 12 times between the years 2007-2011, on three of those occasions teflubenzuron was applied in conjunction with at least one other sea lice treatment and on two of those occasions deltamethrin was applied alongside teflubenzuron (see appendix C; table C.1). Teflubenzuron is highly persistent in the environment (SEPA, 1999) (chapter 2; section 2.3; table 2.1) so even when treatments were not applied within the same month, it is likely that environmental exposure by these treatments will have occurred together when applied even months afterwards (see chapter 2; section 2.3; table 2.1, figures 2.4 – 2.6). For example during the year 2008 teflubenzuron was not used as a sea lice treatment in any of the salmon farms in Scotland, however in 2009 two sediment samples containing teflubenzuron were detected (chapter 2; section 2.3; table 2.4; figure 2.12c). There were 14 occasions in the years 2008-2011 where deltamethrin and cypermethrin were applied to the same farm within the same month (see appendix C). Cypermethrin is a high priority substance, which was added to a UK specific priority watch list (UK Technical Advisory Group, 2008). Monitoring of cypermethrin in rivers in England and Wales found that 7% of rivers tested were above EQS and are therefore in need of greater monitoring. The molecular docking work in this project (chapter 4) indicates that cypermethrin and deltamethrin are highly likely to disrupt the ERα of several aquatic organisms (apart from the negative control *C. gigas*). Teflubenzuron also showed evidence of being a possible ERα disruptor (chapter 4; section 4.3; table 4.6a, 4.6b; figure 4.21). As there is evidence that cypermethrin and deltamethrin have an effect on the ERα of several organisms (chapter 4) (Kojima *et al.*, 2004; Presibella *et al.*, 2005; Sun *et al.*, 2014) and teflubenzuron (chapter 4) may have an effect on the ERα of some organisms, it is proposed that these three VMs be mixture tested to determine whether they have an additive effect on the ERα. It is also recommended that testing of teflubenzuron is specifically targeted on the ERα of *G. aculeatus,* because of its high binding energy (-7.33 kcal/mol) compared with the low energy binding of *D. rerio* and *S. aurata* (-4.84 and -4.95 kcal/mol respectively) (chapter 4; figure 4.21). Such species targeting should be carried out in order to confirm whether this VM is capable of causing variable disruption to the ERα of different aquatic species. There is reasonable evidence that these VMs are entering the environment in mixtures and that they could be having an additive effect on the ERα, which in turn can have downstream impacts on sexual development (Jobling *et al.*, 1998); fertility (Jobling

et al., 2002); gonadosomatic growth index (Filby *et al.*, 2007); osmoregulation (Carrera *et al.*, 2007; Lerner *et al.*, 2012); immune function (Casanova-Nakayama *et al.*, 2011); and embryonic development (Jobling *et al.*, 2003).

6.2.3 Azamethiphos, emamectin benzoate and 3PBA

Mixtures of the sea lice treatments azamethiphos, emamectin benzoate and 3PBA did cause an acute effect (chapter 3; section 3.3.3; table 3.2; figures 3.8-3.11) and the effects could be effectively predicted using IA (chapter 3; section 3.3.3 figure 3.12). Although mixture effects could be predicted, this project has found that the concentrations of sea lice treatments in the UK marine environment are not high enough to cause an acute impact on the bioluminescent bacteria *A. fischeri* when compared with their MECs (chapter 3; section 3.3.1 table 3.1).

6.3 Sea lice treatments and AutoDock

Of the five sea lice treatments tested, emamectin benzoate did not demonstrate affinity to any of the ten ERα receptors (see tables 4.6a and 4.6b). Deltamethrin and α-cypermethrin showed high affinity to all of the ERα, apart from the negative control *C. gigas* (see chapter 4; section 4.3 table 4.6b) and were categorised as having a high likelihood of binding to all species ERα (see chapter 4; section 4.3; tables 4.6a and 4.6b). Teflubenzuron was showed a high likelihood of binding to the ERα of the three-spined stickleback *G. aculeatus* (chapter 4). The use of teflubenzuron dramatically increased from 2002 – 2013 going from 72.73 kg total use in 2002 to 261.96 kg total use in 2013 (chapter 2, section 2.3; table 2.4). *G. aculeatus* is an anadromous fish and its distribution is in and around UK coastal waters, as this is where Scottish salmon farms are based it is likely that *G. aculeatus* is at risk of exposure to concentrations of teflubenzuron that could cause an endocrine disrupting effect.

Azamethiphos showed a medium likelihood of binding to all ten of the tested ERα (chapter 4). Since azamethiphos is the most hydrophilic of the sea lice treatments, and has a half-life of 10.8 days in water, and (see chapter 2; section 2.1.2; table 2.1) levels of exposure to organisms dwelling in the water column is likely to be high. As the exposure is high, and the binding to the ERα is of a medium likelihood then it is recommended that azamethiphos also be tested for oestrogenic activity using an *in vitro* assay and ecotoxicological studies on environmentally relevant species (see chapter 4; section 4.4.4).

Teflubenzuron was not included in the 56 high priority list as the toxicity of teflubenzuron was reported as being low (Boxall *et al.*, 2002). The aquatic toxicity information gathered in the Boxall *et al.* (2002) document for teflubenzuron was based on the toxicity towards fish. However, toxicity data on teflubenzuron available since this was published indicates that concentrations found in the sediment (chapter 2, section 2.3; table 2.4) are higher than the chronic effects for some invertebrates. In a two generational study Tassou and Schulz (2011) found that the EC_{50} for

the fecundity and fertility of *C. riparius* was 112.7 µg/kg and 74.5 µg/kg respectively; which is lower than five of the MECs found around Scottish salmon farms (Chapter 2, section 2.3; table 2.4). Tassou and Schulz (2011) also found that emergence rates of the second generation of *C. riparius* suffered the effects of teflubenzuron at a lower concentration than their parents with the NOEC for emergence being 62.5 µg/kg for offspring compared with a NOEC of 100 µg/kg for parents. Since the life cycle of *C. riparius* is around 25 days it is likely that teflubenzuron would affect more than one generation of this invertebrate and possibly other invertebrates as well (Weltje *et al.*, 2009). Although *C. riparius* is a freshwater sediment dwelling invertebrate, there are no current data on sediment dwelling marine species. Teflubenzuron is more persistent in the environment than previously reported, although the degradation information varies vastly depending on the information source. The SEPA (1999) data indicates that in a 'worst case scenario' the half-life in marine sediment should be set to 115 days (see chapter 2, section 2.4.2). In this 'worst case scenario', however, the amount of teflubenzuron added to the salmon farm was 19.6 kg, which is almost 4 times lower than what has been applied on the site SLO1 (UK NGR HU28508270) in December 2010 (see chapter 2; section 2.3; table 2.3 and figure 2.6a). The impact of teflubenzuron applied at such a high concentration has not been fully investigated and therefore this is an area that needs to be reviewed. It appears from the results displayed in chapter 2, that teflubenzuron does persist for longer than the estimated half-life there 19 sediment MECs which have a detectable concentration of teflubenzuron which are more than 2 km away from the nearest farm that used the treatment in the previous year (see table 2.4). There are two sites in 2008 where the sediment MEC for teflubenzuron was higher than the sediment MAC and the nearest salmon farm using teflubenzuron was more than 94 km away. In 2008 teflubenzuron was measured at concentrations as high as 170.1 µg/kg and 6.36 µg/kg, 94.8 km and 95.1 km away from the nearest salmon farm declaring the use of teflubenzuron respectively (see chapter 2; section 2.3; table 2.4; figure 2.6a; figure 2.9). There is also evidence that teflubenzuron causes physical deformities to the American lobster *H. gammarus* as described in chapter 2 (section 2.4.2) (Samuelsen *et al.*, 2014). Since the salmon culture industry is growing at such a fast pace (see chapter 2; section 2.1), and with the sea lice infection increasing (Costello 2006) it is proposed that the half-life of teflubenzuron is reviewed, as increased use would lead to increased sediment MECs and elevated risk to non-target organisms, especially chitin producing invertebrates (Tassou and Schulz, 2011).

