Molecular genetic studies of pollutant response in the European flounder, *Platichthys flesus* (L.)

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Declaration

I declare that this thesis has been compiled by myself and is the result of my own investigations. It has not been submitted for any other degree and all sources of information have been duly acknowledged.

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Abstract

Effects of man made pollutants on an ecosystem are initiated at the cellular level where a prime determinant for survival of an organism is its ability to metabolise and excrete toxic chemicals or their metabolites, thereby preventing cellular toxicity or damage to germ cell DNA. Cytochrome P450 (CYP) enzymes are responsible (in concert with the remainder of the *Ah* battery enzymes) for the metabolism of numerous xenobiotics and endogenous compounds, including the metabolic activation of most environmental toxic chemicals and carcinogens. Genetic polymorphisms which affect performance of these enzymatic detoxification systems may alter tolerance to pollutants and thus survival in polluted environments. Alterations in the susceptibility of individuals and the development of resistant populations has arisen by forced selection of populations with variant genes, resulting in increased detoxification capacity. There is evidence for such scenarios of variations in activities of pollutant biotransforming enzymes of fish contributing to survival in polluted estuarine environments and several chemically resistant populations have been identified in the USA and Europe. In fish it has been demonstrated that CYP1A enzyme activity is required to activate some carcinogenic xenobiotics to a metabolic state in which they can form DNA adducts. The mechanism of reduced CYP1A expression in highly contaminated populations may therefore represent resistance to chemical stressors. European flounder (*Platichthys flesus*) from some waterways which have a long history of severe sedimentary contamination do not show elevated levels of CYP1A. The aim of the current study was to investigate whether any heritable differences were apparent between offspring from parents inhabiting long-term polluted and pristine areas. Flounder were obtained from a highly polluted estuary in the UK and crossed with fish from a

relatively pristine environment. Offspring were raised in communal tanks in order to standardise environmental conditions, and allow investigations into the genetic variation of CYP1A. To allow identification of offspring to parental fish, polymorphic microsatellite loci were isolated and characterised for the flounder. Novel cDNA probes to transcription factors in the detoxification pathway (AhR2 and ARNT2) were cloned for flounder, and RT-PCR / Southern blot methods were developed for quantitation of gene transcript levels. A novel method of CYP1A quantification using real-time PCR was developed.

PAH and PCB exposure trials were carried out on mixed batch offspring, and CYP1A gene transcript levels assessed using Northern blot and real-time PCR techniques. Offspring were genotyped to their parents using the microsatellites obtained, and CYP1A transcript levels were correlated with clean and polluted areas. CYP1A was further correlated to transcription factor expression, and data are presented. Following exposure to the commercial PCB mixture, Aroclor 1254, CYP1A transcript levels were found to be significantly lower in families whose parents originated from a polluted area. This observation indicates that there is a possible genetic component to variation in CYP1A levels, and that these fish may have acquired a heritable tolerance to polluted areas. The lack of induction, or correlation with CYP1A levels, of AhR2 and ARNT2 expression indicates a possible AhR independent pathway for the metabolism of PCBs in the flounder.

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Abbreviations

g	Gram
l	Litre
M	Molar
m	milli-
µ	micro-
n	nano-
p	pico-
3MC	3-methylcholanthrene
Ah	Aromatic hydrocarbon
AhR	Aryl hydrocarbon receptor
AHRE	*Ah* responsive element
APS	Ammonium persulphate
ARNT	Ah receptor nuclear translocator
BaP	Benzo(a)pyrene
BLAST	Basic local alignment sequence tool
bp	Nucleotide base pair(s)
cDNA	Complementary deoxyribonucleic acid
C_T	Threshold cycle
CYP	Cytochrome P450
dH_2O	Sterile distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxyribo nucleotide tri-phosphate
dsDNA	Double stranded deoxyribonucleic acid

EBI	European Bioinformatics Institute
EMBL	European Molecular Biology Laboratory
EROD	Ethoxyresorufin-O-deethylase
EtBr	Ethidium Bromide
GST(A)	Glutathione S-transferase (A)
HAH	Halogenated aromatic hydrocarbon
HPAH	Halogenated polyaromatic hydrocarbon
Hsp90	Heat shock protein 90
IoM	Isle of Man (UK)
LB	Luria-Bertani (media or broth)
MPC	Magnetic particle concentrator
NTC	No template control
pBSII	Plasmid Blue Script II
PHAH	Planar halogenated aromatic hydrocarbon
PAH	Polyaromatic hydrocarbon
PCB	Polychlorinated biphenyl
PCR	Polymerase chain reaction
PEML	Port Erin Marine Laboratory
PHAH	Polyhalogenated aromatic hydrocarbon
PNK	Polynucleotide kinase
QPCR	Quantitative polymerase chain reaction
QTL	Quantitative trait loci
RE	Restriction endonuclease
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction

SNP	Single-nucleotide polymorphism
SRS	Sequence retrieval service
ssDNA	Single stranded deoxyribonucleic acid
T_a	Annealing temperature
TAE	Tris Acetic acid EDTA
TBE	Tris Boric acid EDTA
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin (dioxin)
TEMED	N,N,N',N',-tetramethylethylenediamine
T_m	Melt temperature
UGT(1B1)	UDP glucoronosyl-transferase (1B1)
VNTR	Variable number of tandem repeats
XRE	Xenobiotic responsive element

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"Try not. Do, or do not. There is no try."

To Bryony, Susan and Sophie, with love.

Section 1 - Introduction

Chapter 1 General Introduction

1.1 Introduction

Throughout their evolution, animals have been exposed to plant and microbial secondary metabolites, which they cannot completely metabolise and utilise for their own life processes (George, 1994). To meet this challenge organisms have developed inducible enzymatic defences with broad substrate specificities to facilitate the biotransformation, elimination and hence detoxification of toxic compounds encountered in the environment (Nebert, Petersen and Fornace, 1990; Korzekawa and Jones, 1993; Hahn, 2002). Many thousands of xenobiotic compounds have been produced in the 20th century as a result of the exploitation of petroleum and increasing usage of pesticides. The ultimate sink for many of these industrially derived xenobiotics is the aquatic environment, thus increasing the exposure to aquatic organisms (Leaver, 1993; George, 1994). The lipophilic and persistent nature of many of these xenobiotics result in their bioaccumulation and magnification during passage up the food chain ultimately to humans, and methods of monitoring these substances are therefore of utmost importance (Peakall, 1992). The difficulty of predicting a toxicological hazard for the aquatic community in an area, using water and sedimentary chemistry, has been well documented in the literature, although chemical measurements are not *per se* indicators of toxicity (Vigano, Arillo, Falugi, Melodia and Polesello, 2001). Enzymatic defences are discussed below, and presented alongside their application to the field of biomonitoring.

1.2 Defence genes

In bacteria many genes are organised in tandem, and expression is regulated from a single site in the genome in response to a single specific endogenous or exogenous signal. This permits a co-ordinated response to changes in gaseous or chemical (including nutritional) composition of their environment (Nebert, Roe, Dieter, Solis, Yang and Dalton, 2000); these organisational units are called regulons. In higher animals (e.g. vertebrates) the organisation of genes is more complex, and whilst they can be co-ordinately regulated by a single stimulus, this is generally achieved by the presence of a recognition motif for the regulatory protein (which is often duplicated) in each individual responsive gene. The term "gene battery" has been coined for this functional unit and it is defined as "a group of (generally non-linked) genes that exhibit cross-talk, having an intricate inter-relationship with regard to up- and down-regulation, in response to a particular endogenous or exogenous signal; the battery's response is mediated by certain regulatory proteins whose effects may be combined in nature" (McKnight and Tjian, 1986; Nebert *et al*., 2000). One such gene battery implicated in carcinogenesis, mutagenesis and oxidative stress is the aromatic hydrocarbon responsive (*Ah*) gene battery.

1.2.1 The Ah gene battery

The *Ah* battery is a collection of genes which are transcriptionally regulated by interaction of a cytoplasmic xenobiotic binding protein (the aryl hydrocarbon receptor (AhR)) with an Ah response element (AHRE, also known as a xenobiotic or dioxin responsive element, XRE / DRE) in the 5' region of the responsive gene, and is an enzymatic defence system which has evolved to counter environmental

adversity (Nebert, Petersen and Fornace, 1990). This gene battery has been most comprehensively studied in mice, and shown to comprise of 6 enzymes: 2 cytochrome P450s, CYP1A1 and CYP1A2, and four non-P450 enzymes, namely: NAD(P)H quinone oxidoreductase (*Nqo1*); a cytosolic aldehyde dehydrogenase 3 (*Aldh3a1*); a UDP glucuronosyl transferase (*Ugt1a6*); and a glutathione transferase (*Gsta1,*Ya) (Vasiliou, Buetler, Eaton and Nebert, 2000).

1.2.1.1 History of the *Ah* gene battery

The *Ah* locus was first identified as a difference in responsiveness to polyaromatic hydrocarbons (PAHs) among inbred strains of mice (Nebert, Goujon and Gielen, 1972; Thomas, Kouri and Hutton, 1972). A genetic polymorphism in strains of these mice showed that 3-methylcholanthrene (3MC) was capable of inducing aryl hydrocarbon hydroxylase (AHH) (now known as Cytochrome P4501A1, or CYP1A1) activity in a certain inbred strain (C57BL/6), but not in another (DBA/2) inbred strain. In genetic crosses and backcrosses between these strains the trait of *Ah* responsiveness (induction of AHH by 3MC) was noted to be inherited in a simple autosomal mode. The locus was designated the *Ah* locus, and the alleles Ah^b (from C57BL/6) for responsive and Ah^d (from DBA/2) for unresponsive mice (Poland and Bradfield, 1992). Further studies using a more potent agonist, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), found non-responsive mice required a 10 fold greater dose of TCDD than the PAH responsive strains (Poland and Glover, 1975). It was postulated that the 3-MC and TCDD bound to the same receptor, a product of the *Ah* locus, and in mice strains homozygous for the non-responsive allele the receptor has a diminished affinity for ligands resulting in a complete insensitivity to weak agonists such as 3-MC, and a lowered sensitivity to the more potent agonist

TCDD (Poland and Bradfield, 1992). The mouse model remains one of the best characterised examples of the *Ah* gene battery in eukaryotes. In the mouse, this battery is known to represent the AhR mediated control of at least six genes, and possibly several more, that are co-ordinately induced by dioxin and PAHs such as benzo(a)pyrene. These enzymes, with particular emphasis on the cytochrome P450s and associated transcriptional factors, are discussed below.

1.2.1.2 Xenobiotic metabolism via the *Ah* battery

Whilst hydrophilic compounds can be excreted directly, less hydrophilic and non-polar compounds require modification before they can be excreted into (or via) an aqueous environment. Enzymes which metabolise drugs, phytoalexins (plant stress metabolites), carcinogens, other plant metabolites and environmental pollutants (the *Ah* battery enzymes) have classically been divided into two broad categories: phase I and phase II biotransformation enzymes (Nebert, Petersen and Fornace, 1990; Sipes and Gandolfi, 1994). Figure 1.1 (below) is a representative diagram of Phase I and Phase II biotransformation of a theoretical xenobiotic.

Phase I metabolism

The first phase of metabolism, unmasking or adding reactive functional groups, involves oxidation, reduction or hydrolysis (George, 1994). Phase I metabolism is carried out by enzymes which are almost exclusively cytochrome P450s which function by the insertion of one atom of atmospheric oxygen into a relatively inert substrate (Nebert, 1991). One of the most important processes in xenobiotic biotransformation is oxidation of a compound to a reactive intermediary which is more water soluble, but can be more carcinogenic, mutagenic and more toxic than the parent compound (Nebert, Petersen and Fornace, 1990; George, 1994; Pirrit,

Healey, Assheton, Leaver and George, 1995). The main focus of this thesis is the phase I metabolic pathway.

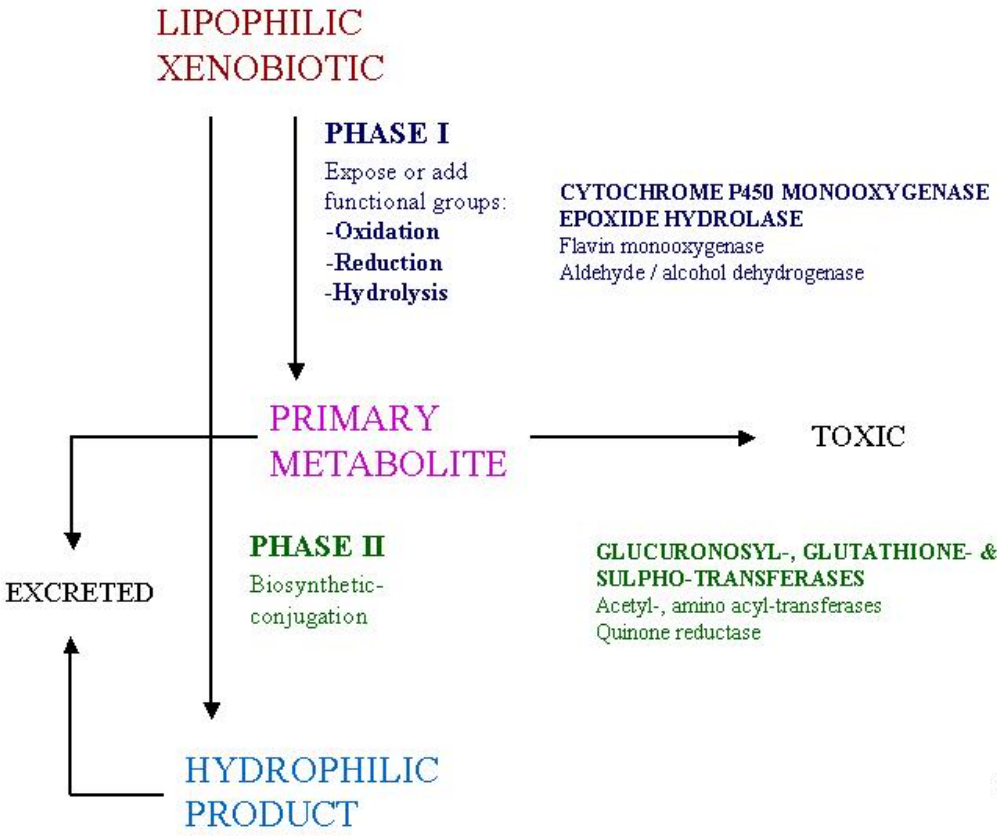

Figure 1.1: Phase I and Phase II pathways of xenobiotic metabolism (based on George, 1994), as described in text.

Phase II metabolism

Phase II enzymes act on oxygenated substrates, including products of phase I metabolism, usually by conjugating them with various endogenous moieties (Nebert, 1991). In phase II reactions, activated water soluble groups are conjugated directly with xenobiotic compounds or their metabolites by transferase enzymes, especially UDP-glucuronosyltransferases (UGTs), sulphotransferases (STs) and glutathione S-transferases (GSTs) to produce polar, hydrophilic derivatives which

can be readily excreted via the bile, urine or gills (Leaver and George, 1995; Pirrit *et al.*, 1995).

1.2.2 Cytochrome P450

The cytochrome P450 mono-oxygenase system (also termed the mixed function oxidase (MFO) system) is a coupled electron-transport system composed of two enzymes: a cytochrome and a flavoprotein, NADPH-cytochrome reductase (Peakall, 1992). The cytochrome P450s are an ancient group (or superfamily) of catalysts (enzymes) involved in the oxidative metabolism (biosynthesis and degradation) of endogenous steroids, fatty acids, prostaglandins, leukotrienes, pheromones, and biogenic amines. They also metabolise foreign chemicals including the activation and inactivation of countless drugs, chemical carcinogens and mutagens, plant metabolites and other environmental contaminants (Stegeman, 1989; Nebert, Petersen and Fornace, 1990; Nebert, 1991). Because of the significance of CYP1A1 in the activation of procarcinogens, there have been active efforts to link polymorphisms of the CYP1A1 gene with individual susceptibility to chemically induced cancers in humans (Nebert, 1991; Hukkanen, 2000) and tumors in fish (Roy, Courtenay, Yuan, Ikonomou and Wirgin 2001). The cytochrome P450 family originally evolved as a defence to detoxify naturally occurring toxic compounds e.g. phytoalexins encountered by eating plant material, but now plays an important role in the detoxification of man-made compounds such as drugs and chemicals (xenobiotics) (Nebert, Nelson and Feyereisen, 1989; Gonzalez and Nebert, 1990). Cytochrome P450s play key roles in the toxicology and pharmacology of xenobiotics (Stegeman, 1989) including poly-aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) (George, Christiansen, Killie and Wright, 1995;

Elskus, Monosson, McElroy, Stegeman and Woltering, 1999; Nebert *et al*., 2000) by biotransformation. In order to fully appreciate the complexity of the cytochrome P450s it is first necessary to have an understanding of the diversity of this superfamily.

Diversity of the cytochrome P450 superfamily

At present, there are more than 270 different CYP gene families documented, with 18 recorded in mammals (Nebert and Russell, 2002). Diversity in plants is enormous, and in the genome of the mustard plant *Arabidopsis thaliana*, 249 active CYP genes and 24 non-functional pseudogenes (comprising 1% of its total gene number) have been discovered. The genome of the rice plant is similar with at least 324 functional genes reported to date (Nebert and Russell, 2002). By contrast, the human cytochrome P450 superfamily comprises 57 CYP genes, and 33 pseudogenes arranged into 18 families and 42 subfamilies (Nebert and Russell, 2002; Nelson, DR, online). With such a huge superfamily to categorise, much confusion was apparent in the nomenclature of these enzymes, and this is discussed below.

Nomenclature of the cytochrome P450s

The complex nomenclature of the cytochrome P450 superfamily has changed several times before reaching its current (logical) format based on divergent evolution. The nomenclature system (recommended by Nebert, Adesnik, Coon, 1987) now stands as follows: The root symbol *CYP* (*Cyp* for mice) denoting cytochrome P450 followed by an Arabic numeral designating the P450 gene family (e.g. CYP*1*), a letter indicating the subfamily (e.g. CYP1*A*), and a second Arabic numeral representing the individual gene (e.g. CYP1A*1*). Cytochrome P450 proteins are thus arranged into families based on percentage amino acid sequence

identity: enzymes which share ≥40% identity are assigned to a particular family (e.g. CYP*1*), whereas those sharing ≥55% identity make up a particular sub-family (e.g. CYP1*A* and CYP1*B*); if further enzymes are discovered which share ≥55% homology, they are assigned as multiple members of a sub-family (e.g. CYP1A*1* and CYP1A*2*) (Nebert and Gonzalez, 1987; Nebert, Adesnik, Coon, 1987; Nebert, Nelson, Adesnik, Coon, Estabrook, Gonzalez, Guengerich, Gunsalus, Johnson, Kemper, Levin, Phillips, Sato and Waterman, 1989; Nebert, Petersen and Fornace, 1990; Nebert and Russell, 2002). This system has been in place for 16 years, and continues to be developed on the internet at http://drnelson.utmem.edu/cytochromeP450.html (Nelson, D.R., online; Nebert and Russell, 2002).