6.4 The use of molecular docking in regulation

The sequencing of proteins has increased almost exponentially from 1952 when Fred Sanger first sequenced the peptide protein insulin to the present day. There are now over 63 million protein sequences available on the database UniProt as of 11th April 2016 (http://www.uniprot.org/). This information can be used to gain a bigger picture on which molecular pathways other than the

MOA might be affected by different drugs. As more information becomes available on species that are threatened or endangered molecular docking will be a useful tool to predict the effects of VMs and pharmaceuticals on the more vulnerable species without the need for testing. Finding species specific sensitivities is of upmost importance. The vulnerability of *Gyps* spp. to diclofenac was not anticipated from the ecotoxicology testing of model species (see chapter 1, section 1), as tolerance of other species of birds to diclofenac was much higher than exposure levels (Naidoo *et al.*, 2007; Taggart *et al.*, 2007). It is not always feasible to undertake ecotoxicological testing on animals, especially vulnerable and endangered species, or practically possible to use species that are representative of often very distinct aquatic habitats. Moreover, sensitivities of native species may not accurately be predicted by using model species alone (Naidoo *et al.*, 2007). Recently there has been some research supporting the sensitivity of bees to neonicotinoids with the aid of molecular docking (Li *et al.*, 2015; Selvam *et al.*, 2015). This highlights how molecular docking can find sensitive species and aid ecotoxicology in the future. In a molecular docking study of the effects of imidacloprid and thiacloprid on the target α6 nicotinic acetylcholine receptor Selvam *et al.* (2015) found that the two neonicotinoids bound more selectively to the cockroach receptor than the honeybee receptor. In a separate study, Li *et al.* (2015) investigated the binding of imidacloprid to the honeybee *Apis cerana,* using mixed method *in silico* and *in vitro* investigations. They found that imidacloprid bound to the odorant binding protein, causing possible chronic effects of the olfactory system, which could cause implications in foraging behaviour thereby contributing to the colony collapse. This emphasises the importance of chronic testing as lethal ecotoxicology testing does not give any indication on the downstream consequences that chronic effects can have on a whole population. Chronic testing often focuses on reproductive testing which can be easier to measure than other systems such as the olfactory system. If molecular docking can assist in identifying impacts that environmental pollutants can have on an individual species level, insights on population dynamics can be anticipated.

Since beginning this project there has been a great deal more focus on the use of molecular docking in regulatory ecotoxicology. The US EPA developed an *in vitro* high throughput screening of chemicals called ToxCast in 2007, this work has developed to include *in silico* work and in 2014 DockScreen was developed by Goldsmith *et al.* (2014), a database which docks chemicals to known 3D structures of receptors. This database focuses on pathways and does not aim to compare the binding of ligands to receptors for different species.

6.5 Veterinary Medicines of Concern

From this study five of the VMs investigated stood out as being of high concern as xenoestrogens. These were deltamethrin, cypermethrin, fenbendazole, teflubenzuron and amitraz (chapter 4). Three of these VMs, deltamethrin, cypermethrin and teflubenzuron, are prominently used in aquaculture and therefore their release into the environment is direct. Fenbendazole has a high

use but there is relatively little ecotoxicology information about it, it also appears on a number of high priority lists (Boxall *et al.*, 2003; Capleton *et al.*, 2006; Kim *et al.*, 2008). The evidence for fenbendazole as an endocrine disruptor has only been determined for invertebrates where it has weak interactions with the ribosomal protein gene RpS3 in *C. riparius*, which is essential for cellular growth and regulation (Park and Kwak, 2012). Amitraz has also had relatively little attention in its potential for interactions with the ERα but in this study interaction with the ERα of four of the species investigated showed there was a high likelihood of binding (see table 4.6a and 4.6b; figure 4.23).

Diclofenac has recently been added to the priority substances list under directive (2008/105/EC). This list also includes E2 and EE2. According to the information gathered in chapter 4, diclofenac may pose a risk to the ERα of aquatic species, especially the common roach *R. rutilus* (see chapter 4, table 4.6a). Cypermethrin and diazinon were added to a UK specific list on pollutants where under the WFD their MACs were revaluated due to evidence of their toxic effect towards invertebrates (UK Technical Advisory Group, 2008). Diclofenac was showed a medium likelihood of binding to the ERα for all of the tested species apart from *R. rutilus* where it was considered a to have a high likelihood of binding (chapter 4). Since its inclusion in the WFD priority substances watch list it is recommended, to eliminate any doubt, *in vitro* studies on the ERα of diclofenac also should be carried out. In other studies it has been shown experimentally to have an effect on reproduction in *O. mykiss* and *D. rerio* at a concentration of 3200 µg/L with no effect at 1000 µg/L (Memmert *et al.*, 2013). Memmert *et al.* (2013) recommends that the NOEC of diclofenac in the aquatic environment to be 320 µg/L, which is magnitudes higher than many recorded concentrations of the drug in sewage effluent in the UK where it has been recorded at a maximum of 2349 ng/L (Ashton *et al.*, 2004).

Both diazinon and cypermethrin have the ability to cause toxic effects at concentrations lower than their current MECs and also there is evidence that cypermethrin has the ability to cause an endocrine disrupting effect through the ERα as shown in chapter 4 (table 4.6a and 4.6b) and indicated in other reports (Kojima *et al.*, 2004; Sun *et al.*, 2014). There is evidence that diazinon has an effect on the endocrine system (Moore and Waring, 1996; Maxwell and Dutta, 2005), and an estimation of binding likelihood made in this thesis (chapter 4; table 4.6a and 4.6b) suggests it has a medium likelihood of binding to all of the species investigated. It is therefore advised that the effect on the ERα is investigated further for diazinon.

6.6 Conclusion

This project has demonstrated that sea lice controls are being used in mixtures, specifically cypermethrin, deltamethrin and teflubenzuron that could potentially disrupt the ERα. Teflubenzuron is more persistent in sediment than previously thought, and could persist in the

environment for longer than its half-life of 115 days (see chapter 2, section 2.4.2). Currently, teflubenzuron is being applied at concentrations that are almost four times higher than the worst-case scenario proposed in 1999 (19.6 kg in 1999 compared with 75 kg in December 2010 (chapter 2; section 2.3.1 figure 2.6a and figure 2.16); it is therefore proposed that teflubenzuron persistence in sediment is re-examined to establish a more accurate picture of its possible environmental impacts. By mapping the use of sea lice treatments (chapter 2) and comparing these with MEC data, it is apparent that either sea lice treatments are more persistent in the environment than previously thought or that salmon farms are using treatments without declaring their use.