Biochemical function of cytochrome P450

Cytochrome P450s are b-type heme proteins containing iron protoporphyrin IX imbedded in a hydrophobic environment in the active site. The ferric prosthetic heme group of the P450s are characterised by having a cysteinyl thiolate as a fifth ligand, and a water molecule placed as the sixth ligand (Halkier, 1996). Cytochrome P450 participates in phase I reactions and catalyses a number of processes including O-dealkylation, N-dealkylation, S-dealkylation, hydroxylation and epoxidation. CYP1A is part of an electron transport chain where two electrons are transferred from NADPH via NADPH-cytochrome-reductase to cytochrome P450 (demonstrated below in Figure 1.2).

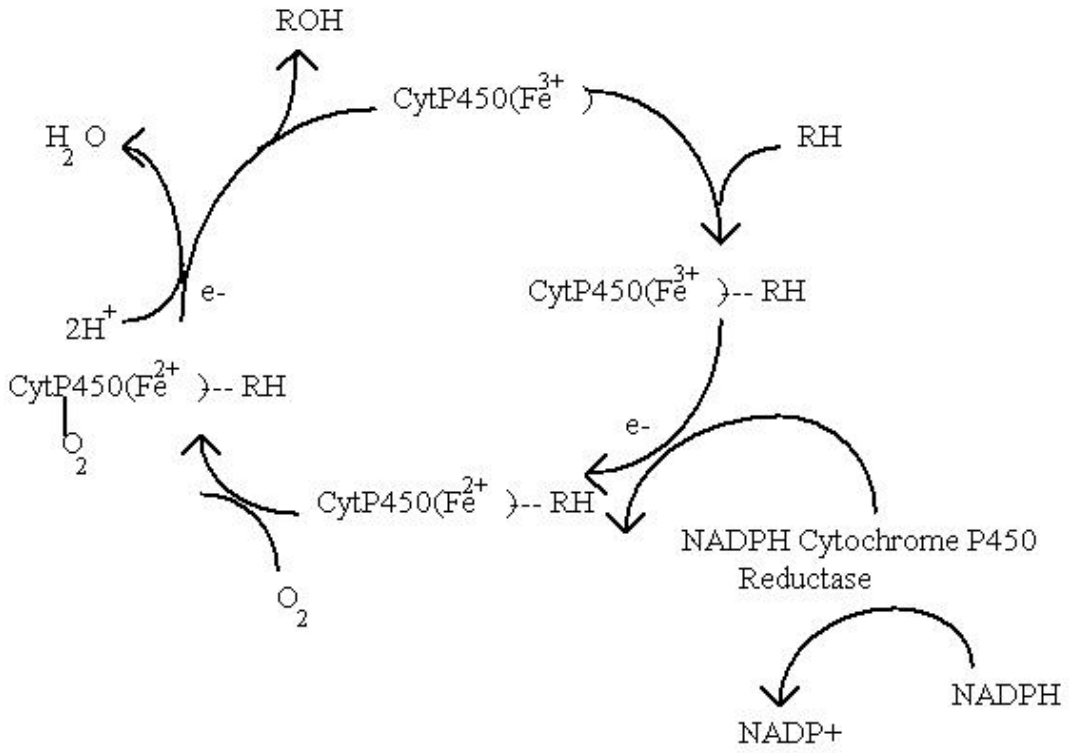

Figure 1.2: The mixed function oxidase electron transport chain: RH is substrate, CytP450 is cytochrome P450 (American Chemical Society, online)

A summary example of a Cytochrome P450-dependent Mixed Function Oxidation (MFO) (Figure 1.2) is presented below (R is a substrate which becomes hydroxylated and thus more water soluble):

$$NADPH + H^+ + O_2 + RH \rightarrow NADP^+ + H_20 + ROH$$

Cytochrome P450s in fish

In fish only one cytochrome P4501A isoenzyme has been discovered, and this has been termed CYP1A, however several 'genes' have been identified in the trout which have further confused the issue of nomenclature. The trout genes have been named CYP1A3 and CYP1A4 somewhat arbitrarily (Heilmann, Sheen, Bigelow and Nebert, 1988; Berndston and Chen, 1994), however their recent divergence

precludes the possibility that one of these genes is orthologous to mammalian CYP1A and the other separately orthologous to CYP1A2. The fish CYP1A gene is responsible for all functions carried out by mammalian CYP1A1 and CYP1A2, and is thought to be an ancestral form of the mammalian isoenzymes which appeared before the split between fish and mammals (Leaver, Pirrit and George, 1993; Morrison, Weil, Karchner, Sogin and Stegeman, 1998). A second CYP1 family (CYP1B) of genes has also been discovered in mammals and fish (Leaver and George, 2000), and in common with CYP1A genes, they are transcriptionally activated by PAH, and their protein products metabolise PAH (Savas, Carstens and Jefcoate, 1997; Leaver and George, 2000). The piscine CYP1A gene structure consists of six introns, and seven exons; intronic sequence is not conserved between fish. Exon one contains no initiation codon, so is untranslated. The ATG initiation codon is contained in exon 2, and the TGA termination codon is found in exon seven (e.g. Morrison *et al*., 1998; Williams, Lee, Sheader and Chipman, 2000). This structure follows that of all other CYP1As identified, in all taxa investigated the length of the 2nd and 7th exons far exceeds that of all other exons, and exons 3-6 are identical in length in almost all species (Roy, Kreamer, Konkle, Grunwald, and Wirgin, 1995). CYP1A is responsible for the metabolism of PAHs and PCBs in fish, (Pirrit *et al*., 1995) and induction of this isoenzyme is widely proposed as a biomarker of exposure to PAHs, PCBs, chlorinated dibenzo-*p*-dioxins and dibenzofurans (Payne, Fancey, Rahimtula and Porter, 1987; Haux and Forlin, 1988; Sulaiman, George and Burke, 1991; Forlin and Celander, 1993; Eggens, Vethaak, Leaver, Horbach, Boon and Seinen, 1996; Besselink, Denison, Hahn, Karchner, Vethaak, Koeman and Brouwer, 1998; Bogovski, Sergeyev, Muzyka and Karlova, 1999). Indeed CYP1A can be specifically induced up to 100-fold by compounds

with a planar molecular structure (Stegeman and Kloepper-Sams, 1987). *Biomonitoring* is 'the study of living organisms to elucidate the effect(s) of a particular chemical (or group of chemicals) upon the environment inhabited by the organism in question'. A *biomarker* is 'a xenobiotically-induced variation in cellular or biochemical processes, structures, or functions that is measurable in a biological system or sample' (NRC, 1987), and therefore *biomarkers* are used as indicators in *biomonitoring* studies. *Biotransformation* (of *xenobiotics*) is the sum of the processes by which a foreign chemical is subjected to chemical change by living organisms. *Biotransformation* pathways are therefore used as *biomarkers* in *biomonitoring,* and these methods may represent the most sensitve and specific early warning indicators for assessing the effects of contaminents on wild fish populations (Haux and Forlin, 1988). One of the most common markers used in biomonitoring is CYP1A expression measured as either gene transcript (mRNA) expression (Leaver, Pirrit and George, 1993), protein assay (Forlin and Celander, 1993), or enzyme activity assays (ethoxyresorufin-o-deethylase, or EROD) (Viarengo, Lafaurie, Gabrielides, Fabbri, Marro and Romeo, 2000). There is increasing documentary evidence that in the aquatic ecosystem alterations in the responses of CYP1A (and the *Ah* gene battery) to xenobiotic assault can lead to a form of tolerance developing. This assumed 'tolerance' is discussed further later in this introduction.

1.2.3 The CYP1A transcription pathway: AhR and ARNT

A transcription factor can be defined as any molecule participating, alone or as part of a complex, in biding to a gene's enhancer response element or promoter with the ultimate outcome being the up- or down-regulation of expression of that gene

(Nebert, 2002). The Aryl hydrocarbon Receptor (AhR) is a member of the bHLH-PAS (basic-helix-loop-helix, Per-ARNT-Sim) superfamily of transcriptional regulatory proteins whose members play key roles in development, circadian rhythmicity, and environmental homeostasis. However the normal cellular function of the AhR is not yet known (see also 1.2.3.1) (Hahn, Karchner, Shapiro and Perera, 1997; Hahn, 1998; Mimura and Fujii-Kuriyama, 2003). Results of several studies have led to the belief that the AhR is a phylogentically ancient protein that exists in bony and cartilaginous fish, as well as the most 'primitive' living vertebrate, the lamprey (Hahn *et al.,* 1998).

In concert with the ARyl hydrocarbon Nuclear Translocator (ARNT), AhR is known to be responsible for the differential regulation of detoxification enzymes, including CYP1A, in response to PAHs such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, or dioxin) (Hahn, Poland, Glover and Stegeman, 1992; Mimura and Fujii-Kuriyama, 2002) via recognition of a large range of exogenous chemicals (Hahn, Woodin, Stegeman and Tillitt, 1998; Hahn, 2002). In the absence of ligand, AhR is present in the cytosol in a complex with heat-shock protein 90 (Hsp90), immunophilin-like protein XAP2 and co-chaperone p23 proteins, which are thought to maintain the stability of the complex (Mimura and Fuji-Kuriyama, 2003). Upon binding to a ligand, the AhR complex translocates into the nucleus and the AhR dissociates from Hsp90 complex to form a heterodimer with its partner molecule, ARNT. Thus, the formed AhR/ARNT heterodimer recognizes an enhancer DNA element designated xenobiotic responsive element (XRE) sequence located in the promoter region of *CYP1A(1)* gene, resulting in the enhanced expression of the gene (see Figure 1.3, below) (Pollenz, Sullivan, Holmes, Necela and Petersen, 1996; Powell and Hahn, 2000; Mimura and Fuji-Kuriyama, 2003).

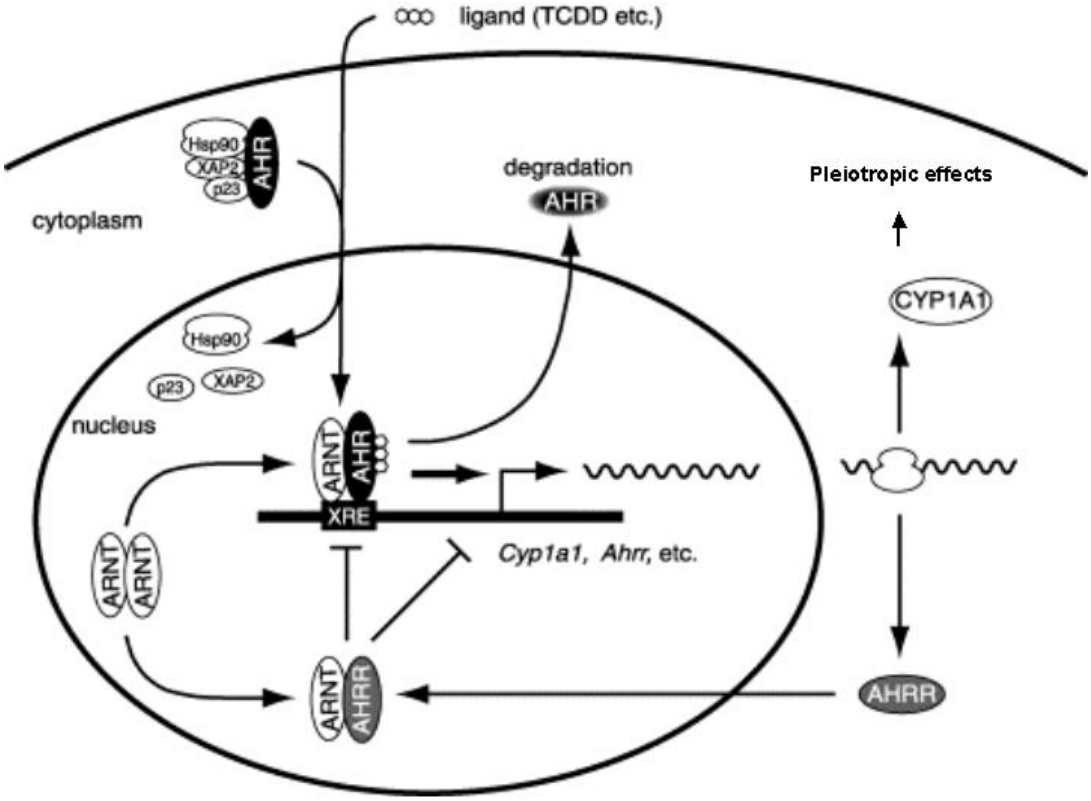

Figure 1.3: Mechanism of transcriptional activation of CYP1A by a xenobiotic (TCDD) following by the AhR and negative feedback regulation of AhR by AhRR (from Mimura and Fujii-Kuriyama, 2002). XREs are xenobiotic responsive element, also termed 'aromatic hydrocarbon responsive element' (AHRE). Hsp90 (heat shock protein 90). The AhR is responsible for pleiotropic effects (the control by a single gene of several distinct and seemingly unrelated phenotypic effects) due to its involvement in a multitude of pathways (see Chapter 6 for further discussion).

The AhR and ARNT form a transcription factor complex which then mediates induction of responsive genes, including CYP1A, by planar halogenated aromatic hydrocarbons e.g. TCDD (Landers and Bunce, 1991; Powell and Hahn, 2000). Evidence of the above mechanism which confirmed that CYP1A(1) induction was dependent upon both the AhR and ARNT was gained from studies in various strains of mice, namely "AhR receptorless", "ARNT deficient", and "CYP1A1 metabolism defective" (Nebert *et al*., 2000). Differences in the characteristics or expression of

the aryl hydrocarbon receptor could contribute to species differences in PHAH responsiveness (Karchner *et al.,* 2000). A third member of the AhR family, the Aryl Hydrocarbon Receptor Repressor (AhRR) has also been reported in mice (Mimura, 2001; Mimura and Fujii-Kuriyama, 2003) and *Fundulus heteroclitus* (Karchner, 2002). The AhRR is an evolutionary conserved, TCDD (dioxin) inducible repressor of AhR1 and AhR2 function (Karchner, 2002), which competes with AhR for dimerizing with ARNT and binding to the xenobiotic responsive element (XRE) sequence (Mimura, Ema, Sogawa and Fujii-Kuriyama, 1999). A description of the structure of the AhRR is also provided in section 1.2.3.1 below. Detailed reviews of the transcription factors are numerous e.g. AhR (Hahn, 1998; 2002; Mimura and Fujii-Kuriyama, 2003), ARNT (Powell and Hahn, 2000) and AhRR (Pollenz, 2002).

1.2.3.1 AhR, ARNT and AHRR structure

In the very NH_2-terminal region, these proteins contain a bHLH motif, which is shared by other transcription factors, and involved in DNA binding and hetero- or homodimerization. The sequence consisting of about 250 amino acids adjacent to the COOH-terminus of the bHLH region constitutes the PAS domain, which was initially identified as a conserved sequence among *Drosophila* PER, human ARNT and *Drosophila* SIM (Mimura and Fujii-Kuriyama, 2003). The PAS domain contains two imperfect repeats of 50 amino acids, PAS A and PAS B, and is considered to function as an interactive surface for hetero- or homo-dimer formation. The PAS domain has recently been known to be distributed in a wide variety of proteins involved in circadian rhythm (PER, CLOCK, BMAL1), hypoxia response (HIF-1α, HIF-2α/HLF, HIF-3α), neurogenesis (SIM), and coactivation of transcription (SRC-1, TIF2) in the animal kingdom and is also found in bacterial

proteins functioning as light and oxygen sensors (Taylor and Zhulin, 1999; Mimura and Fujii-Kuriyama, 2003).

1.2.3.2 Current knowledge of AhR and ARNT sequences in teleost fish

There are two forms of the Ah receptor documented in fish, AhR1 and AhR2, the most predominant being AhR2 (Karchner *et al*. 2000; Hahn, 2002). Two AhR genes have been found to date in fish, and the degree of sequence divergence between two AhR genes from within a fish species is at least as great as the difference between the fish and mammalian AhR genes. This observation suggests that the two genes may have arisen from an ancient gene duplication event that preceeded the divergence of fish and the tetrapod lineages (Hahn, Karchner, Shapiro and Perera, 1998). Although two genes encode different forms of ARNT in rodents, ARNT1 (widely expressed) and ARNT2 (limited tissue expression pattern) (Powell and Hahn, 2000), only the ARNT2 isoform has been discovered in teleosts. The Rainbow trout appears to be an exception to this and has an ancestral form of the ARNT gene that was reported peculiar to salmonids (Powell and Hahn, 2000) in which two forms of the ARNT protein are produced as a result of alternative splicing of the ARNT gene (Pollenz, Sullivan, Holmes, Necela and Petersen, 1996). At the time of writing, there are (at least partial) AhR sequences published in GenBank for five species of teleost fish: the zebrafish, *Danio rerio*; Japanese medaka, *Oryzias latipes*; the killifish, *Fundulus heteroclitus*; rainbow trout, *Oncorhynchus mykiss*; and the European flounder, *Platichthys flesus*. In these species three AhR1 and three AhR2 isoforms, as well as two separate forms in rainbow trout termed ‘a’ and ‘b’ (see Table 1.1) are reported. ARNT sequences have been reported for four species: *D. rerio*; *F. heteroclitus*; *O. mykiss*; and scup,

Stenotomus chrysops. The rainbow trout expresses a divergent form of the ARNT protein (termed 'a' and 'b'). (Table 1.2 summarises published ARNT data in teleosts).

1.2.4 Resistance to chemical stressors – reduced CYP1A expression

Metabolism of foreign chemicals frequently results in successful detoxification (of the irritant) by P450 enzymes, however these enzymes can also generate toxic metabolites that contribute to increased risks of cancer, birth defects and other toxic effects (Nebert and Russell, 2002). In fish it has been demonstrated that CYP1A enzyme activity is required to activate some carcinogenic xenobiotics to a metabolic state in which they can form DNA adducts (Smolarek, Morgan, Moynihan, Lee, Harvey and Baird, 1987), and the mechanism of reduced CYP1A expression in highly contaminated populations may represent resistance to chemical stressors (Elskus, Monosson, McElroy, Stegeman and Woltering, 1999). Aquatic organisms frequently inhabit areas where biochemical adaptions to harsh chemical conditions may be essential for their survival (Van Veld and Westbrook, 1995), and several well documented examples exist of marine populations resistant to the toxicity of pollutants at the site from which they originate. In a population of Atlantic Tomcod (*Microgadus tomcod*) from the highly polluted Hudson River, NY, individuals showed lower induction of CYP1A than fish from the cleaner Miramichi River (Wirgin, 1999; Roy, Courtenay, Yuan, Maxwell, Ikonomou and Wirgin, 2001). The killifish, *F. heteroclitus,* has also been studied extensively in the United States, and several populations of chemically resistant individuals have been identified at various locales, including: New Bedford Harbour, MA (Nacci, Kohan, Pelletier and George 2002), the Elizabeth River, VA (Mulvey, Newman, Vogelbein and Unger,

Table 1.1: A summary of currently published AhR nucleotide sequences in teleost fish following a search for 'Aryl Hydrocarbon Receptor' and '*Teleostei*' in the EMBL (SRS) database.