Molecular docking is a tool that can aid ecotoxicology testing and can provide insight into potentially sensitive species non-target species, as well as identifying molecular targets, which could cause chronic effects. Equations that predict the effects of mixtures, such as IA (chapter 3; equation 3.2; figure 3.12) and CA (chapter 3; equation 3.1; figure 3.13) can serve as useful tools for the prediction of mixture toxicity. Ideally it would be beneficial to combine molecular docking results with equations for mixture toxicity in order to predict mixture effects. While molecular docking appears to be able to differentiate between VMs which bind to the ERα and those which do not, there is no evidence that it can be used to differentiate between agonists and antagonists. The oestrogenic potential of the VMs in this study has not been tested *in vitro* and therefore determining whether molecular docking can assist with mixture toxicity would, at this stage, be speculative. This is an area that would benefit from further investigation.

6.7 Future Work

In order to effectively answer hypothesis 2 (chapter 1; section 1.9.1[2]) the VMs that were examined in chapter 4 need to be tested using an ERα *in vitro* reporter assay to test for both agonistic and antagonistic effects. This could be achieved using a yeast two hybrid system such as the YES described in chapter 5, section 5.2. As molecular docking evolves and develops, along with sequencing data libraries, databases of receptors can be prepared in order to dock chemicals of interest to various receptors from several species. Docking new chemicals to libraries of receptors would allow for rapid identification of biological pathways involved in chronic effects and also would be able to differentiate between possible species sensitivity. Testing can also become more targeted to populations that are likely to be subjected to released VMs rather than the model species that are currently the focus of testing. Once areas of interest have been identified (either specific biological pathways or sensitive species) further confirmation should be carried out with *in vitro* or *in vivo* work. Presently, molecular docking can predict what may cause an effect, rather than the extent of the effect and the possible consequences. To improve this it is suggested that more research is carried out with more possible target receptors such as cytochrome p450 and compared with pharmacology information to optimise the use of molecular docking. However,

predicting the actual concentration that would cause an effect would be difficult using molecular docking as there is a lot of contention on the scoring mechanism of these tools (Leach *et al.*, 2006). This is why it is proposed to be used as a tool for predicting species sensitivity and possible chronic effects rather than a tool to suggest doses and NOECs. The use of molecular docking as a tool for predicting mixture toxicity is not recommended until further testing using *in vitro* assays has been completed and directly compared.

As mentioned above (section 6.3 and 6.6, this chapter), it is highly recommended that the biodegradation and persistence of teflubenzuron is re-tested to fit in with current use of teflubenzuron in aquaculture, which has exceeded the 'worst case scenario' of 1999 by almost 4 times in an application in (SEPA, 1999). It is also highly recommended that fenbendazole is tested for its effect on the ERα of several aquatic species, as this study (chapter 4, table 4.6a and 4.6b) suggests that fenbendazole is either an ERα agonist or antagonist. This has not previously been tested for endocrine disrupting effects on vertebrates.

Appendix A

Salmon farms using treatments on SACs

Table A.1 Salmon farms that are located on special areas of conservation (SACs), which used sea lice treatments in 2007.

Name and NGR of SAC	Name of Farm	NGR of Farm	Treatments used	Reason for designation
Firth of Lorn NM696089	FFMC53	NM71400780	Emamectin benzoate	Reefs
Loch Creran NM945428	FFMC19	NM93074182	Emamectin benzoate; cypermethrin	Reefs
Sunart NM665605	INV1	NM73316102	Emamectin benzoate	Reefs; *Lutra lutra*
	GCD1	NM67606070	Emamectin benzoate; cypermethrin	
	CAG1	NM64425884	Emamectin benzoate; cypermethrin	
Lochs Duich, Long and Alsh Reefs NG845261	SRO1	NG78402535	Emamectin benzoate	Reefs
	ARDT1	NG82412420	Emamectin benzoate	
	DUI1	NG89172320	Emamectin benzoate	
Loch nam Madadh NF924703	CLP1	NF94866945	Emamectin benzoate	Reefs; *L. lutra;* coastal lagoons; shallow inlets and bays; mudflats and sandflats; sandbanks cover by seawater
Loch Laxford NC198501	ARD1	NC18895014	Emamectin benzoate; cypermethrin	Reefs; shallow inlets and bays
	EAM1	NC19864961	Emamectin benzoate; cypermethrin	
	FOI1	NC19904920	Cypermethrin	
Yell Sound Coast HU467755	SWI2	HU45717193	Emamectin benzoate	*L. lutra; P. vitulina*
	SETW1	HU47427068	Emamectin benzoate	

Table A.2 Salmon farms that are located on special areas of conservation (SACs), which used sea lice treatments in 2008.

Name and NGR of SAC	**Name of Farm**	**NGR of Farm**	**Treatments used**	**Reason for designation**
Loch Creran NM945428	FFMC20	NM93804220	Emamectin benzoate	Reefs
Sunart NM665605	INV1	NM73316102	Emamectin benzoate; cypermethrin; deltamethrin; azamethiphos	Reefs; *Lutra lutra*
	GCD1	NM67606070	Emamectin benzoate; cypermethrin	
	CAG1	NM64425884	Emamectin benzoate; cypermethrin; deltamethrin; azamethiphos	
Lochs Duich, Long and Alsh Reefs NG845261	SRO1	NG78402535	Emamectin benzoate; cypermethrin	Reefs
	ARDT1	NG82412420	Emamectin benzoate; cypermethrin	
	DUI1	NG89172320	Emamectin benzoate; cypermethrin	
Loch Laxford NC198501	ARD1	NC18895014	Emamectin benzoate; cypermethrin	Reefs; shallow inlets and bays
	EAM1	NC19864961	Emamectin benzoate; cypermethrin	
	FOI1	NC19904920	Emamectin benzoate; cypermethrin	
Yell Sound Coast HU467755	SWI2	HU45717193	Emamectin benzoate	*L. lutra; P. vitulina*

Table A.3 Salmon farms that are located on special areas of conservation (SACs), which used sea lice treatments in 2009.

Name and NGR of SAC	**Name of Farm**	**NGR of Farm**	**Treatments used**	**Reason for designation**
Firth of Lorn NM696089	FFMC53	NM71400780	Emamectin benzoate	Reefs
Loch Creran NM945428	FFMC20	NM93804220	Emamectin benzoate; cypermethrin; deltamethrin	Reefs
Sunart NM665605	INV1	NM73316102	Emamectin benzoate; deltamethrin	Reefs; *Lutra lutra*
	CAG1	NM64425884	Emamectin benzoate; deltamethrin	
Lochs Duich, Long and Alsh Reefs NG845261	SRO1	NG78402535	Emamectin benzoate	Reefs
	ARDT1	NG82412420	Emamectin benzoate	
	DUI1	NG89172320	Deltamethrin	
Eileanan agus Sgeiran Lios mór NM888471	FFMC40B	NM86104520	Emamectin benzoate	
Loch Laxford NC198501	FOI1	NC19904920	Azamethiphos	Reefs; shallow inlets and bays
Yell Sound Coast HU467755	SWI2	HU45717193	Emamectin benzoate; azamethiphos	*L. lutra; P. vitulina*

Table A.4 Salmon farms that are located on special areas of conservation (SACs), which used sea lice treatments in 2010.