Species	Sequence description	Sequence length (bp)	Accession number
D. rerio	cDNA clone 5' similar to AhR mRNA	409	AW827062
D. rerio	cDNA clone 5' similar to AhR mRNA	557	BI671938
D. rerio	cDNA clone 5' similar to AhR mRNA	334	BI671938
D. rerio	AhR2 mRNA complete cds.	7126	AF063446
D. rerio	AhR1 mRNA complete cds.	2873	AF258854
Ictalurus punctatus	ARNT		BE469171
O. latipes	AhR1 mRNA for AhR1 alpha, complete cds.	3333	AB065092
O. latipes	AhR2 mRNA for AhR1 beta complete cds.	3564	AB065093
O. latipes	AhR3 mRNA for AhR1 alpha, complete cds.	3653	AB065094
F. heteroclitus	AhR1 mRNA for AhR1*1 allele, complete cds.	3371	AF024591
F. heteroclitus	AhR1 beta mRNA, alternatively spliced, partial cds.	872	AF190654
F. heteroclitus	AhR2 mRNA, complete cds.	3343	U29679
O. mykiss	AhR alpha mRNA, complete cds.	3348	AF065137
O. mykiss	AhR beta mRNA, complete cds.	3348	AF065138
P. flesus	AhR2 mRNA, partial cds.	636	AF034412

Table 1.2: Currently published ARNT nucleotide sequences in teleost fish following a search for 'Aryl hydrocarbon Receptor Nuclear Translocator' and '*Teleostei*' in the EMBL (SRS) database.

Species	Sequence description	Sequence length (bp)	Accession number
D. rerio	CDNA clone 5' similar to ARNT2 mRNA	563	BM082753
D. rerio	CDNA clone 5' similar to ARNT2 mRNA	118	AA497351
D. rerio	ARNT2a mRNA, complete cds.	1624	AF155066
D. rerio	ARNT2a mRNA, complete cds. Alternatively spliced	1580	AF219987
D. rerio	ARNT2b mRNA, complete cds. Alternatively spliced	2294	AF219988
D. rerio	ARNT2c mRNA, complete cds. Alternatively spliced	2420	AF219989
F. heteroclitus	ARNT2 mRNA, complete cds.	3045	AF079311
F. heteroclitus	ARNT2 mRNA, complete cds; alternatively spliced	3093	AF402781
O. mykiss	ARNT isoform A mRNA, complete cds.	3112	U73840
O. mykiss	ARNT isoform B mRNA, complete cds.	3488	U73841
*S. chrysops*t	ARNT similar to Rainbow trout isoform	1037	AF155146

2002; Meyer, Nacci and DiGiulio, 2002; Meyer and DiGiulio, 2002; Van Veld and Westbrook, 1995; Nacci, Kohan, Pelletier and George, 2002) and Newark Bay, NJ (Elskus *et al*., 1999). The authors reported that the decreased induction of CYP1A is caused by an adaptation to high levels of pollution, and that suppression of the CYP1A system conferred an advantage to survival of the species. Although individuals would develop a higher incidence of tumors, they would survive to reproductive age, and acclimation would be passed on to offspring (Van Veld and Westbrook, 1995). The reduced inducibility of hepatic CYP1A1 mRNA in adult tomcod is most consistent with a single generation acclimation (Roy *et al.*, 2001). The effects of decreased CYP1A expression in killifish are highly heritable for one generation, but heritable to a lesser degree over subsequent generations (Meyer and Di Giulio, 2002). The Newark Bay population of killifish has acquired an altered response which is both persistent and possibly heritable, and that the use of CYP1A as a biomarker in fish taken from areas of chronic or extreme exposure may provide misleading data (Elskus *et al.*, 1999). For this reason it is of critical importance to investigate this possibility among different species, and indeed individuals within a species, if they are to be employed in biomonitoring studies.

1.2.5 The European flounder

The European flounder (*Platichthys flesus*) is among the most common fish species in Northern European estuaries. Juvenile flatfishes reside in nearshore sediments, and are particularly vulnerable to effects of pollution due to direct contact with sedimentary pollutants (Moles and Norcross, 1998) from which it also finds most of its food (Hylland *et al*., 1996). Along with dab (*Limanda limanda*), flounder is the most widely used species for environmental monitoring in the North Sea (Leaver *et*

al., 1993). Based upon criteria such as abundance, limited movements of juvenile fish and benthic feeding habit, flounder have been adopted as the sentinel species for most estuaries in the northern temperate zone (Pirrit *et al*., 1995). Several biomonitoring studies have demonstrated elevated levels of CYP1A in the livers of several fish species which are directly correlated with pollution gradients of sedimentary PAHs (Van Veld, Westbrook, Woodin, Hale, Smith, Huggett and Stegeman, 1990) including flounder (e.g.Sulaiman *et al*., 1991; Beyer *et al*., 1997). Thus, increases in ethoxyresorufin-*o*-deethylase (EROD) activity or in CYP1A mRNA content are an indication that an organism has been in contact with certain pollutants (Eggens, Vethaak, Leaver, Horbach, Boon and Seinen, 1996).

In mesocosm experiments with sediments from Rotterdam harbour, CYP1A levels in flounder from this area were not induced, whilst plaice (*Pleuronectes platessa*) CYP1A levels were induced following twelve months exposure. This may have indicated a species specific effect rather than a chemically mediated suppression of induction (Eggens *et al*., 1996; George and Vethaak, unpublished data). A similar phenomenon was also demonstrated following exposure to the commercial PCB mix Clophen A50 (Besselink, van Beusekom, Roex, Vethaak and Koemann, 1996). The reasons for this low responsiveness of CYP1A, in flounder, are not fully understood. They may be a consequence of an inefficient AhR pathway for induction of CYP1A activity by PCBs, or may be a consequence of interference of PCB congeners with the catalytic activity of CYP1A in flounder (Besselink *et al*., 1998). Another possibility is that the flounder have been chronically exposed to pollutants, and have developed a similar heritable defence mechanism of reduced CYP1A expression as described in tomcod (Wirgin, 1999) and killifish (Meyer and Di Giulio, 2002), and it is this angle which is covered by this thesis. A possible

reason for decreased induction (of CYP1A) in these animals is that it is the pathway responsible for transcriptional activation, the AhR and the ARNT signalling pathway which varies between populations (Karchner, Kennedy, Trudeau and Hahn, 2000). It is well documented in toxicological studies that clinical differences in drug responses reflect each individual's underlying genetic predisposition to the way in which each drug is metabolised (Nebert, 2000). Indeed, large inter-individual variability in xenobiotic biotransformation is observed in many organisms. In a study characterising the AhR cDNAs in birds and an amphibian, (Karchner et al., 1996) suggested that differences in the characteristic or expression of the AhR could contribute to species differences in PHAH responsiveness. This possible cause of CYP1A variation was investigated in this study, and is presented in Chapters 4 and 5.

1.3 Tools developed for study

In order to investigate variation in CYP1A expression in flounder, it was first necessary to develop several tools for the study, including microsatellite markers for genetic fingerprinting, and a novel method of CYP1A mRNA measurement via real-time PCR. Background on these is provided below.

1.3.1 Microsatellite DNA markers

In order to study genetic variation in the *Ah* gene battery detoxification pathway, environmental conditions were standardised by rearing flounder in communal tanks. Thus, to identify the offspring of the breeding trials to their parents, genetic markers were required. As no genetic markers were available in this species at the time of

the study, they were first isolated and characterised. These markers are discussed below.

Nuclear DNA (nDNA) Markers

Any site in the genome where there is variation in the DNA sequence is of potential use as a system for genetic marking. If the difference is very rare it is termed a mutation, if more common it becomes a polymorphism (Wright and Bentzen, 1994). Nuclear DNA marker systems have been commonly used for tracing parentage, and in population studies for many years. Examples of these include restriction fragment length polymorphisms (RFLPs), randomly amplified polymorphic DNA (RAPDs) (Ward and Grewe, 1994), and the more recently developed satellite DNA markers (O'Reilly and Wright, 1995). The present introduction will deal with a particular class of satellite DNA markers: simple sequence repeats, commonly referred to as microsatellites.

Satellite DNA markers

Dispersed throughout the genome of most, if not all, eukaryotic organisms are tandemly repeated blocks of DNA of identical or similar sequence (Tautz, 1989; O'Reilly and Wright, 1995). The length of these blocks may range from two to thousands of base pairs (Tautz, 1993). DNA consisting of long repeat units (hundreds to thousands of base pairs) was termed satellite DNA because of the manner in which it was first identified. Due to the abundance of the repeats and their slightly different base pair composition compared to bulk genomic DNA, this class formed a separate 'satellite' band distinct from the main genomic band in equilibrium density centrifugation (O'Reilly and Wright, 1995; Estoup and Angers, 1998). Satellite sequences were named before they were known to consist of

tandemly repetitive DNA, but the term 'Satellite DNA' has now become a synonym for any tandemly repeated DNA sequence (Tautz, 1993). The last decade has seen the increasing use of satellite DNA as molecular markers for a wide range of applications in fisheries related research, including population studies (e.g. Fontaine and Dodson, 1999; Hansen *et al*., 1999), parental analysis (Herbinger *et al*., 1995; Shaw and Boyle, 1997b) and genome mapping (Lee and Kocher, 1996). Most interest has focussed on small repeat regions whose detection of size differences is feasible using the PCR technique.

These loci include two sub groups of satellite DNA, minisatellites and microsatellites (Wright, 1993; Park and Moran, 1994). Each group is named somewhat arbitrarily according to the length of the repeat unit. Minisatellites contain repeats of 6 to >50 base pairs (bp) in length (Jeffreys *et al.*, 1985), but the microsatellite repeat contains only 1-6 bp (Tautz, 1993). The total length of minisatellite loci can also be much longer than microsatellites, the former being from a few hundred to several thousand bp, and the latter around 10-200 bp (Estoup and Angers, 1998). The number of repeats within both mini- and microsatellites is often found to be highly variable among individuals, even within family groups (Tautz, 1993). Minisatellites are reported to be clustered in telomeric regions of the chromosome (O'Connell and Wright, 1997). Unlike minisatellites, microsatellite loci appear to be distributed throughout the genome of eukaryotes at very high frequencies (10^3 to 10^5 copies, dispersed at 7-10^{100} kbp intervals) (Wright, 1993), therefore providing an almost ideal tool for genetic mapping and kinship studies (Tautz, 1989; Wright and Bentzen, 1994). Tautz (1989) reported on the differences between mutational processes of simple sequence length polymorphisms (SSLPs or microsatellites) and minisatellites. He stated that the most probable mechanism for

the polymorphisms in microsatellites is slippage mutation, and not due to a recombination mechanism, as found at minisatellite loci. This leads to increased variability at microsatellite loci, allowing higher powers of resolution between individuals, and therefore the ideal choice for this study. The high level of polymorphism at microsatellite loci is demonstrated throughout the literature. Although minisatellites have successfully addressed a number of fishery related questions, this class of satellite DNA has not been widely adopted by fishery geneticists and most of the current research effort is concentrated on the smaller microsatellites (O'Connell and Wright, 1997).

Microsatellites

Microsatellites are generally regarded as being either di-, tri- or tetra-nucleotide repeats (O'Connell and Wright, 1997; Tautz, 1993; Herbinger *et al.*, 1995). They are often highly polymorphic for repeat number (Tautz, 1989), with many alleles at any given locus. Good examples of this include Brooker *et al.* (1994) in the Atlantic cod (*Gadus morhua*) with seven loci having more than 25 alleles each, and McConnell *et al.* (1995) reporting 52 alleles at one locus in Atlantic salmon (*Salmo salar*). Microsatellites are normally embedded in unique stretches of DNA (Tautz, 1989), and therefore if the regions flanking this sequence are known, PCR primers can be designed which allow amplification of the desired fragment by the polymerase chain reaction (PCR) (Wright and Bentzen, 1994). Microsatellite flanking regions are generally conserved among closely related species (O'Connell and Wright, 1997). Therefore, once microsatellites are isolated from one species, they may often be used to amplify sequences in other related species (Estoup and Angers, 1998). For example, many primers designed for the Atlantic cod (*G.*

morhua) have been found to amplify microsatellite loci in haddock (*Melanogrammus aeglefinus*) and pollock (*Pollachius virens*) (Brooker *et al*., 1994). Microsatellite repeat motifs are found to be common between species; the most abundant and widely used examples of these are the di-nucleotide sequences $(GT)_n$ and $(GA)_n$ (Brooker *et al*., 1994). However, Brooker *et al*. (1994) go on to state that there is a significant difference between microsatellites from teleost fish and mammals, the longest to be isolated from cod and rainbow trout being more than twice the length of any reported for the mammalian genome. These repeat motifs can be described as either 'perfect' (i.e. uninterrupted repetitive sequence), 'imperfect' (repetitive sequences interspersed with bases which break up the repeat motif), or 'compound' (neighbouring blocks of differing repeat sections) (Brooker *et al*., 1994). For a review of this including further definitions see Weber (1990).

Use of microsatellites

Polymorphism at microsatellite loci was first demonstrated by Tautz (1989), and Weber and May (1989), and in the two years following this microsatellites were being employed as genetic markers in medical, genome mapping and forensic studies of humans (O'Reilly and Wright, 1995). By the mid-late 1990s the use of microsatellite markers had increased dramatically. In 2001, *Molecular Ecology* released a sister journal *Molecular Ecology Notes* to cope with the vastly increasing number of primer notes. Many hundreds of references to their use are available for many different species, with over 30,00 entries posted on the EMBL database at present (*www.ebi.ac.uk*). The following list provides just a few recent examples of the diversity of the use of microsatellite DNA markers:

Birds: *Phylloscopus* warbler (Bensch *et al*., 1997); Fish: Tilapia (*Oreochromis niloticus* (Lee and Kocher, 1996)), Atlantic salmon (*Salmo salar* (Tessier and Bernatchez, 1999)); Reptiles: the northern water snake (*Nerodia sipedon sipedon* (Prosser *et al*., 1999)); Amphibians: *Bufo calamita* (Rowe *et al*., 1997); Cephalopods: *Loligo forbesi* (Shaw and Boyle, 1997a;b); Mammals: the bottle-nose dolphin (*Tursiops truncatus* (Shinohara *et al*., 1997) and humans (Litt and Luty, 1989; Armour *et al.,* 1994).

Microsatellite Markers in Fisheries and Aquaculture

Microsatellites exhibit many attributes that make them particularly suitable as genetic markers in aquaculture and fisheries research. Good summaries of this are provided by Wright and Bentzen (1994); O'Reilly and Wright (1995); and O'Connell and Wright, (1997). The following list is taken from these reviews:

1) Microsatellites are abundant in the genome;
2) They display varying levels of polymorphism;
3) Alleles exhibit codominant Mendelian inheritance;
4) Minute amounts of tissue are required for assay (e.g. dried scales or otoliths);
5) Loci are conserved in related species, and primers developed for one species frequently amplify polymorphic loci in related species;
6) After initial development, the potential for automated assay when compared to other DNA markers is very high.

One of the first species of fish to be investigated for within and among population variability using microsatellites was rainbow trout (Nielsen *et al*., 1994 *in* O'Connell and Wright, 1997). Elucidating pedigrees of mixed family groups reared

in a common environment is generally not possible without DNA markers because physical tags cannot be applied to newborn fish (O'Connell and Wright, 1997). Microsatellite marker techniques are, therefore, beneficial to small farm operations for stock identification, avoiding the need for separate tanks or tags (e.g. Volckaert and Hellemans, 1999). Moreover, the conclusions drawn from the selection regime are more likely to show reduced environmental bias, in that potential environmental effects are reduced because the progeny from all crosses have been treated in a single tank (O'Connell and Wright, 1997). Herbinger *et al.* (1995) demonstrated that a pedigree of a mixed rainbow trout population could be satisfactorily determined using as few as four microsatellite markers, even though the fish could have originated from 100 possible parental crosses. Similar conclusions were drawn by Beacham and Wood (1999), although they suggest that the use of six microsatellite DNA loci should provide the ability to provide relatively accurate and precise estimates as stock composition when utilised for fishery management applications, and therefore good for this study.

Isolating Microsatellite DNA Markers

Standard techniques for isolating microsatellite DNA markers are common (Wright and Bentzen, 1994; Strassmans *et al*., 1996; O'Connell and Wright, 1997):

Briefly: Genomic DNA is digested with a selection of restriction endonucleases (RE) and size fractionated on an agarose gel. A particular size range is excised (usually around 300-600 base pairs (bp)), and the recovered DNA is ligated into a vector (e.g. pUC18). The ligated fractions are in turn used to transform bacteria (usually Escherichia coli), and allow construction of a partial genomic library. The library is screened with a labelled repeat unit probe (which is complementary to the

sequence required) e.g. (GT)n. The clones that hybridize to the probe are sequenced, and primers are designed to complement unique sequences which flank either side of the repeat array.

1.3.2 A novel method of investigating CYP1A mRNA levels: real-time PCR

A Rotorgene thermocycler (Model 2000, Corbett Research, Australia) was acquired by the Institute of Aquaculture during the course of this study, and provided the opportunity of producing an alternative quantitative assay for detoxification gene expression at the mRNA level. Reverse transcription polymerase chain reaction (RT-PCR) is the most sensitive method for detection of low-abundance mRNA, and the recent introduction of fluorescence based kinetic RT-PCR procedures significantly simplifies the process of producing reproducible quantification of mRNAs (Bustin, 2000), as required by this study. Real-time Quantitative PCR is increasingly used to quantify copy numbers of nucleic acids for clinical applications, and uses a cDNA template generated using the same methods as for traditional quantitative RT-PCR (Wilhelm, Hahn and Pingoud, 2000). Real-time PCR, however, allows the PCR product to be tracked as it is being generated, providing an advantage over traditional quantitative RT PCR in that the product can be followed as it enters, and passes through, the exponential phase, without the need to remove aliquots at different stages of the reaction, or stop the reaction at different time points. All reactions are monitored as product is generated, and as product reaches a specified threshold value (C_T) measurements are taken. The C_T is the fractional cycle number at which the fluorescence signal reaches an arbitrary but defined threshold value within the early exponential phase of the reaction,

(Wilhelm, Hahn and Pingoud, 2000) and is proportional to the logarithm of the initial copy numbers of the target, which are used to determine the initial copy numbers of unknown samples (Yin et al., 2001).

An increasing number of real-time PCR thermocyclers are appearing on the market, and a comparison of technologies is offered in (Bell and Ranford-Cartwright, 2002). The Rotorgene confers several advantages over other machines: by using air heating / cooling, ramping times are accelerated, and by using a rotating carousel system, samples can be assumed to be homogeneously mixed. Another advantage of the rotating system is that it helps to overcome the problem of non-uniform temperature throughout samples which can have a large effect on reaction efficiency (discussed by Wilhelm, Hahn and Pingoud (2000)). In the case of a regular PCR, such discrepancies would probably go unnoticed, but as real-time PCR is such a sensitive process, and is monitored throughout, such factors become increasingly influential on the final result. There are five major chemistries available which offer methods of quantification in the real-time PCR reaction. These chemistries can be split into two groups: 1) Sequence non-specific probes (DNA binding fluorophores) e.g. SYBR® Green I (Wittwer, Herrmann, Moss and Rasmussen, 1997) and ethidium bromide (Higuchi, Dollinger, Walsh and Griffith, 1992) and 2) Sequence specific probes (Linear oligoprobes, 5' oligoprobes, hairpin oligoprobes, and self fluorescing amplicon) (see Bell and Ranford-Cartwright (2002) and Mackay, Arden and Nitsche (2002) for reviews). Yin, Shackel, Zekry, McGuinness, Richards, Van der Putten, McCaughan, Eris and Bishop (2001) provide a report comparing some of these different technologies, and identifying limitations associated with both techniques. The technique of real-time PCR is described further in chapter five.

1.4 Aims of the present study

Development of tools and methodologies for investigation

DNA markers were required for use in parental analysis in order to carry out cross breeding experiments between European flounder from a polluted and a clean site. Crosses were carried out, and offspring were raised in communal tanks to minimise any environmental impact on phenotype. In this way it was possible to investigate variability in detoxification enzyme gene transcript expression (via the *Ah* battery) following exposure to pollutants, and attribute phenotypic variation to genotype. Probes for investigating *Ah* battery gene expression were also required. Chapter three and four concentrate on developing the necessary microsatellite markers, and probes and quantitative methodologies of *Ah* battery gene expression, respectively. Chapter five provides results of exposure of flounder to xenobiotics, and analysis of gene transcript expression.

Overall aims

The first aim of this study was to ascertain interindividual variability levels in expression and induction of flounder CYP1A by xenobiotics, particularly PCBs. The second aim was to ascertain whether maternal pollutant exposure history affects inductive response of CYP1A in xenobiotic (PCB) exposed offspring, as has been proposed in studies of several species including the European flounder in previous studies (Eggens 1996). Approaches undertaken to study these aims are described in section 2.

Section 2 - Experimental chapters

Approaches to study

1.5 Breeding trials

At the outset of the study it was anticipated that flounder would be obtained from the Rotterdam estuary, Netherlands, an area subjected to long term pollution, and crosses carried out with flounder obtained from a clean area. As described earlier, studies carried out on fish from this area had demonstrated little induction of CYP1A despite high sedimentary PAH and PCB concentrations, and this lack of induction was further confirmed by experimental mesocosm exposure studies (Eggens *et al.*, 1996). Several suggestions were put forward by the authors as to causes of these depressed levels including other compounds in the sediment. (Roy, Courtenay, Yuan, Maxwell, Ikonomou and Wirgin, 2001) have postulated that down regulation of CYP1A1 mRNA inducibility may occur through two mechanisms: 1) genetic adaption, indicating a multigenerational response independent of locale, or 2) physiological acclimation which would suggest a single generational response. A further proposed theory involves saturation of receptors in the CYP1A transcription pathway.

Due to unforeseen refusal of an import licence by the Ministry due to disease transmission regulations, it was not possible to obtain fish from the Rotterdam waterway for these studies. The Mersey estuary in the UK also has a long historical record of high petrochemical and PCB pollution, and levels of all PCBs in 6 species of fish (including flounder) collected from this area were 20 to 200 times higher than in fish from the North-west Atlantic and Solway Firth areas (Woodhead, Law and Matthiessen, 1999; Leah, Johnson, Conner and Levene, 1997). Therefore we

crossed flounder from the Mersey with fish from a relatively pristine area of the UK (Port Erin Bay, Isle of Man) in order to investigate whether any variation between individuals is observed in expression of detoxification enzyme gene transcript level, and also whether any of this variation is inherited from parent to offspring. The original study aims were modified to examine whether there were any differences in CYP1A expression levels between offspring produced from 'clean' and 'polluted' parents, and to ascertain what the interindividual variability levels were in flounder. These data would be of relevance to biomonitoring studies whose sample size questions have not been fully investigated.

In order to ensure that it was possible to carry out artificial breeding trials of European flounder, and that offspring could be successfully raised from crosses, an initial small scale trial was carried out. A small number of Port Erin Bay (Isle of Man) flounder were crossed to provide offspring to test genotyping possibilities, and carry out some initial exposure trials. Once the system had been tested it was then possible to carry out trials with fish from polluted × clean area crosses. This is covered in Chapter 2.

1.6 Parentage assignment

In order to carry out mixed offspring breeding trials, and eventually identify offspring raised communally to their parents, a method of 'tagging' larval fish was required. As it is not possible to tag larval fish using a traditional tagging system, molecular markers were the method of choice. Microsatellite markers are available for a large number of teleost species including Atlantic salmon (*Salmo salar*)(O'Reilly and Wright, 1995; Cairney *et al.*, 2000; Tessier and Bernatchez, 1999), tilapia (*Oreochromis niloticus*) (Lee and Kocher, 1995), Atlantic cod (*Gadus*

morhua) (Brooker *et al.,* 1994) and plaice (*Pleuronectes platessa*) (Watts *et al.,* 2000). At the outset of the project there were no such markers available for the European flounder (*Platichthys flesus*). Thus, one of the primary aims of this study was to isolate and characterise sufficient microsatellite DNA markers for the European flounder that would allow parental analysis of half-sib crosses for ten families.

Screening for Microsatellites

Despite their usefulness for many applications, the difficulties, expense and time involved in obtaining microsatellite markers are a major hindrance to their use (Kijas *et al*. 1994), for example the amount of clones required for screening. Kijas *et al.* (1994) suggest an alternative method of library construction which involves an enrichment step of the desired repeat sequence previous to cloning. The result is a microsatellite-enriched sequence population suitable for cloning and screening in the conventional manner. Kijas *et al*. (1994) obtained a success rate of c.20% positive clones in the citrus genome, and Rowe *et al*. (1997) found 68 positives from 665 clones in the natterjack toad using enrichments in comparison to non-enriched methodologies. Prosser *et al.* (1999) obtained 48 positives from 6000 clones screened in the northern water snake, and Bensch *et al.* (1997) found 9 positives from c.40,000 clones in the *Phylloscopus* warbler, using non-enriched methodologies. Many other examples of enrichment success are documented which demonstrate a substantial improvement on non-enriched library construction (Armour *et al,.*1994; Lee and Kocher, 1996).

Microsatellites have generally been investigated by radioactively labelling one of two primers that are complementary to the flanking sequence on either side of the

repeat unit array. These primers are used in a PCR reaction to amplify the repeat unit array. Length differences, due to the variable number of repeats among samples, are resolved by running out the amplification products on a sequencing gel. The gel is then dried, exposed to X-ray film and usually developed overnight. Reference to standards (e.g. M13 and samples with known genotype) and/or an allelic ladder commonly determine allele sizes (O'Connell and Wright, 1997). An alternative method which has become increasing popular due to not using isotopes is that of fluorescent primers. PCRs with these primers are resolved on automated sequencers which also give the advantage of high throughput, semi-automated genotyping. For initial characterisation of loci, isotopically labelled primers were used and run out on manual sequencing gels, however the latter method using fluorescently labelled primers was employed in this study for routine genotyping.

1.7 Gene expression studies: Developing probes and assay methods for study

Probes were developed to allow quantitative studies of the expression of genes involved in the pleuronectid PAH / PCB metabolic pathway (namely AhR2, ARNT2, CYP1A, UGT1B1 and GSTA). Several probes were already available in our laboratory, and several novel probes were developed, and this is discussed in Chapter 4.1.

Quantitative PCR for genes in the PAH metabolic pathway

There are two main methods of quantifying gene expression at the molecular level, quantitative and semi-quantitative PCR based assays (including traditional RT-PCR and real-time PCR), and hybridisation analysis (Northern blot, or Southern blot of

RT-PCR products) involving labelled probes (e.g. isotopic or fluorescent). A Rotorgene real-time thermocycler (Model 2000, Corbett Research, Australia) was acquired by the Institute of Aquaculture during the course of this study, and provided the opportunity of producing an alternative quantitative assay for detoxification gene expression at the mRNA level. The main advantage of the real-time quantitative PCR system over traditional methods is that no post-reaction (e.g. image analysis) analysis is necessary, and results can be tracked in 'real-time' (MacKay, Arden and Nitsche, 2002). Other advantages conferred by real-time PCR are that very small tissue samples can be assayed, the system also allows a high throughput method to be used, and direct quantification from a known standard can be used. Section 3 of chapter 4 focuses on developing methods of real-time PCR quantification using SYBR® Green 1 intercalating dye for measuring detoxification enzyme expression. The aim of the real-time PCR study was to produce a novel method of mRNA (via cDNA) quantification which could provide a sensitive method of measuring the expression of detoxification genes in flounder. The main focus of this work was measurement of CYP1A mRNA transcript level. Further work was attempted for UGT1B1, and also the transcription factors, AhR2 and ARNT2. The development of standard curves for the transcription factors (AhR2 and ARNT2) and of UGT1B1 is reported, along with initial trials on quantification of samples of unknown quantity.

Blot studies

An alternative method of quantification of gene expression using traditional Northern and Southern blotting is presented in Chapter 4.3.

Chapter 2 Fish breeding

2.1 Collection of broodstock fish

Initial breeding trials were carried out using five male and five female flounder obtained from Port Erin Marine Laboratories (PEML), Isle of Man (IoM) during January 1999, and selected to provide ova and sperm for crosses. Fish were then kept in holding tanks until ready for crossing later that year.

To provide experimental crosses of flounder for the main breeding trial, parental fish were collected using standard trawling methods. The initial aim was to collect 10 female and 10 male fish from each area (Port Erin Bay, IoM, and the Mersey Estuary), and produce reciprocal half-sib crosses of 40 families. Several problems were encountered whilst obtaining fish due to limited trawler availability, small numbers of fish being caught during the separate trawls, and low survival of the broodstock fish during transport. Only six female and four male fish were available from the Mersey Estuary in March / April 2000. Further problems resulted from the lack of viability of several of the Mersey fish, and limited spawning windows caused difficulties in synchronicity of spawning times for the males and females collected from different areas. For the final trial only two of the female (and no male) fish from the Mersey Estuary could be used for breeding. A further eight fish were collected in Port Erin Bay (IoM) in March / April 2000, and crosses were set up as detailed below.

2.1.1 Stripping / crosses

All of the breeding experiments were carried out at PEML, University of Liverpool, IoM. Fish were stripped manually, and gametes transferred to holding vials. For the

initial trial in March 1999, sperm was collected from five male flounder, and ova from five female flounder. Each parent was tagged with a fin tag, and fin clips were taken from each parent and retained for future DNA analysis. For the initial trials, parents were 'partnered' to create five families of fish, and a single cross was made between each set of parents. Ova were mixed with the appropriate sperm according to group / cross desired (see below). Fertilisation success was determined from a sub-sample taken from each cross. Twelve hours post-fertilisation, eggs were transferred to 10 l holding buckets, and incubated at 8^{o}C in 32 ±1 ppt aerated seawater. Eggs were monitored, and post-hatching, survival rate of offspring was recorded.

For the experimental trials in April 2000, four male and five female fish were stripped from the clean site, and two female fish were stripped from the polluted site, and crosses were set up using the methods described above.

2.1.2 Offspring produced in initial trial

At day 14 of both trials, offspring were transferred to communal tanks. Post-metamorphosis, the juvenile flounder were transferred from holding tanks at PEML, IoM, to the biological sciences aquarium, University of Stirling. As 2-3,000 fish were produced in each set of crosses, the offspring were randomly divided into three equal groups. Fish were maintained on an artificial pellet diet (Trouw Commercial Number 3) at 8^{o}C in 70 l holding tanks in 32 ±1 ppt aerated seawater. Every family of the initial crosses which was set up yielded viable offspring.

2.1.3 Offspring for experimental trials

The initial aim of the study was to set up four groups of crosses (Table 2.1).

Table 2.1: Intended crosses for use in the study (for 'area' IoM is clean, and Mersey is polluted)

Group	Area x Area
A	Polluted x Polluted
B	Polluted Male x Clean Female
C	Clean Male x Polluted Female
D	Clean x Clean

Unfortunately due to problems encountered as described in section 2.1 above, only limited crosses were possible due to lack of males obtained from the polluted (Mersey) site. Table 2.2 gives details of the crosses which were actually produced in the breeding experiments, including: tag numbers of fish; source of parent (IoM is the clean site; Mersey Estuary is the polluted site); number of offspring which survived through to post-metamorphosis stage from each cross.

Table 2.2: Offspring produced from breeding experiments

Cross	Female	Male	Female source	Male source	Offspring ongrown in each tank
6	7225	7234	Mersey	IoM	No hatch
9	7225	7235	Mersey	IoM	No fertilisation
5	7228	7230	Mersey	IoM	800
7	7228	7234	Mersey	IoM	800
2	7239	7237	IoM	IoM	800
1	7240	7235	IoM	IoM	800
8	7240	7234	IoM	IoM	200
3	7241	7234	IoM	IoM	850
4	7242	7230	IoM	IoM	60
10	7242	7235	IoM	IoM	300

Of the 10 families set up, eight produced viable offspring. Of the eight families, two were from polluted x clean crosses, and in both cases the same Mersey female was involved. No sexually mature males with viable milt were obtained from the Mersey estuary. In the other two polluted x clean families, large numbers of offspring were produced. Of the two families whose crosses did not survive for on-growing, the same female was used, in cross six, eggs were fertilised, but did not hatch, and in cross nine, fertilisation success was zero.

2.1.4 Use of offspring

A sub-sample of offspring was removed from each cross in the initial trial shortly after fertilisation of ova. These sub-samples of offspring were then preserved in 100% ethanol for subsequent DNA (microsatellite) analysis. The sub-samples were necessary in order to confirm that a) the microsatellites employed were heritable in a Mendelian fashion, and b) it was possible to match known offspring with their parents via microsatellite analysis. The offspring were also used to study initial effects of exposure to model pollutants (see Chapter 5).

All offspring produced from the experimental crosses were reared communally to minimise environmental variability, and used in this study to measure levels of detoxification enzyme expression in relation to pollutant exposure. As all offspring were reared communally, all fish were genotyped and assigned to a family group using microsatellites (see chapters 3 and 5.3).

2.2 Discussion

Initial trials of flounder from IoM × IoM crosses demonstrated that cross breeding trials could be carried out successfully. A successful trial was established between

flounder obtained from a clean and polluted environment. No such trial has been documented in the literature for flounder, although the idea has been successfully used in the US with populations of killifish and tomcod, as described in the introduction. The families which were generated from the Mersey × IoM crosses provided a large number of offspring, and represented a large enough proportion of the total population to provide statistically viable results. Although the trial was successful, a few problem areas were encountered in the trials, and these are discussed below.

Fish could not be obtained from the original collection area (Rotterdam waterway) due to the imposition of a newly introduced import restrictions to prevent disease spread, and a new area for focus had to be identified. High levels of chronic PAH and PCB pollution have been identified in the Mersey estuary as exemplified recently by Leah *et al*. (1997) and this was selected as a suitable site for obtaining fish with a pollutant burden history. Trawls were organised to collect fish from these areas in March / April 2000, and several separate trawls were conducted. A limited time window (due to limited trawler availability) put pressure on fish availability and by the end of trawling only a low number of suitable flounder were caught which led to obtaining low numbers of fish from a polluted area. A lack of spawning synchronicity between fish from the two areas (IoM and Mersey) further reduced numbers, and some of the Mersey fish caught were viable prior to the IoM fish becoming viable. One female parent which did produce eggs for use in the trial, when crossed with a male with known quality milt, did not produce viable offspring. Studies have indicated that fecundity is related to past pollutant burden (Janssen, Lambert and Goos, 1995) and PAHs and PCBs can reduce reproductive success by interacting directly with the gonads and germ cells (Armstrong, 1986). The lack of

fecundity of some of the Mersey fish, and problems with fertilisation may be caused by the high levels of pollutants in the Mersey estuary, which could be investigated further by setting up other comparison crosses as discussed later in this chapter. By contrast, all of the fish obtained from IoM waters for both the initial trials, and the experimental crosses produced viable ova and milt, and all offspring from all crosses of clean × clean fish survived to metamorphosis.

Future suggestions for study would be to obtain a quantity of male and female flounder from two distinct areas, and maintain them in an aquarium system for a period of one year to allow spawning synchronicity to develop by standardising environmental factors. Parents would then generate viable ova and milt at roughly synchronised times of the year, and allow crosses to be set up, as intended for this trial. A mixed batch of offspring could be generated which would provide an approximately equal mix of offspring from all areas, and thus any experimental trials should use an equal representation of each area / cross. Unlike model species of fish used in other laboratory based studies (e.g. zebra fish, *Danio rerio* and the killifish, *Fundulus heteroclitus*) flounder have a relatively long generation time. If future trials did confirm any possible genetic component to pollutant responses, as has been demonstrated for other species, it would be informative to continue breeding trials for subsequent generations from the F1 crosses to follow this genetic component further. These F2 generation offspring would also assist in resolving any questions of single generational acclimation, and remove factors such as pollutants in the yolk sac, in addition to providing data on heritability levels. This type of trial has been carried out in the US on *F. heteroclitus*, and useful data has been produced.

Chapter 3 Isolation and characterisation of microsatellite DNA markers in the European flounder

3.1 Methods of microsatellite isolation

In order to genotype mixed batch offspring to their parents, molecular (microsatellite) DNA markers were developed as follows. The first method of identification of microsatellite loci involved the investigation of currently available sequences. This was carried out via online database searches to identify any flounder microsatellites, or any from closely related species, which would have been potentially useful for application in flounder, however none were found. In addition, several microsatellite loci were found by chance in plaice (*Pleuronectes platessa*) the closest relative of the European flounder, during other sequencing studies in the laboratory (M. Leaver, unpublished data). Primer sets flanking the microsatellite regions were designed to investigate the possibility that they may detect polymorphic loci in the flounder. Two experimental methods were also investigated. Both methods involved the initial construction of a small insert genomic library to isolate small fragments (300-800bp) of DNA. Small insert libraries require no sub-cloning steps, and provide a target which can be sequenced in a single run. Using inserts of <800 bp is considered to be the optimal size range for generating sequences for PCR assays (Pulido and Duyk, 1994). For a marker to be used in routine automated studies, it must be easy to amplify by PCR, and easy to score which favours microsatellites below 300bp in size. The first experimental method involved carrying out an enrichment for tri- and tetra-nucleotide microsatellite

repeat motifs. Tri- and tetra-nucleotide repeat loci generally exhibit higher levels of polymorphism than di-nucleotide repeats (Armour, Neumann, Gobert and Jeffreys, 1994) and are less prone to stutter bands and therefore scoring difficulties (O'Connell and Wright, 1997). Enrichment has been extensively proven to reduce time in screening, and increase the chances of finding the reasonably uncommon tri- and tetra-nucleotide repeat motifs (Kijas *et al.,* 1994; Dawson *et al.,* 1997; Hansen, Taggart and Meldrup, 1999; Iyengar *et al.,* 2000; Cairney *et al*., 2000). The second experimental method was a more random approach to isolating microsatellites, and was employed due to difficulties found during the enrichment process. This second method focused on the more common di-nucleotide repeats. Four or five polymorphic loci should be adequate to differentiate between individuals from crosses of five sets of parents, to be raised in the same tank (Herbinger *et al.*, 1995) so the initial aim was to characterise this number as a minimum.

Isolation of microsatellites

The enrichment method which was used in this study is presented below. It is a modified version of a protocol obtained from Dr. P. Prodohl, Queens University, Belfast (pers com.) based upon Kijas *et al.* (1994) using hybridisation of biotinylated oligonucleotides of repetitive sequence bound to streptavidin coated magnetic beads. Five different rounds of enrichment were carried out to construct libraries with a variety of different repeat motifs. The general method for all enrichments was identical, but different biotinylated oligos were used for each (see section 3.3 below).

3.2 General Method

High molecular weight genomic DNA was extracted from liver tissue of three individual flounder (*Platichthys flesus*), using a modified phenol-chloroform extraction technique. This technique was based upon (Taggart *et al.,* 1992) and 'Mullins traditional DNA extraction' (from mouse tail), but was extensively optimised to produce good quality DNA from European flounder whilst offering rapid throughput (see Appendix I.1 for full method). The extracted DNA was checked on an agarose gel to confirm that it was of high molecular weight, then pooled to provide material for the construction of a small insert genomic library.