Name and NGR of SAC	**Name of Farm**	**NGR of Farm**	**Treatments used**	**Reason for designation**
Loch Laxford NC198501	ARD1	NC18895014	Emamectin benzoate; azamethiphos	Reefs; shallow inlets and bays
	FOI1	NC19904920	Emamectin benzoate; azamethiphos	
Loch Creran NM945428	FFMC19	NM93074182	Emamectin benzoate; deltamethrin	Reefs
Sunart NM665605	INV1	NM73316102	Emamectin benzoate; deltamethrin; azamethiphos	Reefs; *Lutra lutra*
	GCD1	NM67606070	Emamectin benzoate; deltamethrin; azamethiphos	
	CAG1	NM64425884	Emamectin benzoate; deltamethrin; Azamethiphos; cypermethrin	
Lochs Duich, Long and Alsh Reefs NG845261	SRO1	NG78402535	Deltamethrin	Reefs
	ARDT1	NG82412420	Deltamethrin	
Eileanan agus Sgeiran Lios mór NM888471	FFMC40B	NM86104520	Emamectin benzoate	*P. vitulina*
Loch nam Madadh NF924703	CLP1	NF94866945	Emamectin benzoate; deltamethrin	Reefs; *L. lutra;* coastal lagoons; shallow inlets and bays; mudflats and sandflats; sandbanks cover by seawater
	FERR1	NF93806990	Emamectin benzoate; deltamethrin	
Yell Sound Coast HU467755	SWI2	HU45717193	Emamectin benzoate	*L. lutra; P. vitulina*
	BOA1	HU49507100	Emamectin benzoate; deltamethrin	

Table A.5 Salmon farms that are located on special areas of conservation (SACs), which used sea lice treatments in 2011.

Name and NGR of SAC	**Name of Farm**	**NGR of Farm**	**Treatments used**	**Reason for designation**
Loch Laxford NC198501	ARD1	NC18895014	Emamectin benzoate; azamethiphos; deltamethrin	Reefs; shallow inlets and bays
	FOI1	NC19904920	Emamectin benzoate; azamethiphos; teflubenzuron	
Loch Creran NM945428	FFMC19	NM93074182	Deltamethrin	Reefs
Sunart NM665605	INV1	NM73316102	Emamectin benzoate; deltamethrin	Reefs; *Lutra lutra*
	CAG1	NM64425884	Emamectin benzoate; deltamethrin	
Eileanan agus Sgeiran Lios mór NM888471	FFMC40B	NM86104520	Deltamethrin	*P. vitulina*
Loch nam Madadh NF924703	CLP1	NF94866945	Emamectin benzoate	Reefs; *L. lutra;* coastal lagoons; shallow inlets and bays; mudflats and sandflats; sandbanks cover by seawater
Firth of Lorn	FFMC53	NM71400780	Emamectin benzoate; azamethiphos	Reefs
Yell Sound Coast HU467755	SWI2	HU45717193	Emamectin benzoate	*L. lutra; P. vitulina*
	BOA1	HU49507100	Emamectin benzoate	

Appendix B

Number of applications of treatments

Table B.1 The number of applications of each treatment for the year 2007. Number of individual farms using each treatment also shown.

Treatment	Number of applications	Number of farms
Azamethiphos	0	0
Cypermethrin	308	87
Deltamethrin	0	0
Emamectin benzoate	308	147
Teflubenzuron	8	6

Table B.2 The number of applications of each treatment for the year 2008. Number of individual farms using each treatment also shown.

Treatment	Number of applications	Number of farms
Azamethiphos	68	34
Cypermethrin	149	71
Deltamethrin	77	41
Emamectin benzoate	294	154
Teflubenzuron	0	0

Table B.3 The number of applications of each treatment for the year 2009. Number of individual farms using each treatment also shown.

Treatment	Number of applications	Number of farms
Azamethiphos	138	55
Cypermethrin	91	49
Deltamethrin	280	104
Emamectin benzoate	209	125
Teflubenzuron	3	3

Table B.4 The number of applications of each treatment for the year 2010. Number of individual farms using each treatment also shown.

Treatment	Number of applications	Number of farms
Azamethiphos	124	57
Cypermethrin	61	32
Deltamethrin	237	94
Emamectin benzoate	242	123
Teflubenzuron	1	1

Table B.5 The number of applications of each treatment for the year 2011. Number of individual farms using each treatment also shown.

Treatment	**Number of applications**	**Number of farms**
Azamethiphos	153	69
Cypermethrin	14	10
Deltamethrin	326	114
Emamectin benzoate	274	137
Teflubenzuron	8	7

Appendix C

The occurrence of chemical mixtures of sea lice treatments on salmon farms in Scotland 2007 -2011

Table C. 1 The number of times each sea lice treatment was applied in conjunction with at least one other treatment within the same month on the same fish farm.

Treatment combinations	Year					
	2007	2008	2009	2010	2011	Total
Cypermethrin and teflubenzuron	1	0	0	0	0	1
Cypermethrin and emamectin benzoate	43	26	7	3	0	79
Cypermethrin and deltamethrin	0	5	3	3	3	14
Cypermethrin and azamethiphos	0	15	25	38	5	83
Deltamethrin and teflubenzuron	0	1	0	0	0	1
Deltamethrin and emamectin benzoate	0	8	21	21	50	100
Deltamethrin and azamethiphos	0	0	31	34	82	147
Emamectin benzoate and teflubenzuron	0	0	0	1	0	1
Emamectin benzoate and azamethiphos	0	0	11	12	32	55
Azamethiphos and teflubenzuron	0	0	0	0	0	0
Cypermethrin, azamethiphos and emamectin benzoate	5	0	0	3	0	8
Deltamethrin, azamethiphos and emamectin benzoate	0	0	0	2	15	17
Deltamethrin, cypermethrin, azamethiphos and emamectin benzoate	0	2	0	0	0	2
Total	49	57	98	117	187	508

Appendix D

Media and agar constituents

Table D.1 Media constituents for broths and agar.