Genomic DNA for library construction was digested with appropriate restriction enzymes. Both blunt end and cohesive end strategies were used. Restriction enzymes were selected for several characteristics:

1. The enzyme was compatible with the cloning strategy to be used i.e. blunt end cutting, or production of cohesive termini.
2. Frequent (4 base) cutting characteristics were required to produce small fragments (i.e. <1kb).
3. Enzyme restriction sites were not similar to the targeted microsatellite motifs.
4. Enzymes were selected that had been successfully used in previous isolation studies within this, and other, laboratories.

Separate 10µg aliquots of the pooled genomic DNA were digested to completion with excess of four restriction enzymes - *Alu*I, *Hae*III, *Rsa*I and *Mbo*I (Promega, UK). Several enzymes were used in order to generate a random pool of fragments.

Three units of each restriction enzyme (RE) were used per μg of DNA in each reaction to ensure excess of the enzyme.

Restriction digests were performed by addition of 10μg of genomic DNA, 5μl 10× manufacturer's supplied RE buffer, 30 units of RE, and dH_2O to a total volume of 50μl to a 1.5ml microfuge tube. Each reaction was incubated at 37°C for two hours. A phenol extraction step was then included to remove enzyme and salts from the reaction. Three of the purified digests (restricted by *Alu*I, *Hae*III, *Rsa*I) were then pooled to use as template for further cloning (blunt ended cutting REs), and the *Mbo*I fragments were used separately (cohesive end cutting RE).

A total of 15μg of the pooled blunt cut, and 5μg of the *Mbo*I, digested flounder DNA was size fractionated by gel electrophoresis (1.2% agarose, 0.7× TAE) for one hour at 85mA, flanked by 200ng of ϕX174 *Hae*III DNA marker. Gel sections at 200-600bp size fractions were removed and purified using a Quik-Pik™ electroelution capsule (Stratagene) according to the manufacturer's instructions. The size fractionated restriction digested genomic DNA was then ligated into *Eco*RV cut (for blunt end ligations) or *Bam*HI cut (for cohesive ends) dephosphorylated pBluescript II KS- vector (Institute of Aquaculture stock) as described below at a insert : vector molar ratio of 2 : 1 (see below). A large excess of T4 DNA ligase (>20U) was used for blunt-end ligations. The phagemid vector pBlueScript II (KS-) was used throughout [Stratagene # 212208] as it has been shown to work well in other studies (i.e. has been demonstrated as retaining the repeat sequences) and the T3 and T7 promoter sequences at either end of the multiple cloning site of this vector provided convenient primer sites for PCR amplification using T3 and T7 17-mer sequencing primers.

Ligations were carried out by addition of the following:

Component	Volume	Final concentration / amount
100ng/µl dephosphorylated *Eco*RV cut pBluescript II KS- vector	1µl	[100ng]
50ng/µl insert DNA	2µl	[100ng]
10× T4 DNA Ligase buffer	1µl	[1×]
High conc.T4 DNA Ligase	xµl	[20 units] (blunt end) [2 units] (cohesive end)
dH_2O	yµl	to total 10µl vol.

Reactions were incubated overnight at 14°C for blunt ends, and 16°C for cohesive ends.

3.3 Approach 1: Enrichment

Oligonucleotides were designed consisting of various tri- and tetra-nucleotide repeat motifs. The particular motifs were chosen due to success achieved in previous studies on teleosts (see Table 3.1 for details of oligos used for each library). Oligonucleotides used in this study were $(GACA)_6$, $(GATA)_6$, $(GGAT)_6$, $(AAGG)_6$, $(GGA)_8$, $(TAA)_8$ and $(AGC)_8$. 5' biotinylated oligonucleotides were ordered from MWG Biotech Ltd. Enrichment for repeat units (microsatellites) was then carried out as detailed below using the biotinylated oligos bound to streptavidin-coated magnetic beads (Dyna-beads, Dynal Inc. UK).

The protocol used for the microsatellite enrichment was a modification of the detailed methodology described by Kijas *et al*. (1994), following modifications made by Dr P. Prodöhl, Queens University, Belfast (pers comm.) and in our laboratory. Figure 3.1 below is a summary diagram of the enrichment methodology employed.

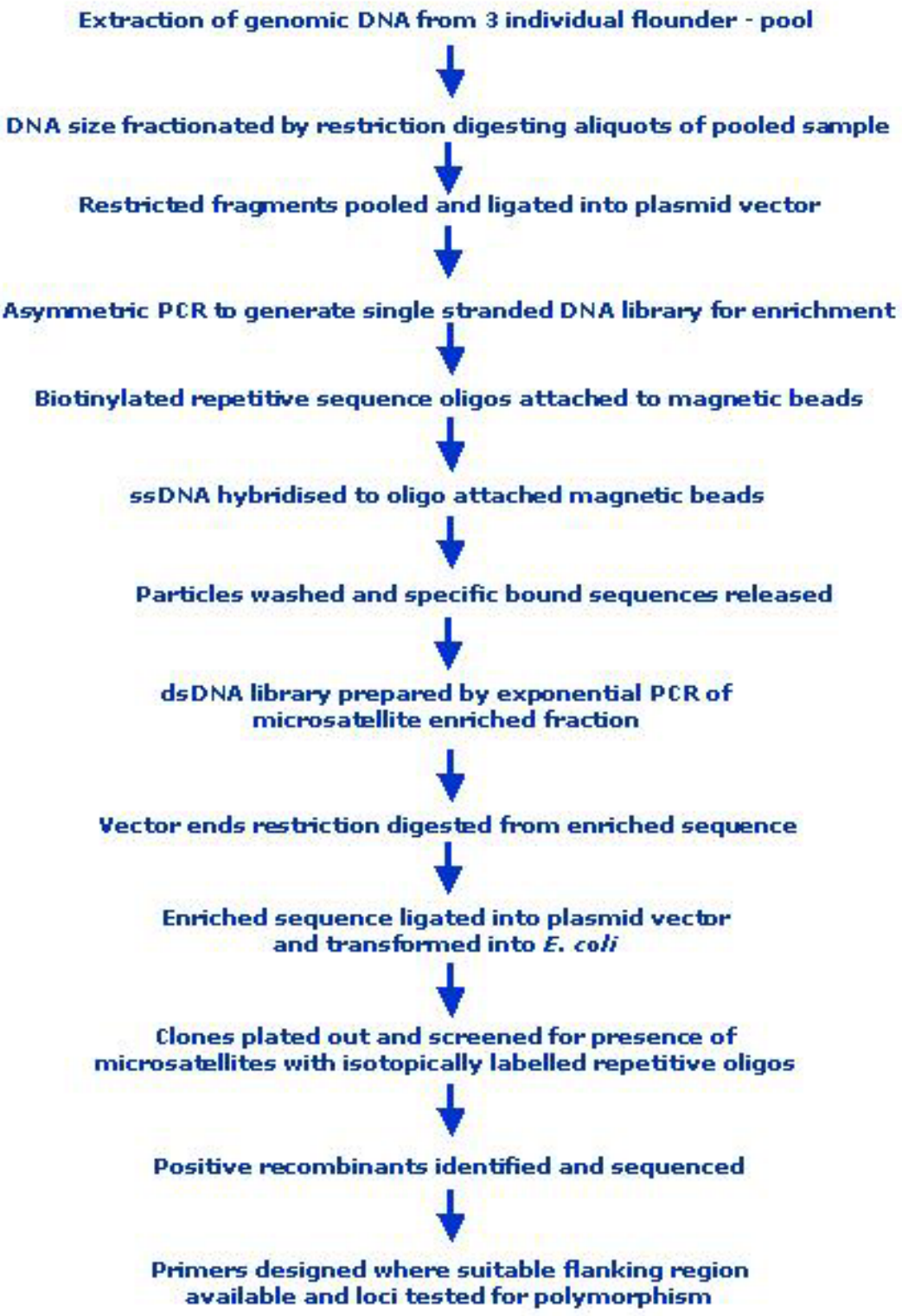

Figure 3.1: A flow diagram describing the main methodology used in construction and screening of an enriched microsatellite DNA library.

Table 3.1: Summary of enriched library construction detailing size of insert fragments, REs, and oligos used in enrichment

Library	Size fragments	Enrichment
1	300-600	$(GACA)_6$/ $(GATA)_6$ / $(GGAT)_6$
2	600-800	$(GACA)_6$/ $(GATA)_6$ / $(GGAT)_6$
3	600-800	$(TAA)_8$ / $(AGC)_8$
4	600-800	$(AAGG)_6$ / $(GGA)_8$
5	600-800	$(GACA)_6$/ $(GATA)_6$

Once ligated into pBluescript vector, the following steps were carried out:

1. **Asymmetric PCR** of ligation mix was carried out using a 10 : 1 molar excess of one of the two vector specific primers (T3 or T7) in order to generate single stranded DNA for the enrichment step. (Promega *Taq* DNA polymerase and supplied 10× buffer (50mM KCl, 10mM Tris-HCl pH 9.0, 0.1% Triton X-100) - # M1861 - were used throughout)

PCRs were set up to contain the following components in each tube

Component	Volume	Final concentration / amount
10× *Taq* polymerase buffer:	5µl	[1×]
25 mM $MgCl_2$:	3µl	[1.5 mM]
5 mM dNTP:	2µl	[200µM]
10 ng / µl primer 1 (T3):	5µl	[50 ng]
10 ng / µl primer 1 (T7):	0.5µl	[5 ng]
Taq polymerase (5 U / µl):	0.2µl	[1 U]
Ligation (from step 4):	xµl	[50 ng]
H_2O	yµl	to total 50µl volume

Thermal cycling was carried out on a Hybaid Omnigene using the following PCR cycling conditions: An initial denaturation at 94ºC for 4 min, followed by 32 cycles of denaturation at 94ºC for 1 min, annealing at 50ºC for 1 min and extension at 72ºC for 1 min, followed by a final extension step at 72ºC for 3 min.

2. **Microsatellite Enrichment (Magnetic Particle Separation)**

Streptavidin coated magnetic beads (Dynabeads, Dynal Inc.) were used in conjunction with a magnetic particle concentrator (MPC) (Promega, UK).

- **Preparation of beads:** Magnetic particles (50µl suspension for each enrichment) were washed twice in 0.1× SSC and resuspended in 100µl 5× SSC (pH 7.0). Following washes, the reaction was inserted into the MPC for a period of 40 seconds to allow concentration of the beads. The solution was carefully pipetted out of the tube whilst in the MPC.
- **Attaching biotinylated oligonucleotides to magnetic beads:** 1µg of biotinylated oligo was attached to the magnetic particles. Seven biotinylated oligonucleotides were used (all at a concentration of ≈250 ng/µl): $(GATA)_6$, $(GGAT)_6$,$(GACA)_6$, $(ATT)_8$, $(AGC)_8$, $(GGA)_8$, $(AAGG)_6$. For each enrichment 2µl (i.e. 500ng) of the appropriate biotinylated oligo was added to 100µl magnetic particles in 5× SSC. Each mixture was incubated at room temperature for 15 min with regular gentle mixing by hand to allow the biotin labels to attach to the streptavidin coated magnetic particles. The oligo-bound magnetic particles were then concentrated by placing the reaction tube into the MPC for 40 secs, and the supernatant removed by pipetting. The particles were washed three times in 5× SSC to remove unbound oligos and finally resuspended in 35µl of 10× SSC.
- **Hybridisation of flounder DNA and oligo probe / magnetic particles:**

Ten µl of asymmetric PCR product (step 1) were added to 55µl dH_2O (65µl total volume). The DNA was denatured for 10 mins at 100ºC, then the tube was placed on ice for 1 min. The contents were spun down briefly and the 35µl of oligo attached magnetic particles were added. The tube was then incubated with gentle agitation at 30ºC for 30 min.

- **Wash magnetic particles:** The tube was re-placed into the MPC and hybridisation supernatant removed. The beads were resuspended in 100µl of low stringency washing solution (2× SSC; 0.5 ng/µl each primer (T3 and T7)), and incubated for 5 min at room temperature. The wash was repeated three times (i.e. four times in total). Beads were then resuspended in 100µl of high stringency washing solution (1× SSC; 0.5 ng/µl each primer (T3 and T7)), then incubated at 30ºC for 5 mins. The wash was then repeated three times (i.e. four times in total).
- **Release specific bound sequences:** Magnetic beads were resuspended in 100µl 0.2× SSC, and incubated at 78ºC for 10 min. The contents were pulse spun in a microfuge (10 sec) and placed into the MPC. The supernatant (microsatellite enriched ssDNA) was then transferred to a clean tube.

3. **Microsatellite-enriched supernatant desalted**: The reaction was desalted using a QIAquick PCR clean up kit (QIAgen, UK) according to the manufacturers' instructions.

Double stranded DNA preparation by exponential PCR was carried out as shown below in order to prepare the DNA for cloning:

Each PCR reaction was set up to contain:

Component	Volume	Final concentration
10× *Taq* polymerase buffer	10µl	[1×]
25 mM $MgCl_2$	6µl	[1.5 mM]
5 mM dNTP	4µl	[200 µM]
100 ng / µl primer 1 (T3)	0.7µl	[70 ng]
100 ng / µl primer 1 (T7)	0.7µl	[70 ng]
Taq polymerase (5 U / µl)	0.4µl	[2 U]
Template (from step 3)	20µl	[50 ng]
H_2O	58.2µl	to final vol. 100µl

PCR cycling conditions (using an Hybaid Omnigene thermocycler) were as follows: an initial denaturation at 94ºC for 4 min, followed by 32 cycles of denaturation at 94ºC for 1 min, annealing at 50ºC for 1 min and extension at 72ºC for 1 min, followed by a final extension at 72ºC for 3 min.

4. A further **desalting** of the reaction was then carried out (see step 3).
5. **Vector arm fragments were removed** from PCR products by digestion with appropriate restriction enzyme(s) (i.e. for cohesive-end libraries, *Mbo*I; for blunt-end libraries *Eco*RI and *Hind*III).
6. **Desalting** was again repeated (as step 3).
7. **Enriched DNA was quantified** (by comparison with standard DNAs on agarose gel) and a standard ligation set up with 50-100 ng dephosphorylated BlueScript phagemid vector (molar ratio of insert:vector of 2:1). *Bam*HI digested phagemid was used for cohesive-end libraries and *Eco*RI / *Hind*III digested phagemid used for blunt-end libraries.

Transformation

Phagemid constructs (as prepared in step 8 above) were transformed into Epicurian coli™ SoloPack Gold Supercompetent cells (Stratagene™®) according to the manufacturer's protocol (see Appendix I.8). Transformations were plated out onto LB agar (100µg/ml ampicillin) plates and colour selection was carried out according to standard protocols (Sambrook *et al.*, 1996)(see Appendix I.10 for details). The following day, colonies containing insert (i.e. white) were picked from the agar plates using cocktail sticks, and colony PCRs (PCR of a single bacterial colony – see Appendix I.15 for protocol) were carried out on a selection to ensure that insert selection was successful. White colonies were picked individually and transferred to 96 well LB microtitre plates (100µg/ml ampicillin). The microtitre plates were incubated for 7 hours at 37°C. After 7 hours, an equal volume of LB medium containing 30% glycerol was added to preserve colonies. The plates were sealed with adhesive plastic tape and stored at -20°C.

3.3.1 Identification of microsatellite containing clones

96 well plates were removed from -20°C and centrifuged for 1 minute at 1,200×g. Hybond 'N' (Amersham, UK) hybridisation membrane was cut into 290mm squares, and laid onto 300mm square pre-prepared LB agar plates (100µg/ml ampicillin). Colonies from 96 well plates were replicated onto the membrane using a 96 pin plate replicator, and incubated over-night at 37°C. Following incubation, standard protocols were used to lyse colonies (see Appendix I.11.1), and DNA was fixed to the membrane by baking for 2 hours at 80°C.

Ordered array screening

Probing of filters was carried out according to standard protocols (see Sambrook *et al.*, 1996) as detailed below using γ^{32}P ATP (4000 Ci/mmol; 10µCi/µl) end labelled oligonucleotides (i.e. of identical sequence to those used in enrichment, see Table 3.2 for details).

Table 3.2: Probes used for enriched library screening

Library	γ^{32}P ATP end-labelled probe
1	$(GATA)_6$, $(GACA)_6$, $(GGAT)_6$
2	$(GATA)_6$, $(GACA)_6$, $(GGAT)_6$
3	$(ATT)_8$, $(AGC)_8$
4	$(GGA)_6$, $(AAGG)_6$
5	$(GATA)_6$, $(GACA)_6$

Isotopic end-labelling of probes

The following mixture was prepared, and the contents incubated at 37°C for 30 min. The reaction was stopped by heating to 95°C for 10 min, and the tube immediately transferred to ice until required.

Amount	Component
0.4µl	Probe (100ng/µl) [40ng]
1.0µl	10× T4 poly-nucleotide kinase (PNK) buffer [×1]
0.5µl	T4 PNK [5U]
0.5µl	γ^{32}P ATP (4000 Ci / mmol; 10µCi/µl)
7.6µl	dH_2O (to 10µl)

Pre-hyb. and hybridisation conditions

50ml of hybridisation solution (6× SSC, 6× Denhardt's solution (see recipes), 0.1% SDS) was prepared and 25ml used for pre-hybridisation for 3 hours at 42ºC. Hybridisation was then carried out using 4 pmol (≈20ng) probe in 25 ml solution, and hybridised overnight at 42ºC in a Techne rotisserie style hybridisation oven. Following probing, transfer membrane was washed to remove excess unbound probe according to the conditions detailed below:

One wash in 6× SSC; 0.1% SDS at 42ºC for 5 min, followed by 2 washes in 5×SSC; 0.1% SDS at 42ºC for 30 min each. Once satisfactorily washed, membranes were wrapped in Saranwrap™, and fixed into autoradiography cassettes with appropriate film (Blue sensitive X-Ray film, GRI, UK). Cassettes were then exposed for the appropriate length of time (between 4-24 hours, with or without an intensifying screen at –70^{o}C, dependant upon signal strength).

Following development of film each positive colony was graded between 1 (lowest intensity / lightest spot) and 10 (highest intensity / darkest spot). An example of a colony probe is provided in Figure 3.2 below.

3.3.2 Microsatellites obtained

1,152 colonies were plated out (12 × 96 well plates, 2 plates per library, except library 5 which consisted of 4 plates), and from this 221 positive recombinants (i.e. any colony with an intensity graded as >2 to avoid background) were identified by probing with the appropriate γ^{32}P ATP end labelled oligonucleotide (See Table 3.3 for probe details).

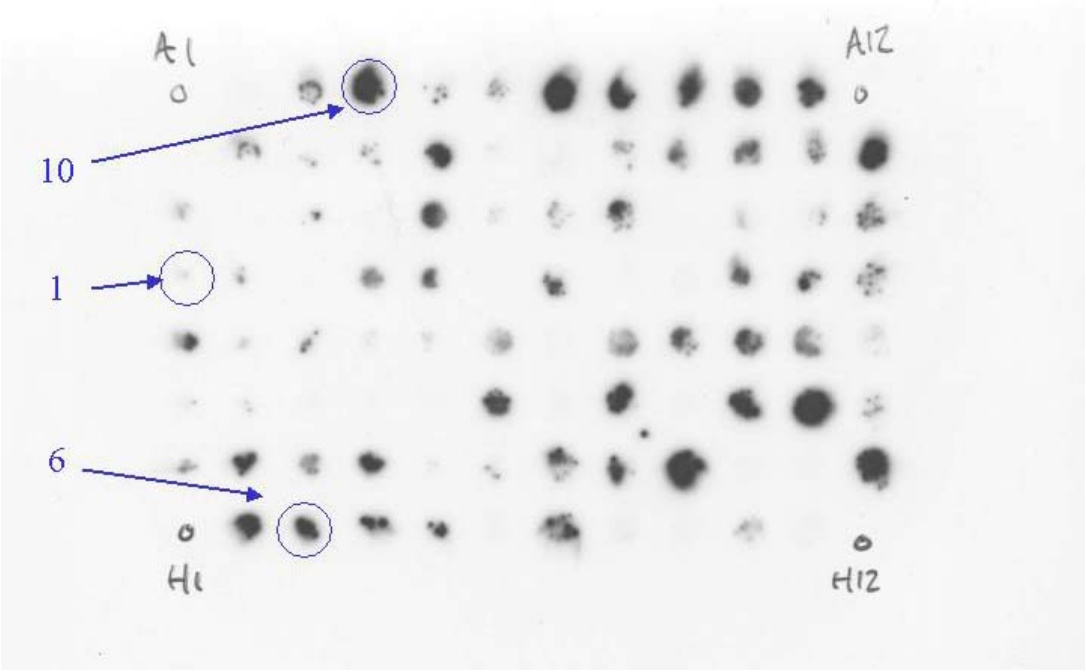

Figure 3.2: An example of an autoradiograph of a colony probing of a 96 well plate containing microsatellite clones from library 1 spotted onto Hybond membrane, and probed with isotopically (^{32}P) labelled oligonucleotide complementary probes of repetitive sequence. Examples of grading values based on intensities are shown in blue, and range from 1 (weakest, background level) to 10 (strongest). Plate orientations are marked on each corner corresponding to clone co-ordinates.

Table 3.3: Positive recombinants present in each library (Total no. colonies per library = 192, except library 5 which contains 384 colonies)

Library	**No. Positives**
1 GGAT / GATA / GACA 600-800 bp	69
2 GGAT / GATA / GACA 300-600 bp	26
3 TAA / AGC 600-800bp	49
4 AAGG / GGA 600-800bp	33
5 GATA / GACA 600-800bp	44

Positive recombinants were picked from 96 well microtitre plates using a single, sterile cocktail stick into universals containing 5ml LB medium, 100µg/ml ampicillin, and grown overnight at 37^{o}C / 250 RPM in a shaking incubator. The phagemid construct was purified using the QIAprep Spin Plasmid Kit (QIAgen, UK) according to the manufacturer's protocol. An aliquot of the purified product was digested with the relevant RE to release the insert, and an aliquot of the digested DNA was run on an ethidium bromide stained electrophoresis gel (1.2% agarose, 0.7× TAE) alongside a ϕX174 *Hae*III marker to estimate quantity and insert size.

3.3.3 Sequencing and sequence analysis

Automated sequencing of the inserts from the positive recombinants using the vector specific T3 and T7 (forward and reverse) primers was carried out using the 'Big Dye Terminator Cycle Sequencing Ready Reaction Kit' (PE Applied Biosystems) according to the manufacturer's protocol (as described in Appendix I.13.1) on an ABI PRISM 377™ DNA sequencer. An example of a sequence trace (generated by ABI PRISM 377™ sequence analysis package version 3.4) containing a GATA microsatellite repeat from locus Stpfl006 is provided below (Figure 3.3).

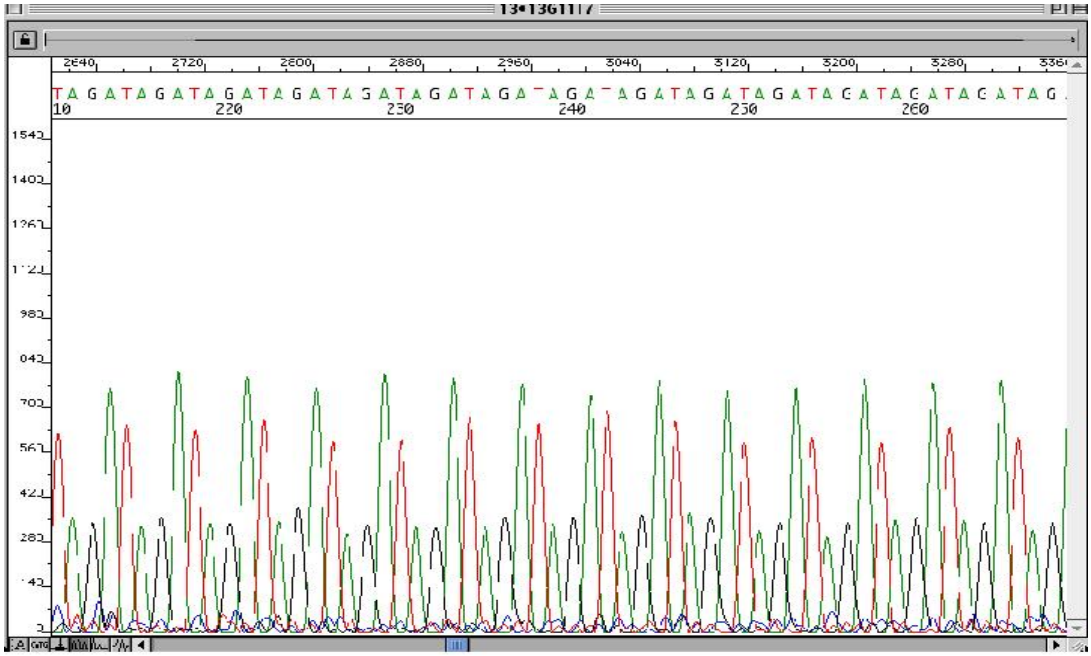

Figure 3.3: A partial repeat section (from 210-270 bps from beginning of construct) of locus StPfl006 as an example sequence trace from an ABI PRISM 377™ DNA sequencer (generated by ABI PRISM 377™ sequence analysis package version 3.4). Note the perfect GATA microsatellite tetranucleotide repeat. 'A' is represented as a green peak, 'T' is represented as a red peak, and 'G' as a black peak (visualised as yellow on sequenceing gel, but black on trace file for ease of viewing).

Sequences were analysed for the presence of a repeat unit using MegAlign (DNAStar) dot-plot option (see below as an example of a MegAlign dot-plot).

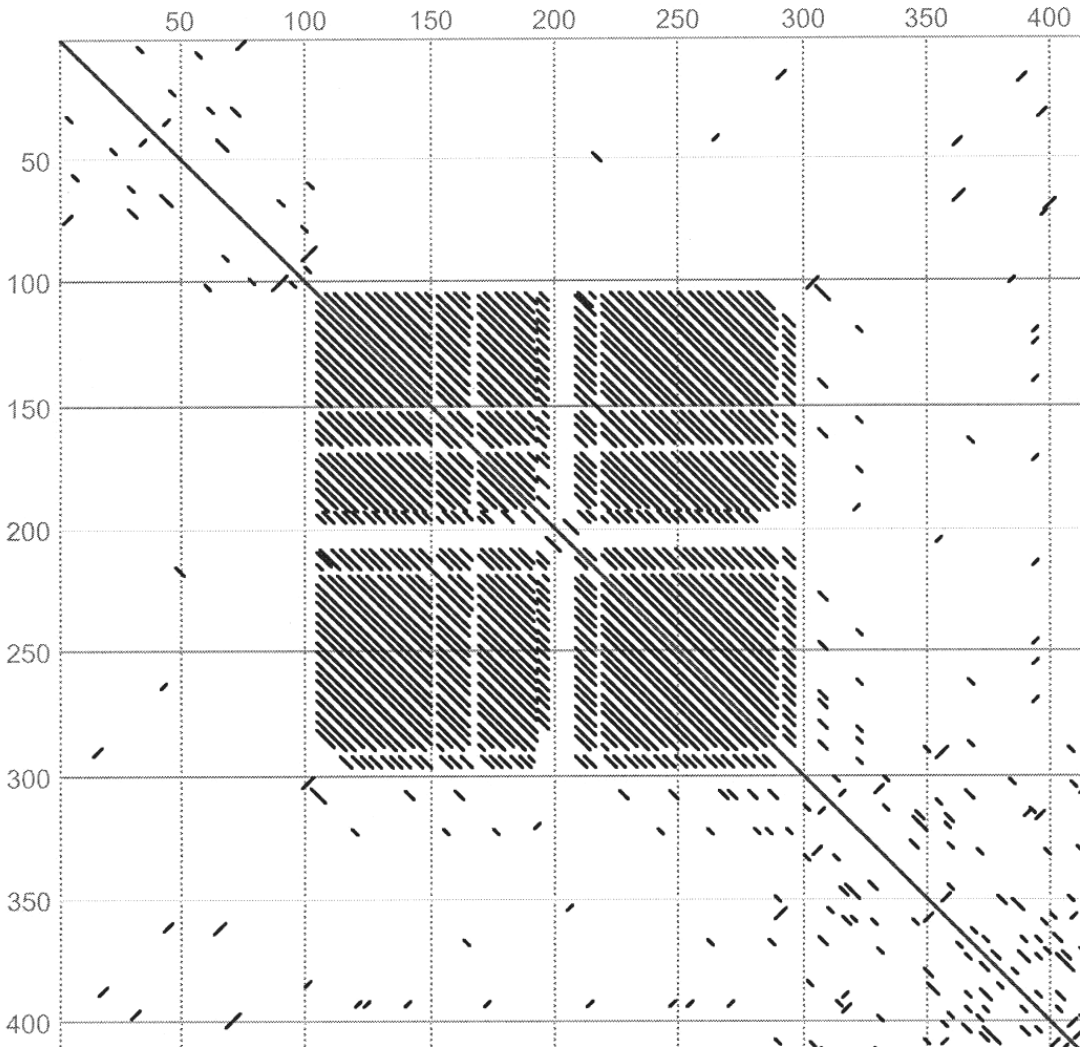

Figure 3.4: Locus Stpfl009 as an example of a MegAlign dot-plot to analyse repeated sections of DNA sequence. Matching sequnce regions appear as shaded areas, and repeat regions are apparent as blocks of shaded area (approximately 105-195 bp, and 210-280 bp in the example above).

3.3.4 Primer design

The longer (>7 unit) repeat motifs were selected and primers designed in the unique regions flanking the repeat unit array (microsatellites). Primer design was assisted by PrimerSelect (DNAStar) software. All primers were ordered from MWG Biotech Ltd. Loci were originally named according to their co-ordinates in the microtitre

plates, and were later renamed for ease of identification. Microsatellite primer sequences designed from the enrichment technique are presented below:

Table 3.4: Microsatellite primers designed including sequence data, and library from which each locus was isolated.

Locus name	Library	Primer sequence, 5'-3'
STPFL001	2	F CATCAAAGCATGAAACCC
		R CTGGCCCAAGTGGAGCAT
STPFL002	2	F ACCCGATCAAGTTGTAGTCAT
		R CATTTCTCCTCTCGGCGTGTT
STPFL003	2	F GTCAAATTAGGGAGGGCAGTG
		R CTCTGACCTTGCACAGGATAAAT
STPFL004	5	F ATGAGGACGTGGATGTTCTTC
		R CCCCTATCTCTGCTTAATGTTCAC
STPFL005	5	F TGCTGCCGAGCTGGGCTTATTATC
		R CACACGGCATCCCAACTGTCACAT
STPFL006	5	F TGTAGATACTCCGCTATAAAAAGT
		R AGCCCCCTGAGCAGCGTCTGT
STPFL007	1	F GAACAGAAGCGGGATAAGAATG
		R CAGACCAAAGGCCAAGAGTG
STPFL008	1	F GAACCAGAGACGAACCAGAGACC
		R ACAGGGGACAGATTTGAGACAGAA
STPFL009	1	F GGAGGAGTTTTCTTTGTTTTAG
		R GTCAGTGTTATGACTATATTATTAG
STPFL010	1	F CTTCACCTTGTTTTTGCTCTC
		R TATGACTGTAGGTTGTATCTGAA
STPFL011	1	F ATCCATCTGTCAGTAAACCTAACC
		R GTGTCCAGAAGGGGGCAG
STPFL012	1	F GTATTATTTCAAACCCCTACAT
		R TTAACAAGACCTCACGCAAATAC
STPFL013	2	F TTGGGAAGTTAAACAGGGAGTATC
		R TGAGCGTTTGGTTGTTTGTCTA
STPFL014	2	F TGCAAGAAGAGCGGAGGAATG
		R ACAGTTGGTGGACGGGACA
STPFL015	2	F GAGGGTTTAGTCTTGTGGTCATTG
		R AGGCCATCAGCTTGCTTTCT
STPFL016	2	F CTTTAATTGCGCCAGACTGACAG
		R CCCTTCGGGGATGAATAAAGT
STPFL017	2	F TCCCTTTCTGCATGTAGTGATT
		R GGATGAGGGAAGACAAAGAGA
STPFL018	2	F TGGTTTGCAAGATATTCATTCA
		R AGCCCTACTGCCCGTTTGTC
STPFL019	3	F GAACGAGGAGGCGACCGACCAG
		R TTGACTCTCCTTCTCCCGATGTTC
STPFL020	5	F ATCGGACCATCTTCAGAACTCAG
		R GGCCTCCTTCCAACATTAGATTAT
STPFL021	5	F GTGCGCACTCCGCGGCTCATC
		R CAGTTTGGGGCGGGGGACACG
STPFL022	5	F GCACTCACCTTTCACTTCAACCTT
		R CAGAAGCAGCGCGAGAAGATAAAG
STPFL023	5	F GATTAGGGCTTCAGGGCAGAGT
		R GAGCACCGGGGATCATTACAG
STPFL024	5	F GCGGCGCCTCCACCACCTC
		R AGCCTGCCGCACCGTCAACAGAG
STPFL025	5	F TTCAACAATGACCAAGGGGCATCA
		R TTTGCAATCTTGTAACACCTGAGA
STPFL026	5	F AGACTTCACATGCCAAGGTCAA
		R TCAGAGAACCCTGAGGTAGAGA

Table 3.5 summarises the numbers of positives identified, total number sequenced, and the number of primer sets designed from a total number of 1,152 original clones (12 × 96 well plates).

Table 3.5: Numbers of microsatellites identified: Numbers of positives identified, sequenced, and primer sets designed.

	Number	% of total clones
Total positives	221	19.2
Sequenced	142	12.3
*Readable sequence	132	11.4
#Significant repeat region	107	9.3
Redundancy	10	0.86
Suitable flanking	26	2.3
Primers sets designed	26	2.3

*Ten of those colonies sequenced appeared to have a double insert (i.e. two plasmids tandemly sequenced). #Significant repeat defined as >7 repeat units)

Table 3.6 provides details of the numbers of primers designed as a percentage of total positive clones from each library.

Table 3.6: Positive colonies with significant repeat and ample flanking region for primer design:

Library	Number	Percentage
1	6	7
2	9	38
3	1	2
4	0	0
5	10	23

3.4 Approach 2: Random isolation of microsatellites

After ligation (see section 3.2), an aliquot of the pooled (*Alu*I, *Hae*III and *Rsa*I cut DNA) RE digests ligated into the phagemid construct was used directly to transform Epicurian Coli® Solopak™ Gold Supercompetent Cells (Stratagene, UK), according to the manufacturer's protocol (see Appendix I.8). Transformations were plated out on 140mm diameter LB agar plates (100µg/ml ampicillin) according to standard protocols (see Appendix I.10). Optimum volumes were established by plating out test plates (1µl and 10µl) prior to plating out whole transformations. The plates were then incubated for 16hrs (overnight) at 37^{o}C. After incubation, 132mm filters for colony lifts (Boehringer Mannheim) were laid onto the plates, and left for 5 minutes. Transfer membranes containing colony spots were gently lifted from the plates and laid onto fresh LB agar plates (100µg/ml ampicillin) colony side up, and grown up overnight at room temperature. Standard protocols were used to lyse colonies, and DNA was fixed to the membrane by baking for 2 hours at 80°C. Filters were probed identically as filters from the enriched library, but using the most common di-nucleotide repeat probes $(GT)_{10}$ and $(CT)_{10}$ following standard protocols (Sambrook e*t al.,* 1996) (see Appendix I.11).

A total of approximately 4,000 clones were produced and screened. Positive recombinants were then selected for sequencing, and primers designed where possible, following the methods described above (Table 3.7 contains details of the primers designed).

Table 3.8, below, describes the number of positive recombinants, number sequenced, and number of primer sets designed. 132 recombinants gave a positive

Table 3.7: Microsatellite primers designed from the random screening method

Locus	Primer sequences, 5' to 3'
STPFL027	F GGTGACGAACTGACCCAACACA
	R AGTTCGGGCTTTGACATCTGCT
STPFL028	F ACCGGAGGAACTCGCTTTTTAAAG
	R GATGCCTGCTGACATGAACATATA
STPFL029	F TTTATCTAGCAACACCTCAATG
	R GGTCCTAGATCAAAGGAATCTT

signal, and 92 of these were transferred to a 96 well micro-titre plate containing LB broth (100µg/ml ampicillin), and grown up overnight at 37°C.

Table 3.8: Numbers of microsatellites identified: Numbers of positives identified, sequenced, and primer sets designed.

	Number	% of total clones
Total positives	132	3.3
Sequenced	20	0.5
Readable sequence	18	0.45
*Significant repeat region	7	0.18
Redundancy	0	0
Suitable flanking	4	0.1
Primers sets designed	3	0.1

*Significant repeat = >7 repeat units. For those loci with suitable flanking region, primer pairs were designed assisted by Primer select (DNAStar) as for section 3.3, above.

3.4.1 Summary

Microsatellites were isolated, and primer sets were designed (see Table 3.4 for details), from both the random isolation, and from the enriched library methods. Primers were synthesised by MWG Biotech Ltd. The next section (3.5) of this

chapter is focussed on optimising amplification conditions for these microsatellites, and assessing the successful candidates for polymorphism and heritability (i.e. 'Characterisation').

3.5 Characterisation of microsatellites

Once microsatellites have been isolated for a particular species, it is necessary to carry out optimisation reactions, and assess primer success and polymorphism for each locus. Each primer set located was therefore stringently tested to deduce optimal conditions for standard PCR (including variations in annealing temperatures and $MgCl_2$ concentrations). DNA was extracted from fin clips of four European flounder using a rapid phenol-chloroform protocol based on Taggart *et al*. (1992) for Atlantic salmon optimised for use in flounder (see Appendix I.1). Fish were chosen which were wild caught and therefore unlikely to be inbred in order to increase likelihood of detecting polymorphism, if present, at each locus. Once extracted, all DNA samples were diluted to 50ng/µl for ease of assay.

3.5.1 Optimisation of PCR conditions for microsatellite loci

PCR reactions were carried out in 10µl final volumes using the following components in each reaction. The use of master mixes in primer optimisations was carried out to ensure consistency between each primer set / template sample (see below for further details).

Table 3.9: Components used in standard PCR

Component	Volume	Working concentration / amount
50ng/µl template DNA	1µl	[50ng]
10× Taq polymerase buffer (Promega)	1µl	[1×]
25mM $MgCl_2$	0.2-0.8µl	[0.5-2.0mM]
10µM dNTPs	0.13µl	[130µM]
10µM forward primer	0.75µl	[0.75 pmoles]
10µM reverse primer	0.75µl	[0.75 pmoles]
5U / µl T*aq* DNA polymerase (Promega)	0.1µl	[0.5 Units]
dH_2O	xµl	to total volume of 10µl

The use of 'master-mixes' in primer optimisations

PCR reactions were set up from two master mixes. By using master mixes (and therefore larger volumes) the likelihood of pipetting error was reduced. Also master mixes allowed any mistakes made in setting up a reaction, or degraded / contaminated samples, to be identified more easily (e.g. if a single DNA sample yielded no product over several loci, it was deduced likely that the DNA sample was at fault).