Media	Reagents	Sterilisation	pH	Agar
Lysogeny broth (LB)	LB powder 37 g (Fisher, Aberdeen, UK)	121°C 15 minutes	7.2	15 g
Oceanbulbus Broth	Sea salts (Sigma-Aldrich, Poole, UK) 14 g Tryptone (Sigma-Aldrich, Poole, UK) 10 g Yeast extract (Sigma-Aldrich, Poole, UK) 5 g NaCl 10 g	121°C 15 minutes	7.2	15 g
Yeast Extract-Peptone-Dextrose (YPD) broth	YPD broth (Sigma-Aldrich, Poole, UK) 50 g	121°C 15 minutes	6.5	15 g
Yeast Nitrogen Base (YNB) without amino acids	YNB powder (Sigma-Aldrich, Poole, UK) 6.8 g Glucose (Sigma-Aldrich, Poole, UK) 5 g	Filter sterilise	5.4	15 g
YNB without uracil (URA)	YNB powder 6.8 g Glucose 5 g Yeast Synthetic Drop-out Medium Supplements without uracil (Sigma-Aldrich, Poole, UK) 1.92 g	Filter sterilise	5.4	15 g
YNB without tryptophan (TRP)	YNB powder 6.8 g Glucose 5 g Yeast Synthetic Drop-out Medium Supplements without tryptophan (Sigma-Aldrich, Poole, UK) 1.92 g	Filter sterilise	5.4	15 g
YNB without URA and TRP	YNB powder 6.8 g Glucose 5 g Yeast Synthetic Drop-out Medium Supplements without uracil, leucine and tryptophan (Sigma-Aldrich, Poole, UK) 1.46 g L-Leucine (Sigma-Aldrich, Poole, UK) 380 mg	Filter sterilise	5.4	15 g

Appendix E

Example grid parameter file (.gpf) for *H. sapiens* ERα

```
npts 60 60 60
gridfld 3ERT.maps.fld
spacing 0.375
receptor_types N C OA NA SA A
ligand_types A OA C HD
receptor 3ERT.pdbqt
gridcenter 29.287 4.108 28.182
smooth 0.5
map 3ERT.A.map
map 3ERT.OA.map
map 3ERT.C.map
map 3ERT.HD.map
elecmap 3ERT.e.map
dsolvmap 3ERT.d.map
dielectric -0.1465
```

Appendix F

Example docking parameter file (.dpf) for *H. sapiens* ERα

```
# Generated with Raccoon v.1.0c
outlev 2
intelec
seed time pid
ligand_types A OA C HD
fld 3ERT.maps.fld
map 3ERT.A.map
map 3ERT.OA.map
map 3ERT.C.map
map 3ERT.HD.map
elecmap 3ERT.e.map
desolvmap 3ERT.d.map
move 3PBA.pdbqt
about 4.452 -2.048 -0.357
tran0 random
quat0 random
axisangle0 random
dihe0 random
rmstol 2.0
ga_pop_size 150
ga_num_evals 2500000
ga_num_generations 27000
ga_elitism 1
ga_mutation_rate 0.02
ga_crossover_rate 0.8
ga_window_size 10
ga_cauchy_alpha 0.0
ga_cauchy_beta 1.0
set_ga
sw_max_its 300
sw_max_succ 4
sw_max_fail 4
sw_rho 1.0
sw_lb_rho 0.01
ls_search_freq 0.06
set_psw1
unbound_model bound
ga_run 100
analysis
```

Appendix G

Tukey's post hoc results for binding energy; two way ANOVA between species

Table G.1 P values showing the statistical difference between species for average binding energy of 3PBA.

Species	*C. gigas*	*D. rerio*	*G. aculeatus*	*H. sapiens*	*O. mykiss*	*P. promelas*	*R. rutilus*	*S. salar*	*S. aurata*	*X. laevis*
C. gigas	-	0	0	0	0	0.011	0.001	0	0	0
D. rerio	0	-	0.72	0.817	0.954	0.057	0.235	0.942	0.899	0.496
G. aculeatus	0	0.72	-	0.555	0.678	0.123	0.407	0.666	0.817	0.747
H. sapiens	0	0.817	0.555	-	0.862	0.033	0.156	0.874	0.72	0.362
O. mykiss	0	0.954	0.678	0.862	-	0.05	0.213	0.988	0.853	0.46
P. promelas	0.011	0.057	0.123	0.033	0.05	-	0.475	0.048	0.076	0.222
R. rutilus	0.001	0.235	0.407	0.156	0.213	0.475	-	0.207	0.289	0.612
S. salar	0	0.942	0.666	0.874	0.988	0.048	0.207	-	0.841	0.451
S. aurata	0	0.899	0.817	0.72	0.853	0.076	0.289	0.841	-	0.58
X. laevis	0	0.496	0.747	0.362	0.46	0.222	0.612	0.451	0.58	-

Table G.2 P values showing the statistical difference between species for average binding energy of α-cypermethrin.

Species	*C. gigas*	*D. rerio*	*G. aculeatus*	*H. sapiens*	*O. mykiss*	*P. promelas*	*R. rutilus*	*S. salar*	*S. aurata*	*X. laevis*
C. gigas	-	0	0	0	0	0	0	0	0	0
D. rerio	0	-	0.806	0.484	0.933	0.531	0.794	0.633	0.888	0.101
G. aculeatus	0	0.806	-	0.65	0.871	0.703	0.988	0.817	0.699	0.163
H. sapiens	0	0.484	0.65	-	0.538	0.942	0.661	0.824	0.4	0.346
O. mykiss	0	0.933	0.871	0.538	-	0.587	0.859	0.694	0.822	0.119
P. promelas	0	0.531	0.703	0.942	0.587	-	0.715	0.881	0.442	0.31
R. rutilus	0	0.794	0.988	0.661	0.859	0.715	-	0.829	0.687	0.167
S. salar	0	0.633	0.817	0.824	0.694	0.881	0.829	-	0.536	0.244
S. aurata	0	0.888	0.699	0.4	0.822	0.442	0.687	0.536	-	0.075
X. laevis	0	0.101	0.163	0.346	0.119	0.31	0.167	0.244	0.075	-

Table G.3 P values showing the statistical difference between species for average binding energy of amitraz.

Species	*C. gigas*	*D. rerio*	*G. aculeatus*	*H. sapiens*	*O. mykiss*	*P. promelas*	*R. rutilus*	*S. salar*	*S. aurata*	*X. laevis*
C. gigas	-	0	0	0	0	0	0	0	0	0
D. rerio	0	-	0.356	0.495	0	0.789	0	0	0.962	0
G. aculeatus	0	0.356	-	0.108	0	0.512	0	0	0.331	0
H. sapiens	0	0.495	0.108	-	0	0.342	0	0	0.526	0
O. mykiss	0	0	0	0	-	0	0	0.92	0	0.835
P. promelas	0	0.789	0.512	0.342	0	-	0	0	0.752	0
R. rutilus	0	0	0	0	0	0	-	0	0	0.001
S. salar	0	0	0	0	0.92	0	0	-	0	0.758
S. aurata	0	0.962	0.331	0.526	0	0.752	0	0	-	0
X. laevis	0	0	0	0	0.835	0	0.001	0.758	0	-

Table G.4 P values showing the statistical difference between species for average binding energy of azamethiphos.