Master mix one contained: DNA, dNTPs, 10× *Taq* polymerase buffer, and dH_2O to a final volume of 5µl per reaction.

Master mix two contained: Primers, $MgCl_2$, *Taq* polymerase, dH_2O to a volume of 5µl per reaction.

Volumes for each master mix were calculated by multiplication of the required components (see Table 3.9 above), by the number of reactions + 5% (to compensate for any pipetting error). By varying amount of $MgCl_2$ contained in 'Master mix two'

it was possible to optimise for $MgCl_2$ concentrations. All reactions were carried out at four different $MgCl_2$ concentrations (0.5, 0.8, 1.0 and 1.5mM) initially. $MgCl_2$ concentrations were optimised for each individual locus and further refined where necessary. Cycling parameters were: initial denaturation at 96°C for 3mins, then 32 cycles of: denaturation at 95°C for 50s, annealing at the *appropriate temperature for 50s, and extension at 72°C for 70s, followed by a hold at 4°C.

*Deduced annealing temperature, first estimated from Equation 3.1.

$69.3 + 0.41 \times \%\ GC\ content - (650/length\ (bp)) = Estimated\ primer\ T_m$

Equation 3.1: Primer T_m calculation (from Sambrook, 1996)

Optimum annealing temperatures and $MgCl_2$ concentrations for each primer set were deduced according to the point of production of a single band of expected size when run on an agarose gel. Optimum annealing temperatures were later deduced using a gradient thermocycler (Biometra Tgrad) which allowed testing several temperatures simultaneously. Examples and explanations of two optimisation gels are provided below (Figure 3.5 is an example of variation caused by $MgCl_2$ concentration, and Figure 3.6 is an example of product production caused by varying annealing temperature).

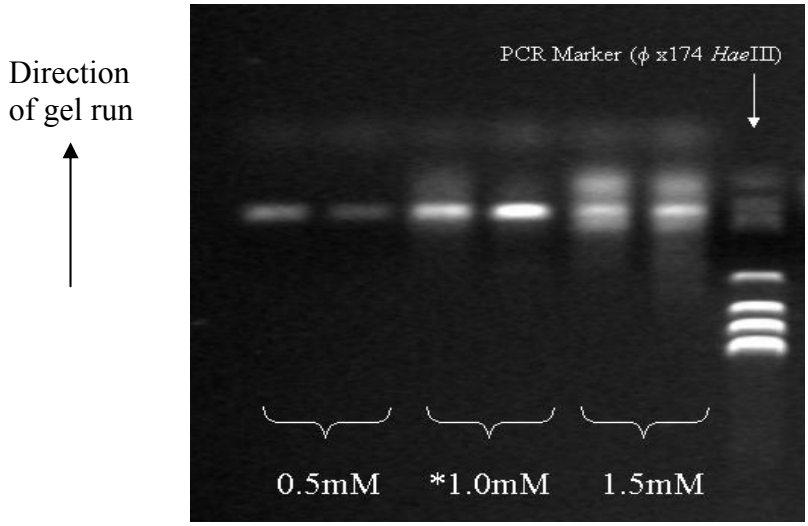

Figure 3.5: An agarose gel showing PCR products of locus STPFL014 of two adult flounder DNA samples at a single annealing temperature as an example of an optimisation gel showing the variation in product production caused by $MgCl_2$ concentration. Deduced optimum (tightest band of expected size) is highlighted (*). Note: as $MgCl_2$ increases, product specificity decreases.

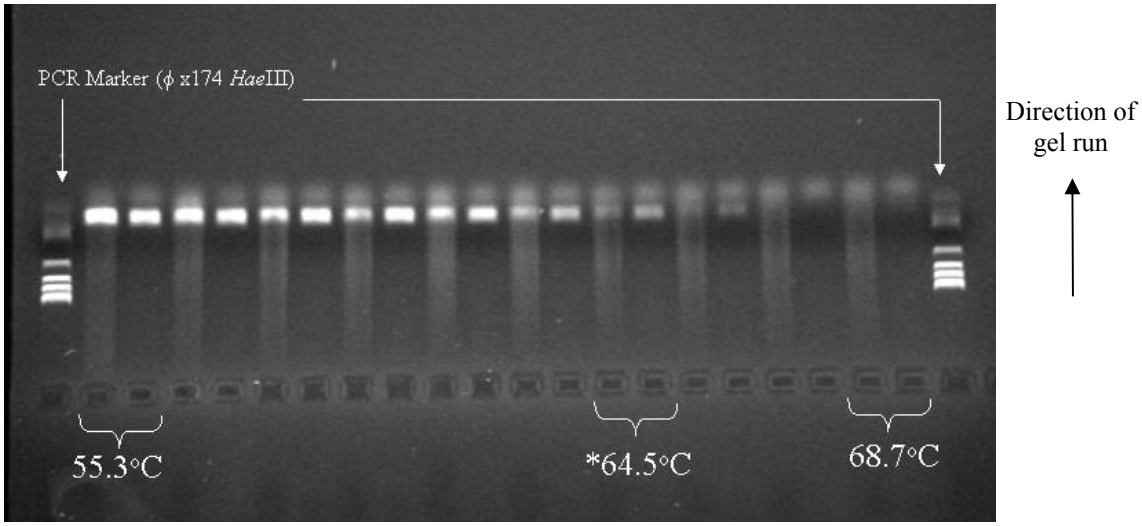

Figure 3.6: Locus STPFL002 as an example of an optimisation gel on agarose showing the variation in product production caused by annealing temperature changes. Temperature is low to high from left to right at roughly 1.2°C intervals. Each temperature is replicated twice (2 different DNA samples). Note: optimal temperature is highest temperature at which product is produced (to minimise possibility of non-specific primer binding). The optimum deduced Ta for this locus, 64.5°C, is highlighted (*).

3.5.2 Optimisation of PCR conditions for microsatellite loci – isotopic PCR

Once a single product (band) of expected size was consistently amplified, each locus was further tested using isotopically labelled primers to resolve potential allelic variability. As T4 PNK buffer contains a relatively high level of $MgCl_2$, and due to the increased sensitivity and resolution of detection levels attainable with polyacrylamide gel electrophoresis and isotopic detection, additional optimisation of PCR reactions with isotopically labelled primer was occasionally necessary. Primers were end-labelled according to standard protocol (as detailed below). Sufficient isotope (γ-^{32}P ATP (4000 Ci / mmol; 10μCi /μl) was added to primers to give ≈12% labelling, and the labelled primer mix was used directly in subsequent PCR

reactions. Components were added to a screw-cap microfuge tube in the order as follows:

Component	Volume	Concentration / amount
10 × T4 PNK buffer	1µl	[1×]
10µM primer	1µl	[1 µM]
5U / µl T4 PNK	0.1µl	[0.5 units]
γ-labelled ATP (4000 Ci / mmol; 10µCi /µl)	yµl	1.2 pmol
dH_2O	xµl	to final 10µl

The tube was then incubated at 37ºC for 45-60 min, followed by a 4 min denaturation at 90ºC to inactivate the PNK. Immediately after denaturation, the tube was transferred to ice, and centrifuged briefly to 'spin down' condensation. The labelled primer was then added to a standard PCR reaction, and upon completion of PCR, 2µl of formamide loading dye (Appendix IV) was added and the reaction denatured for 5mins at 95°C. 1-2µl of each reaction was then loaded onto 6% denaturing polyacrylamide sequencing gels, and visualised via autoradiographic film.

Once optimisation was complete, DNA samples from four different individuals were amplified to check potential polymorphism. Products were run on 6% denaturing polyacrylamide sequencing gels according to standard protocols (see Appendix I.12) and genotyped manually. Where a single non-variant band was observed in all individuals, the locus was scored as monomorphic. Where one or two bands were observed in a single individual which appeared to follow Mendelian and Hardy-Weinberg (HW) proportions, loci were scored as polymorphic. The number of alleles present was recorded, and further samples were included in order

to calculate levels of heterozygosity of each locus. Figure 3.7 (below) is an example of an autoradiograph of an optimised locus, Stpfl1001 exhibiting polymorphism.

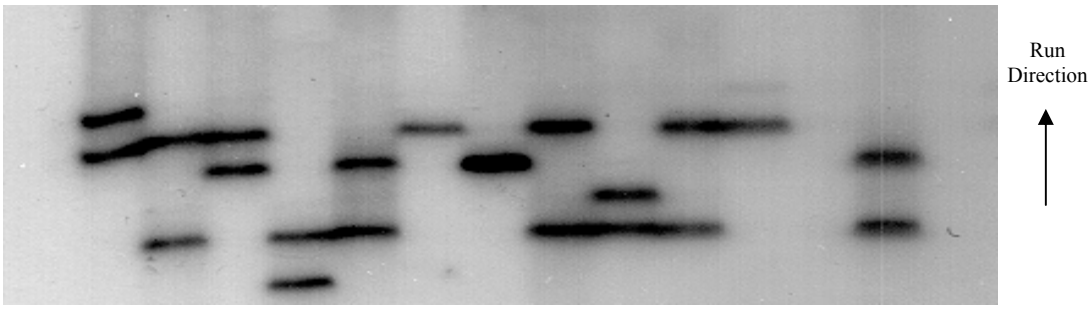

Figure 3.7: Autoradiography of locus STPFL001. PCR was carried out using isotopically labelled (^{32}P) primers, and products resolved on a 6% denaturing polyacrylamide gel following electrophoresis for 1.5 hours. The products demonstrate six clear alleles in twelve individual fish, and serve as an example of a polymorphic microsatellite

Data following optimisation of each locus including microsatellite repeat motifs found in each clone, deduced annealing temperatures and magnesium concentrations, allele size range, number of alleles at each locus are presented below in Table 3.12. Observed and expected (Nei, 1987) heterozygosities were calculated using 'Microsatellite tools' (Park, 2001) in MS Excel®, and are also presented. NB: All primer sets which amplified product are presented in the table. Those loci which were found to be monomorphic in this study may prove valuable in a larger scale population analysis, or similar study (full sequence details are in the GenBank database available at http://srs.ebi.ac.uk). Those which showed multiple or faint bands may be of use if alternative primers were designed

Table 3.10: Number of primer sets designed, number which successfully amplified correct size of product, number polymorphic, and number *finally employed in study.

	Number
Primer sets designed	29
Successfully amplified loci	24
Polymorphic loci	6
Finally used in study	3

*Reasoning provided in the discussion (current chapter).

Once characterisation was carried out, all loci were submitted to GenBank (via Webin at http://srs.ebi.ac.uk/) and Accession numbers were returned (presented in Table 3.11, below). Loci are to be published in Molecular Ecology Notes (Dixon, Taggart and George, in prep).

Table 3.11: GenBank accession numbers

Locus Name	Accession Number
StPfl001	PFL315970
StPfl002	PFL315975
StPfl003	PFL315971
StPfl004	PFL315973
StPfl005	PFL315974
StPfl006	PFL315972
StPfl007-29	AJ538305-27

Table 3.12: PCR primer sequences, repeat motif of original clones, optimised PCR conditions (Ta (^{o}C) and $MgCl_2$ (mM)), polymorphism and heterozygositiesof 26 European flounder microsatellite and minisatellite loci. NB Loci used in current study are highlighted

Locus	Repeat motif of original clone	Ta (^{o}C)	$MgCl_2$ (mM)	*Allele size Range (bp)	No. of alleles (10 individuals)	Heterozygosities Expected (H_e)	Observed (H_o)
StPfl001	$(GT)_6(N)_{12}(CTAT)_{10}(CT)_4AT(CT)_3\ (N)_{10}(CTAT)_3$	57	1.0	235-340	10	0.86	1.0
StPfl002	$GTCT(GT)_4(N)_8(CTGT)_7$	64	1.0	147-154	3	0.63	0.9
StPfl003	$(CCAT)_2CCACCAT(CTAT)_4(CCAT)_3CTAT(CCAT)_4$	64	1.0	163-175	4	0.62	0.8
StPfl004	$(CT)_{18}$			146	4	0.69	0.6
StPfl005	$(GT)_5$ TAG $(GT)_{10}$	-	-	113	6	0.86	0.8
StPfl006	$(CTAT)_{36}$	60	1.0	257 to >500	18	0.97	1.0
Stpfl007	$(GT)_8(GA)_8$ CAGA CG $(CAGA)_{10}$			323	Unoptimised		
Stpfl008	$(TAGA)_8(GGAT)_2(N)_{18}(GGAT)_2$ $(AT)_2(GGAT)_3$	58	1.0	278	Monomorphic		-
Stpfl009	$(GATA)_{11}GACA(GATA)_3GAGA(GATA)_6(N)_{14}(GATA)_2GACA(GATA)_{17}$	55	1.0	286	Not resolvable		-
Stpfl010	$(CTGT)_{19}$	-	-	142	Not resolvable		-
Stpfl011	$(CTAT)_{11}(N)_{14}(GTCT)_9(GT)_2(GTCT)_2$			208	Unoptimised		
Stpfl012	$(GGAT)_4$ GAAT $(GGAT)_2$			117	Unoptimised		
Stpfl013	$(CAGA)_5(GA)_2$ CAGG $(CAGA)_2$	58	1.0	140	Monomorphic		-
Stpfl014	$(CAGA)_5$ N8 $(CAGA)_2$	58	1.0	225	Monomorphic		-
Stpfl015	$(GACA)_7$			132	Unoptimised		

Locus	Repeat motif of original clone	Ta (°C)	$MgCl_2$ (mM)	*Allele size Range (bp)	No. of alleles (10 individuals)	Heterozygosities Expected (H_e)	Observed (H_o)
Stpfl016	$(GATA)_{10}$ GTTA $(GATA)_{10}$	58	1.0	174	Monomorphic		-
Stpfl017	$(GATA)_{17}$ N_{23} $(GATA)_5$	-	-	331	Unoptimised		
Stpfl018	$(GACA)_{12}$	58	1.0	121	Monomorphic		-
Stpfl019	$(GCT)_{14}$ GCG $(GCT)_4$	-	-	231	Multiple bands – unoptimisable		-
Stpfl020	$(CA)_3$ AA $(CA)_{30}$	-	-	256	Unoptimised		
Stpfl021	$(CAT)_2(CCT)_2(CAT)_7(CCT)_4$	-	-	98	Monomorphic		-
Stpfl022	$(CTAT)_{12}$	-	-	194	Multiple, but not resolvable		-
Stpfl023	$(CA)_{18}$ TA $(CA)_3$	-	-	123	Unoptimised		
Stpfl024	$(CCT)_6$	-	-	125	Unoptimised		
Stpfl025	$(GT)_{20}$	-	-	150	4		0.60
STPFL026	$^m(CCCTCTGTCTGT)_{16}$	-	-	398	Unoptimised		

^{m}Minisatellite repeat

*Where allele size range is quoted, those loci with only one value were monomorphic when assayed in this study, and the original clone size is given. For those loci which are polymorphic, but have only a single value in this column, this indicates that no sizing of product was carried out when products were run on radiolabelled sequencing gels due to problems in optimisation etc, and original clone size is given. Ta and $MgCl_2$ are deduced optimums from this study. where no data are included, perfect optimals were not achieved.

3.6 Verification of microsatellites

3.6.1 Sequence analysis

Three microsatellite loci were selected as suitable for use in this study due to adequate polymorphism in parental fish. These three loci were then re-tested via sequence analysis to verify that the desired target was being amplified. DNA from 4 individuals was amplified by PCR using the three sets of primers designed for each of the three loci in use for microsatellite analysis. Each DNA sample used was selected for sequence analysis on the basis that they were homozygous at the locus in question to allow direct sequencing of PCR products. PCRs for direct sequencing were carried out in 20µl volumes using the components and thermocycles described in section 3.5, above.

Each reaction was then purified using GFX gel band and PCR purification kits (Amersham Pharmacia, UK) according to the manufacturer's protocol, and eluted in 30µl of dH_2O. One µl of each purified PCR reaction was run on a gel and quantified relative to known standards (ϕX174*Hae*III 100ng and 50ng). The appropriate volume of each was then used as template in a cycle sequencing reaction, and sequenced on an ABI PRISM 377™ Automated DNA sequencer (PE Applied Biosystems) (see Appendix I.13.2). All microsatellites which were sequenced confirmed that the amplification product matched that of the original clone. By sequencing the microsatellites, confirmation that size polymorphism was due to addition / loss of complete repeat motifs as expected was also obtained from locus STPFL002 (see Figure 3.8, below).

```
Stpfl002A   TGTATTGATATATGAGAGGGGGACCCGATCAAGTTGTAGTCATGTCTGTGTGTGTTAGTG 60
Stpfloo2B   TGTATTGATATATGAGAGGGGGACCCGATCAAGTTGTAGTCATGTCTGTGTGTGTTAGTG 60
            ************************************************************

Stpfl002A  CTGCTGTCTGTCTGTCTGTCTGTCTGTCTGT----TCTACTCGCTGACAGTGGACCTGCA 116
Stpfloo2B  CTGCTGTCTGTCTGTCTGTCTGTCTGTCTGTTCTACTCGCTGACAGTGGACCTGCA 120
           ******************************----************************

Stpfl002A  TTGTTGCTTATCCTGTTTGCCAGAAGTTGCACCCAAAATACTGCCAGGAGGGCAACACGC 176
Stpfloo2B  TTGTTGCTTATCCTGTTTGCCAGAAGTTGCACCCAAAATACTGCCAGGAGGGCAACACGC 180
           ************************************************************

Stpfl002A  CGAGAGGAGAAATGTGCTTCTACATGTGCTTCTACATGTGCTT 219
Stpfloo2B  CGAGAGGAGAAATGTGCTTCTACATGTGCTTCTACATGTGCTT 223
           *****************************************
```

Figure 3.8: A ClustalW alignment of locus STPFL002 as an example of repeat unit difference between microsatellites proved by direct sequencing of PCR products from two homozygous individuals. Note the addition of one 'CTGT' repeat unit in allele B.

3.6.2 Inheritance studies

DNA was extracted from the larvae of four known families based on a rapid incubation extraction, as detailed below. Larvae were obtained from the original IoM × IoM crosses produced (see Chapter 2), and were stored in 99% ethanol. Parental samples were also obtained, and DNA was extracted from fish tissue using the optimised flounder extraction described in Appendix I.1.

Rapid extraction of fish larval DNA

As larval fish were extremely small (≈ 2mm in length- sampled at three to four days post hatching prior to mixing in communal tanks) a separate DNA extraction

protocol was required to minimise handling steps, and prevent loss of material. A single-step protocol was used therefore adopted as detailed below:

Extractions were carried out in a 0.2ml PCR tube (or 96 well plate for high throughput). Fifty µl of TE, pH 8.0 and 2.5µl of 20mg/ml proteinase K were added to each tube. A single larva was blotted on tissue to remove excess ethanol, then added to each tube. The above mixture was incubated for 2 hours at 65^{o}C, then the proteinase K was denatured by incubation at 95^{o}C for 10 minutes. One µl of the above extraction was found to be sufficient as template in a 10µl PCR reaction.