Species	*C. gigas*	*D. rerio*	*G. aculeatus*	*H. sapiens*	*O. mykiss*	*P. promelas*	*R. rutilus*	*S. salar*	*S. aurata*	*X. laevis*
C. gigas	-	0.157	0.545	0.659	0.175	0.939	0.33	0.205	0.117	0.457
D. rerio	0.157	-	0.418	0.331	0.955	0.136	0.659	0.882	0.88	0.502
G. aculeatus	0.545	0.418	-	0.87	0.452	0.496	0.712	0.509	0.337	0.89
H. sapiens	0.659	0.331	0.87	-	0.36	0.605	0.595	0.41	0.261	0.763
O. mykiss	0.175	0.955	0.452	0.36	-	0.152	0.701	0.927	0.835	0.539
P. promelas	0.939	0.136	0.496	0.605	0.152	-	0.294	0.179	0.101	0.413
R. rutilus	0.33	0.659	0.712	0.595	0.701	0.294	-	0.77	0.554	0.818
S. salar	0.205	0.882	0.509	0.41	0.927	0.179	0.77	-	0.765	0.601
S. aurata	0.117	0.88	0.337	0.261	0.835	0.101	0.554	0.765	-	0.411
X. laevis	0.457	0.502	0.89	0.763	0.539	0.413	0.818	0.601	0.411	-

Table G.5 P values showing the statistical difference between species for average binding energy of BPA.

Species	*C. gigas*	*D. rerio*	*G. aculeatus*	*H. sapiens*	*O. mykiss*	*P. promelas*	*R. rutilus*	*S. salar*	*S. aurata*	*X. laevis*
C. gigas	-	0.324	0.639	0.284	0.427	0.779	0.836	0.4	0.326	0.65
D. rerio	0.324	-	0.145	0.931	0.847	0.205	0.233	0.884	0.996	0.15
G. aculeatus	0.639	0.145	-	0.123	0.206	0.851	0.793	0.19	0.147	0.988
H. sapiens	0.284	0.931	0.123	-	0.781	0.176	0.201	0.817	0.928	0.127
O. mykiss	0.427	0.847	0.206	0.781	-	0.282	0.317	0.963	0.851	0.212
P. promelas	0.779	0.205	0.851	0.176	0.282	-	0.941	0.262	0.207	0.863
R. rutilus	0.836	0.233	0.793	0.201	0.317	0.941	-	0.295	0.235	0.805
S. salar	0.4	0.884	0.19	0.817	0.963	0.262	0.295	-	0.888	0.195
S. aurata	0.326	0.996	0.147	0.928	0.851	0.207	0.235	0.888	-	0.151
X. laevis	0.65	0.15	0.988	0.127	0.212	0.863	0.805	0.195	0.151	-

Table G.6 P values showing the statistical difference between species for average binding energy of DDT.

Species	*C. gigas*	*D. rerio*	*G. aculeatus*	*H. sapiens*	*O. mykiss*	*P. promelas*	*R. rutilus*	*S. salar*	*S. aurata*	*X. laevis*
C. gigas	-	0	0	0	0	0	0	0	0	0
D. rerio	0	-	0.025	0.003	0.252	0.045	0	0.978	0.982	0.007
G. aculeatus	0	0.025	-	0.47	0.001	0.818	0.099	0.027	0.027	0.632
H. sapiens	0	0.003	0.47	-	0	0.34	0.355	0.003	0.003	0.807
O. mykiss	0	0.252	0.001	0	-	0.002	0	0.241	0.243	0
P. promelas	0	0.045	0.818	0.34	0.002	-	0.06	0.048	0.047	0.478
R. rutilus	0	0	0.099	0.355	0	0.06	-	0	0	0.242
S. salar	0	0.978	0.027	0.003	0.241	0.048	0	-	0.996	0.007
S. aurata	0	0.982	0.027	0.003	0.243	0.047	0	0.996	-	0.007
X. laevis	0	0.007	0.632	0.807	0	0.478	0.242	0.007	0.007	-

Table G.7 P values showing the statistical difference between species for average binding energy of deltamethrin.

Species	*C. gigas*	*D. rerio*	*G. aculeatus*	*H. sapiens*	*O. mykiss*	*P. promelas*	*R. rutilus*	*S. salar*	*S. aurata*	*X. laevis*
C. gigas	-	0	0	0	0	0	0	0	0	0
D. rerio	0	-	0.876	0.281	0.754	0.059	0.84	0.898	0.795	0.31
G. aculeatus	0	0.876	-	0.217	0.875	0.083	0.72	0.776	0.677	0.241
H. sapiens	0	0.281	0.217	-	0.164	0.003	0.381	0.343	0.414	0.951
O. mykiss	0	0.754	0.875	0.164	-	0.115	0.606	0.658	0.566	0.184
P. promelas	0	0.059	0.083	0.003	0.115	-	0.036	0.044	0.032	0.004
R. rutilus	0	0.84	0.72	0.381	0.606	0.036	-	0.941	0.954	0.416
S. salar	0	0.898	0.776	0.343	0.658	0.044	0.941	-	0.895	0.375
S. aurata	0	0.795	0.677	0.414	0.566	0.032	0.954	0.895	-	0.45
X. laevis	0	0.31	0.241	0.951	0.184	0.004	0.416	0.375	0.45	-

Table G.8 P values showing the statistical difference between species for average binding energy of diazinon.

Species	*C. gigas*	*D. rerio*	*G. aculeatus*	*H. sapiens*	*O. mykiss*	*P. promelas*	*R. rutilus*	*S. salar*	*S. aurata*	*X. laevis*
C. gigas	-	0.003	0.063	0.136	0.005	0.709	0.182	0.006	0.002	0.187
D. rerio	0.003	-	0.281	0.147	0.888	0.01	0.109	0.852	0.902	0.105
G. aculeatus	0.063	0.281	-	0.712	0.348	0.137	0.599	0.372	0.229	0.587
H. sapiens	0.136	0.147	0.712	-	0.191	0.264	0.876	0.207	0.116	0.863
O. mykiss	0.005	0.888	0.348	0.191	-	0.015	0.143	0.963	0.792	0.139
P. promelas	0.709	0.01	0.137	0.264	0.015	-	0.336	0.017	0.007	0.345
R. rutilus	0.182	0.109	0.599	0.876	0.143	0.336	-	0.156	0.084	0.987
S. salar	0.006	0.852	0.372	0.207	0.963	0.017	0.156	-	0.757	0.151
S. aurata	0.002	0.902	0.229	0.116	0.792	0.007	0.084	0.757	-	0.081
X. laevis	0.187	0.105	0.587	0.863	0.139	0.345	0.987	0.151	0.081	-

Table G.9 P values showing the statistical difference between species for average binding energy of diclofenac.

Species	*C. gigas*	*D. rerio*	*G. aculeatus*	*H. sapiens*	*O. mykiss*	*P. promelas*	*R. rutilus*	*S. salar*	*S. aurata*	*X. laevis*
C. gigas	-	0.02	0.002	0.007	0.016	0	0	0.028	0.03	0
D. rerio	0.02	-	0.436	0.706	0.936	0.016	0.013	0.9	0.878	0.022
G. aculeatus	0.002	0.436	-	0.688	0.485	0.101	0.086	0.366	0.351	0.13
H. sapiens	0.007	0.706	0.688	-	0.767	0.041	0.034	0.616	0.596	0.055
O. mykiss	0.016	0.936	0.485	0.767	-	0.019	0.016	0.837	0.815	0.027
P. promelas	0	0.016	0.101	0.041	0.019	-	0.94	0.011	0.01	0.9
R. rutilus	0	0.013	0.086	0.034	0.016	0.94	-	0.009	0.008	0.84
S. salar	0.028	0.9	0.366	0.616	0.837	0.011	0.009	-	0.977	0.016
S. aurata	0.03	0.878	0.351	0.596	0.815	0.01	0.008	0.977	-	0.014
X. laevis	0	0.022	0.13	0.055	0.027	0.9	0.84	0.016	0.014	-

Table G.10 P values showing the statistical difference between species for average binding energy of diflubenzuron.

Species	*C. gigas*	*D. rerio*	*G. aculeatus*	*H. sapiens*	*O. mykiss*	*P. promelas*	*R. rutilus*	*S. salar*	*S. aurata*	*X. laevis*
C. gigas	-	0	0	0	0	0	0	0	0	0
D. rerio	0	-	0.705	0.007	0.764	0.041	0	0.754	0.937	0
G. aculeatus	0	0.705	-	0.02	0.938	0.015	0	0.948	0.765	0
H. sapiens	0	0.007	0.02	-	0.016	0	0	0.017	0.009	0
O. mykiss	0	0.764	0.938	0.016	-	0.019	0	0.989	0.825	0
P. promelas	0	0.041	0.015	0	0.019	-	0	0.018	0.034	0.074
R. rutilus	0	0	0	0	0	0	-	0	0	0.001
S. salar	0	0.754	0.948	0.017	0.989	0.018	0	-	0.815	0
S. aurata	0	0.937	0.765	0.009	0.825	0.034	0	0.815	-	0
X. laevis	0	0	0	0	0	0.074	0.001	0	0	-

Table G.11 P values showing the statistical difference between species for average binding energy of E2.

Species	*C. gigas*	*D. rerio*	*G. aculeatus*	*H. sapiens*	*O. mykiss*	*P. promelas*	*R. rutilus*	*S. salar*	*S. aurata*	*X. laevis*
C. gigas	-	0	0	0	0	0	0	0	0	0
D. rerio	0	-	0.656	0.106	0.628	0.003	0.861	0.628	0.936	0.082
G. aculeatus	0	0.656	-	0.039	0.352	0.001	0.535	0.352	0.599	0.029
H. sapiens	0	0.106	0.039	-	0.258	0.182	0.15	0.258	0.125	0.904
O. mykiss	0	0.628	0.352	0.258	-	0.014	0.757	1	0.686	0.21
P. promelas	0	0.003	0.001	0.182	0.014	-	0.006	0.014	0.004	0.225
R. rutilus	0	0.861	0.535	0.15	0.757	0.006	-	0.757	0.925	0.118
S. salar	0	0.628	0.352	0.258	1	0.014	0.757	-	0.686	0.21
S. aurata	0	0.936	0.599	0.125	0.686	0.004	0.925	0.686	-	0.098
X. laevis	0	0.082	0.029	0.904	0.21	0.225	0.118	0.21	0.098	-

Table G.12 P values showing the statistical difference between species for average binding energy of EE2.

Species	*C. gigas*	*D. rerio*	*G. aculeatus*	*H. sapiens*	*O. mykiss*	*P. promelas*	*R. rutilus*	*S. salar*	*S. aurata*	*X. laevis*
C. gigas	-	0	0	0	0	0	0	0	0	0
D. rerio	0	-	0.377	0.187	0.738	0.042	0.564	0.71	0.926	0
G. aculeatus	0	0.377	-	0.662	0.584	0.252	0.76	0.61	0.429	0.001
H. sapiens	0	0.187	0.662	-	0.324	0.479	0.457	0.343	0.219	0.003
O. mykiss	0	0.738	0.584	0.324	-	0.09	0.808	0.97	0.808	0
P. promelas	0	0.042	0.252	0.479	0.09	-	0.147	0.098	0.053	0.023
R. rutilus	0	0.564	0.76	0.457	0.808	0.147	-	0.837	0.628	0
S. salar	0	0.71	0.61	0.343	0.97	0.098	0.837	-	0.78	0
S. aurata	0	0.926	0.429	0.219	0.808	0.053	0.628	0.78	-	0
X. laevis	0	0	0.001	0.003	0	0.023	0	0	0	-

Table G.13 P values showing the statistical difference between species for average binding energy of fenbendazole.

Species	*C. gigas*	*D. rerio*	*G. aculeatus*	*H. sapiens*	*O. mykiss*	*P. promelas*	*R. rutilus*	*S. salar*	*S. aurata*	*X. laevis*
C. gigas	-	0	0	0	0	0	0	0	0	0
D. rerio	0	-	0.126	0.874	0.46	0.238	0.292	0.438	0.926	0.299
G. aculeatus	0	0.126	-	0.091	0.023	0.727	0.635	0.021	0.151	0.01
H. sapiens	0	0.874	0.091	-	0.562	0.181	0.225	0.537	0.801	0.379
O. mykiss	0	0.46	0.023	0.562	-	0.055	0.073	0.97	0.406	0.764
P. promelas	0	0.238	0.727	0.181	0.055	-	0.9	0.051	0.277	0.027
R. rutilus	0	0.292	0.635	0.225	0.073	0.9	-	0.067	0.336	0.036
S. salar	0	0.438	0.021	0.537	0.97	0.051	0.067	-	0.385	0.793
S. aurata	0	0.926	0.151	0.801	0.406	0.277	0.336	0.385	-	0.258
X. laevis	0	0.299	0.01	0.379	0.764	0.027	0.036	0.793	0.258	-

Table G.14 P values showing the statistical difference between species for average binding energy of nalidixic acid.

Species	*C. gigas*	*D. rerio*	*G. aculeatus*	*H. sapiens*	*O. mykiss*	*P. promelas*	*R. rutilus*	*S. salar*	*S. aurata*	*X. laevis*
C. gigas	-	0.013	0.001	0.001	0.025	0	0.005	0.021	0.02	0.001
D. rerio	0.013	-	0.486	0.476	0.807	0.145	0.73	0.858	0.867	0.36
G. aculeatus	0.001	0.486	-	0.988	0.346	0.448	0.725	0.381	0.387	0.827
H. sapiens	0.001	0.476	0.988	-	0.338	0.457	0.714	0.373	0.379	0.839
O. mykiss	0.025	0.807	0.346	0.338	-	0.089	0.555	0.948	0.939	0.246
P. promelas	0	0.145	0.448	0.457	0.089	-	0.267	0.102	0.104	0.589
R. rutilus	0.005	0.73	0.725	0.714	0.555	0.267	-	0.6	0.608	0.568
S. salar	0.021	0.858	0.381	0.373	0.948	0.102	0.6	-	0.991	0.273
S. aurata	0.02	0.867	0.387	0.379	0.939	0.104	0.608	0.991	-	0.278
X. laevis	0.001	0.36	0.827	0.839	0.246	0.589	0.568	0.273	0.278	-

Table G.15 P values showing the statistical difference between species for average binding energy of sulfadiazine.

Species	*C. gigas*	*D. rerio*	*G. aculeatus*	*H. sapiens*	*O. mykiss*	*P. promelas*	*R. rutilus*	*S. salar*	*S. aurata*	*X. laevis*
C. gigas	-	0	0	0.003	0	0.029	0.024	0.001	0	0.029
D. rerio	0	-	0.991	0.359	0.683	0.08	0.096	0.627	0.758	0.082
G. aculeatus	0	0.991	-	0.365	0.692	0.082	0.098	0.635	0.767	0.084
H. sapiens	0.003	0.359	0.365	-	0.611	0.404	0.454	0.666	0.543	0.411
O. mykiss	0	0.683	0.692	0.611	-	0.179	0.209	0.938	0.92	0.183
P. promelas	0.029	0.08	0.082	0.404	0.179	-	0.932	0.206	0.149	0.991
R. rutilus	0.024	0.096	0.098	0.454	0.209	0.932	-	0.238	0.175	0.941
S. salar	0.001	0.627	0.635	0.666	0.938	0.206	0.238	-	0.859	0.21
S. aurata	0	0.758	0.767	0.543	0.92	0.149	0.175	0.859	-	0.152
X. laevis	0.029	0.082	0.084	0.411	0.183	0.991	0.941	0.21	0.152	-

Table G.16 P values showing the statistical difference between species for average binding energy of sulfapyridine.

Species	*C. gigas*	*D. rerio*	*G. aculeatus*	*H. sapiens*	*O. mykiss*	*P. promelas*	*R. rutilus*	*S. salar*	*S. aurata*	*X. laevis*
C. gigas	-	0.001	0	0.028	0.003	0.065	0.032	0.006	0.001	0.112
D. rerio	0.001	-	0.508	0.317	0.802	0.177	0.293	0.67	0.984	0.108
G. aculeatus	0	0.508	-	0.097	0.362	0.044	0.087	0.277	0.521	0.023
H. sapiens	0.028	0.317	0.097	-	0.454	0.727	0.958	0.566	0.308	0.544
O. mykiss	0.003	0.802	0.362	0.454	-	0.272	0.423	0.861	0.786	0.175
P. promelas	0.065	0.177	0.044	0.727	0.272	-	0.767	0.356	0.171	0.796
R. rutilus	0.032	0.293	0.087	0.958	0.423	0.767	-	0.531	0.283	0.579
S. salar	0.006	0.67	0.277	0.566	0.861	0.356	0.531	-	0.656	0.237
S. aurata	0.001	0.984	0.521	0.308	0.786	0.171	0.283	0.656	-	0.104
X. laevis	0.112	0.108	0.023	0.544	0.175	0.796	0.579	0.237	0.104	-

Table G.17 P values showing the statistical difference between species for average binding energy of tamoxifen.

Species	*C. gigas*	*D. rerio*	*G. aculeatus*	*H. sapiens*	*O. mykiss*	*P. promelas*	*R. rutilus*	*S. salar*	*S. aurata*	*X. laevis*
C. gigas	-	0	0	0	0	0	0	0	0	0
D. rerio	0	-	0	0	0.697	0.789	0.002	0.266	0.801	0
G. aculeatus	0	0	-	0	0	0	0	0	0	0.014
H. sapiens	0	0	0	-	0	0	0	0	0	0
O. mykiss	0	0.697	0	0	-	0.511	0.008	0.469	0.891	0
P. promelas	0	0.789	0	0	0.511	-	0.001	0.167	0.603	0
R. rutilus	0	0.002	0	0	0.008	0.001	-	0.052	0.005	0.089
S. salar	0	0.266	0	0	0.469	0.167	0.052	-	0.39	0
S. aurata	0	0.801	0	0	0.891	0.603	0.005	0.39	-	0
X. laevis	0	0	0.014	0	0	0	0.089	0	0	-

Table G.18 P values showing the statistical difference between species for average binding energy of teflubenzuron.

Species	*C. gigas*	*D. rerio*	*G. aculeatus*	*H. sapiens*	*O. mykiss*	*P. promelas*	*R. rutilus*	*S. salar*	*S. aurata*	*X. laevis*
C. gigas	-	0	0	0	0	0	0	0	0	0
D. rerio	0	-	0	0	0.631	0	0.012	0.564	0.848	0.752
G. aculeatus	0	0	-	0	0	0	0	0	0	
H. sapiens	0	0	0.669	-	0	0	0	0	0	0
O. mykiss	0	0.631	0	0	-	0	0.041	0.922	0.502	0.426
P. promelas	0	0	0	0	0	-	0	0	0	0
R. rutilus	0	0.012	0	0	0.041	0	-	0.051	0.007	0.004
S. salar	0	0.564	0	0	0.922	0	0.051	-	0.442	0.371
S. aurata	0	0.848	0	0	0.502	0	0.007	0.442	-	0.901
X. laevis	0	0.752	0	0	0.426	0	0.004	0.371	0.901	-

Appendix H

Sequence results for pYES2-*LacZ*-ERE

gaCtaCTagcagcTgtaTACGACTCACTATAGGGAATATTAAGCTTGGCACTGGCCGTCGTTTTACAACGTCGT
GACTGGGAAAACCCTGGCGtTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAAT
AGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGCGCTTTGCCT
GGTTTCCGGCACCAGAAGCGGTGCCGGAAAGCTGGCTGGAGTGCGATCTTCCTGAGGCCGATACTGTCGT
CGTCCCCTCAAACTGGCAGATGCACGGTTACGATGCGCCCATCTACACCAACGTGACCTATCCCATTACGGT
CAATCCGCCGTTTGTTCCCACGGAGAATCCGACGGGTTGTTACTCGCTCACATTTAATGTTGATGAAAGCTG
GCTACAGGAAGGCCAGACGCGAATTATTTTTGATGGCGTTAACTCGGCGTTTCATCTGTGGTGCAACGGGC
GCTGGGTCGGTTACGGCCAGGACAGTCGTTTGCCGTCTGAATTTGACCTGAGCGCATTTTTACGCGCCGGA
GAAAACCGCCTCGCGGTGATGGTGCTGCGCTGGAGTGACGGCAGTTATCTGGAAGATCAGGATATGTGGC
GGATGAGCGGCATTTTCCGTGACGTCTCGTTGCTGCATAAACCGACTACACAAATCAGCGATTTCCATGTTG
CCACTCGCTTTAATGATGATTTCAGCCGCGCTGTACTGGAGGCTGAAGTTCAGATGTGCGGCGAGTTGCGT
GACTACCTACGGGTAACAGTTTCTTTATGGCAGGGTGAAACGCAGGTCGCCAGCGGCACCGCGCCTTTCG
GCGGTGAAATTATCGATGAGCGTGGTGGTTATGCCGATCGCGTCACACTACGTCTGAACGTCGAAnACCCG
AAACTGTGGAGCGCCGAAATCCCGAATCTCTATCgtgnnggtgGTTGAACTGCACACCGCCGACGGCACGCT
GATTGAAGCAgaaGCCTGcgaTGTCGGTTtccncgaGgtgcgnaTTGAAAatggnctGCTGCTGCTGAACGGCAan
cCGTTGCTGATTcnagg

Note: Uppercase letters are nucleotides (GATC) confirmed from sequencing; lower case letters are predicted but unconfirmed nucleotides (gatc), the letter n represents a nucleotide that was not viable and could not be sequenced.

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