Inheritance of microsatellites

Between 10 and 18 offspring from each of four known families were genotyped at the three loci isolated, which were suitable for parental analysis, and results compared with parental samples. These data are presented below in Table 3.13, Table 3.14 and Table 3.15 for locus STPFL001, STPFL002 and STPFL003 respectively, and tables following for heterozygosities.

Table 3.13: Locus STPFL001: Offspring no. / genotype for 4 families

Family / No.	**Parent**	**Parental genotype**	**Possible offspring genotypes / No. of offspring in category**			
Family 1	♂	248 290	244 248	244 290	248 290	248 248
N=10	♀	244 248	2	6	1	1
Family 2	♂	248 260	248 252	252 260		
N=12	♀	252 252	5	7		
Family 3	♂	248 253	248 253	248 260	248 248	253 260
N=17	♀	248 260	4	6	4	3
Family 4	♂	260 266	260 256	260 260	256 266	260 266
N=15	♀	256 260	5	4	4	2

Table 3.14: Locus STPFL002: Offspring no. / genotype for 4 families

Family / No.	Parent	Parental genotype	Possible offspring genotypes / No. of offspring in category			
Family 1	♂	146 153	144 146	144 153	146 153	146 146
N=11	♀	144 146	2	4	2	3
Family 2	♂	146 153	144 146	144 153	146 153	146 146
N=12	♀	144 146	4	1	3	4
Family 3	♂	146 148	144 146	144 148	146 153	148 153
N=17	♀	144 153	4	2	6	5
Family 4	♂	148 153	148 153	153 153		
N=18	♀	153 153	6	12		

Table 3.15: **Locus STPFL003: Offspring no. / genotype for 4 families**

Family / No.	Parent	Parental genotype	Possible offspring genotypes / No. of offspring in category		
Family 1	♂	166 170	166 166	166 170	170 170
N=11	♀	166 170	4	5	2
Family 2	♂	162 166	162 162	162 166	166 166
N=12	♀	162 166	3	6	3
Family 3	♂	167 167	165 167	167 167	
N=17	♀	165 167	2	15	
Family 4	♂	165 167	165 167	167 167	
N=18	♀	167 167	2	16	

All offspring from known families matched up to parental samples, and no spurious alleles were detected (see Appendix II). This proves that in these four families, the three microsatellite loci are inherited. Most of the loci conformed to Mendelian

segregation, however there were exceptions to this (see below). Chi-squared probabilities of the above are presented in Table 3.16 below.

Table 3.16: Chi-squared probabilities (P) of allele frequencies to indicate probability of the particular combination of alleles, including locus data and family group.

Locus	Family	P-value (χ^2)
STPFL001	1	0.08
	2	0.56
	3	0.77
	4	0.74
STPFL002	1	0.80
	2	0.57
	3	0.56
	4	0.16
STPFL003	1	0.66
	2	1.0
	3	0.002
	4	0.001

Most values were within an acceptable range, however at locus STPFL003 families three and four returned extremely low results ($P<0.01$), indicating that these results were not by chance (discussed in section 1.7).

3.7 Discussion

This study successfully reported the isolation and characterisation of a set of novel microsatellite loci in the European flounder, for which (at time of writing) there are no other data published. In addition to the loci which were polymorphic in the individuals tested in this study, monomorphic loci are valuable for future studies,

and this is discussed further below. Although the enrichment methodology was successful, there are several limitations to using such a technique.

Redundancy of microsatellites

Three types of redundancy were encountered with loci during this study:

i) lack of suitable flanking regions surrounding the repeat unit (i.e. redundancy due to lack of primer sites);

ii) multiple cloning of a single locus (including multiple alleles of a single locus.

iii) presence of flanking region, but lack of amplification of locus

i) Although relatively low in this study, the increased rate of redundancy generated by enrichment techniques could be the result of several factors. By selecting (enriching) for known regions, similar regions in the flanking area may also be preferentially selected. If these flanking regions bind to any of the oligonucleotides (e.g. a high percentage of 'C' and 'G' nucleotides is found in the flanking regions which enriching for e.g. a 'GC' repeat, or the repeat exhibits a compound format around the main microsatellite as has been found in this study with several loci), they are likely to cause a higher degree of redundancy, as primer sites are difficult to find in repetitive elements. A much higher percentage of microsatellites obtained using the enrichment method contained no suitable primer binding sites compared to the microsatellites obtained in the random cloning approach (although the number of loci isolated was limited in the latter, and may be biased). An alternative approach using 6bp cutting REs may increase flanking region, and would be an area worth exploring further (McGowan and Reith, 1999). Another possible cause of redundancy could be due to carry over of the oligonucleotides used in enrichment,

which could act as primers in initial PCR steps. This could lead to microsatellites being amplified from the centre of the repeat region, and thus leaving no flanking DNA from which to design primers. This problem could be overcome by increasing stringency of reaction washes to further remove any excess probe oligonucleotide.

ii) The second type of redundancy involves multiple cloning of a single locus (or multiple alleles at the same locus). As PCR can lead to an increased bias in the final product through favourable amplification of certain fragment sizes, any redundancy which may be present in the original sample would be amplified exponentially. One suggestion to counter this high level of redundancy would be to begin the initial selection on a much larger quantity of DNA, and reduce the number of PCR steps involved. However, this would require very large quantities of genomic DNA, and although not a problem when isolating microsatellites from higher animals, this approach could prove problematic when working on small organisms. A cause of multiple cloning of the same locus could also arise from multiple copies of a locus being present in the genome (Rallo, Dorado and Martin, 2000). Small genome size or low numbers of microsatellites in the genome are also linked with an increased redundancy of this type (Zane *et al.,* 2002). The relatively low abundance of redundancy in the this study, may suggest that the flounder genome has a high level of microsatellites.

iii) A third type of redundancy involving lack of amplification of a locus at which there is suitable primer bind region was encountered in several loci (e.g. Stpfl009). This may be due to the primer design process, however it may also be caused by concatamer formation (i.e. two insert sequences, from unrelated areas of the genome, joined together during ligation, and ligated into the plasmid construct appearing as a single insert) during the ligation process (Zane, Bargelloni and

Patarnello, 2002). If primers were designed from such a sequence, then one primer will bind to one area of the genome, and the other to an entirely separate area, and therefore no (double stranded) product would be produced. Alternative primer pairs were designed for some loci and no amplification product was detected, which may indicate that concatamer formation had taken place for these constructs. Rather than pursue these loci during the course of this study, other loci were investigated as an alternative.

Polymorphism among loci

The level of polymorphism obtained from loci in this study has been found to be relatively low when compared to other such studies in fish species, however a low level of polymorphism has also been found in markers isolated from other flatfish species including the closely related plaice (pers. comm.. P. Watts, University of Liverpool). Many of the loci isolated which turned out to be monomorphic had many highly repetitive motifs (e.g. Stpfl016, $(GATA)_{22}$, and Stpfl018, $(GACA)_{12}$), and it was surprising to find such a lack of variability, as tetra-nucleotide loci that have in excess of 10 repeat units are usually found to be polymorphic (Cairney *et al*., 200). Part of this reason may be due to an inherent low level of genetic variation within flounder from the areas which we tested, and low levels of polymorphism have also been documented in loci isolated in the closely related plaice (pers. comm. P. Watts, University of Liverpool). However, it must be appreciated in this study that levels of polymorphism were estimated from relatively few fish from only two geographic locations. Heterozygosities tested in the offspring raised from the 1999 fish breeding trials revealed a significant divergence from Mendelian inheritance at one locus (Stpfl003). This may have been a result of the families being raised from

a mixture of brood stock fish bred for several generations in captivity, and a decrease in genetic variability has been described in several species of farmed fish in previous studies (Coughlan, Imsland, Galvin, Fitzgerald, Naevdal and Cross, 1998). However the findings were more likely to be due to the small sample sizes tested in the trials, as heterozygosities were within acceptable levels in other families.

Further polymorphism (in some of the loci identified as monomorphic in this study) has been found in other studies: loci isolated in this study are currently being employed in population genetic studies of Northern European flounder populations in Denmark (pers. comm. Jakob Hemmer-Hansen, University of Aarhus, Denmark), and also in Sweden, and thus far at least 2 of the loci listed here as monomorphic have been found to be polymorphic (pers. comm. M. Fornbacke, University of Uppsala, Sweden). As this study necessitated highly polymorphic markers due to obtaining fish from relatively geographically similar areas (Irish Sea populations) the bias toward monomorphism is probably likely to be exaggerated due to being more genetically similar populations.

All of the microsatellites found to be polymorphic in these investigations were isolated using enrichment methodologies, and all loci isolated from the random screening approach were monomorphic. Loci designed from plaice sequences were also found to be monomorphic when tested in flounder, but polymorphism was observed in plaice (data not presented).

Through direct sequencing and inheritance studies, these markers are shown to be amplified correctly, and inherited from parent to offspring in a Mendelian fashion in most of the families which were tested (two families indicated a non-Mendelian inheritance at one locus which may be due to occurrence of null alleles, reduced

genetic variation or, more probably, the small sample size available). In addition, this study isolated a number of other microsatellites which may be useful to future studies of European flounder, but were only tested for polymorphism in a limited number of individuals due to the requirements of this study (to find highly polymorphic markers for use in family assessment). These apparently monomorphic loci are also published on the GenBank database to allow their use in further studies. Three of the microsatellite loci isolated in this study (StPfl001-003) were employed in a small scale population study of flounder inhabiting UK estuarine waters, and found a significant genetic differentiation of populations of fish from at least one area tested (unpublished data and Garvey, 2001), however, due to low sample numbers these data are not presented here.

Family identification

Three microsatellite loci were informative in the experimental families used for the current study, and a further two were found to be polymorphic enough to be useful for possible application to future studies, however were not informative in the families involved in this study (Dixon *et al*., in prep). Another locus isolated in this study was highly polymorphic, with alleles ranging from 256 to >500bp in length. The locus was assayed in several flounder DNA samples using isotopic methods which detected these large alleles. This locus was tested in parental fish for the trial, and three parents had loci above 500bp, which unfortunately was outside of the range of analysis by Genescan (employed in semi-automated genotyping, maximum feasible allele size measured by Genescan marker standards employed was 500bp). Markers which allow analysis of DNA of >500bp in size are available, however they are not commonly used in microsatellite analysis, and consequently it would

prove difficult to assay markers alongside others. The three microsatellites which were informative in this study were used as tools to match offspring / parental relationships (see chapter 5.3 for details). All of these were found as a result of enrichment analysis, and all were tetra-nucleotide compound or imperfect repeats. In addition to these loci, two loci became available during the course of the study which were isolated in plaice and demonstrated amplification of flounder loci (Watts *et al.,* 2001). These loci were employed to improve resolving power. Loci designed from plaice sequences were found to be polymorphic in plaice, but not informative in the flounder, and data are not included.

Future directions

Due to the large time costs involved with screening microsatellites found in this study, it may be more effective for future studies to choose a large scale screening technique (e.g. screening clones by colony lifts, as described for the random cloning approach, as opposed to first picking the colonies into 96 well microtitre plates). Enrichment does not in itself take a vast amount of time, however the percentage redundancy is often elevated when compared to randomly cloned approaches (current study; Zane *et al.,* 2002). At the later stages of identification, sequencing and identification of these redundant clones can be highly consuming in terms of both time and consumables involved. Screening approaches using colony lifts from large agar plates instead of picking recombinant colonies into 96 / 384 well plates can also cut back on the time involved, and as long as measures are taken to ensure orientation of plates and membranes can be easily traced, and also ensuring that colonies are spread at a reasonable separation, screening times can be reduced still further. The more polymorphic tetra-nucleotide loci are around 100-200× less

common in the genome than less-polymorphic di-nucleotide repeats (O'Reilly and Wright, 1995), and isolation of these repeats therefore justifies the additional time costs involved in enrichment. According to the time constraints imposed by any study of this nature, and to increase the number of loci detected, for future directions this study would suggest an enrichment methodology followed by a large scale screening approach.

Summary

Microsatellites are applicable to numerous fields of research, and successes in isolation studies using both random and enriched library construction approaches have been widely documented. Enrichment methods have previously been presented as a method of choice when searching for rarer microsatellites (e.g. tri- and tetra-nucleotide), and the comparison of methodologies employed in this study, and several others in our laboratory, has proved this to be the case also. The apparent shift towards enrichment methodology from a random isolation approach in the literature further supports this finding, and many new methods of enrichment are continually being developed (see Zane, 2002 for a review). Tetra-nucleotide microsatellites are more polymorphic than their di-nucleotide counterparts (O'Reilly and Wright, 1995), and this study supports this finding. Imperfect and complex repeats are more common in teleost fishes than in mammals (Brooker *et al.*, 1994), and the majority of microsatellites in this study were of a complex / imperfect type.

Several polymorphic (and many more monomorphic) microsatellite markers have been isolated and characterised during this study, which are now published on the GenBank database (accessible using accession numbers listed in this chapter). Markers are employed later in this study (Chapter 5.3) as tools in parental

identification as an attempt to elucidate some of the problems associated with potential genetic variation of pollutant responses in European flounder.

Chapter 4 Preparation of probes and assay methods for study

4.1 Preparation of probes

4.1.1 Genes of the PAH metabolic pathway

Probes for investigating mRNA expression levels for the PAH metabolic pathway in the European flounder (CYP1A, UGT1B1 and GSTA) were available from within our laboratory. The CYP1A probe held in our laboratory (clone PpP450BNF2 containing the full length plaice CYP1A cDNA) demonstrates 98% homology to the flounder sequence, and has been employed in previous studies to quantitate flounder mRNA expression (Eggens *et al.*, 1996). GSTA and UGT1B1 plasmids derived from plaice have also been used in studies to quantitate flounder GSTA and UGT1B1 mRNA. Details of all clones are provided in Table 4.1 below.

Table 4.1: Probes initially available for investigation of mRNA expression levels of the PAH metabolic pathway, including references and GenBank sequence accession numbers

Gene	GenBank accession	Reference
CYP1A	X73631	(Leaver, Pirrit and George, 1993)
UGT1B1	AJ249081	(George, Leaver and Wright, 1998)
GSTA	X95200	(Leaver, Wright and George, 1997)

Insert fragments (to be used as probe) were isolated from phagemid constructs by restriction digestion (see Table 4.2 for specific enzymes used) in OPA ('one phor all') buffer (Amersham Pharmacia UK) for two hours at 37°C. Each reaction was electrophoresed on a 1% agarose gel at 4V/cm to separate the probe fragments from

the plasmid DNA, and insert DNA was then excised from the agarose with a sterile scalpel, and purified using the 'GFX Gel Band purification kit' (Amersham Pharmacia, UK).

Table 4.2: Conditions specific to each probe. Plasmid from which each probe was obtained, and restriction enzymes used to isolate probe fragments are described.

Probe	Plasmid	Restriction enzyme
GSTA	PpGSTA	*Eco*RI / *Hind*III
UGT1B1	PpUGT1B1	*Eco*RI / *Nco*I
CYP1A	PpP450BNF2	*Eco*RV / *Hind*III

4.1.2 The CYP1A transcription pathway – AhR and ARNT

Current knowledge of the CYP1A transcription factor pathway in the European flounder (AhR2 and ARNT2 genes) was discussed in Chapter 1. No probes were available in our laboratory for the AhR2 and ARNT2 genes, thus it was necessary to generate novel probes by amplifying and cloning fragments of both genes. A partial sequence for AhR2 (636bp) was reported for European flounder (Besselink, Denison, Hahn, Karchner, Vethaak, Koeman and Brouwer, 1998): GenBank accession number AF034412 and a full-length ARNT2 sequence for the teleost *Fundulus heteroclitus* (3045 bp) was reported by Powell, *et al.*, (1999): GenBank accession number AF079311.

4.1.2.1 Sequence analysis and primer design

PCR primer pairs were designed with the aid of PrimerSelect (DNAStar) at several locations in the PAS domain of the published AhR2 European flounder sequence

and also from several regions of the published *F. heteroclitus* ARNT2 sequence. (Gene diagrams of ARNT2 and AhR2 from the killifish are provided below indicating regions of interest in Figure 4.2 and Figure 4.3, respectively). Four primer pairs were designed for *F. heteroclitus* ARNT2, and three sets to *P. flesus* AhR2, to allow a choice of fragments to be amplified. Initial optimisation of PCR primer pairs was carried out on a Biometra T-Grad thermocycler using a variety of annealing temperatures in order to determine preferential annealing conditions. The following reactions were used in optimisations and components added as follows:

Component	Volume	Working concentration / amount
50ng/µl template cDNA	2µl	[100ng]
10x *Taq* polymerase buffer (Promega, UK)	2µl	[1×]
25mM $MgCl_2$	0.8µl	[1.0mM]
5mM dNTPs	0.52µl	[130µM]
10µM forward primer	1.5µl	[0.75µM]
10µM reverse primer	1.5µl	[0.75µM]
5U/µl T*aq* DNA polymerase (Promega, UK)	0.2µl	[0.05U/µl]
dH_2O	Xµl	to total 20µl

The reactions were run on the Biometra T-Grad using the following cycling parameters: an initial denaturation at 96°C for 3min, followed by 32 cycles of denaturation at 95°C for 50s, annealing at *Ta°C for 50s, then extension at 72°C for 70s, followed by a hold at 4°C (*Ta is the annealing temperature of which several were tested (55-67°C)). Table 4.3 gives details for primer pairs designed which produced products of correct target size.

Once optimal conditions were obtained for each primer set (i.e. each PCR yielded one discrete band of expected size when visualised on an agarose gel), further PCRs were carried out to give sufficient product for cloning.

Table 4.3: AhR2 and ARNT2 primers successfully used to amplify fragments from the European flounder, including amplicon regions as nucleotide locations of corresponding killifish sequences ('F' denotes forward primer, 'R' denotes reverse primer, last number denotes primer set number).

Primer sequence (5' > 3')	Primer Name	Original target sequence
ACAGCAGCAGGCAGAGTTAGAGGTTCATCA	ARNT2 /F3	1583-2085
TGTAGGAGGCCGGGTCAGTCTGGTAGTTG	ARNT2 /R3	
GTTTTATGCCTCTCCCACAATCAA	PflAHR2 /F1	440-990
TAGCCGGAGCCTTTCATACACA	PflAHR2 /R1	
TGTGTCGCTTCCGCTGCCTCCTG	PflAhR2 /F3	685-945
GATTATTTTCCCCCTGCTGTCAA	PflAhR2 /R3	

PCR was carried out on European flounder 1st strand cDNA previously prepared from mRNA (see Appendix I.3 for protocol), and products were verified as approximately the right size (approximate sizes were used to allow for any differences between the flounder cDNA amplified and the killifish sequence from which it was designed). An appropriate volume of 6× loading buffer (3.5µl per 20µl reaction) was then added to each PCR reaction, and the complete reaction was electrophoresed on a 1.0% agarose, 0.7 × TAE, 0.5mg/ml EthBr gel for 30min at 4V/cm. Gels were visualised on a UV transilluminator, and bands were excised from the gel using a sterile scalpel, making sure to remove excess agarose. DNA was purified from the gel slices using a 'GFX DNA and Gel Band purification kit' (Amersham Pharmacia) according to the manufacturer's protocol.

Once purified, 5µl of the eluent was used as a template in a direct PCR sequencing reaction using Big Dye™ Terminator Cycle Sequencing Ready Reaction mix (PE

Applied Biosystems) using the appropriate forward and reverse primer to sequence in both directions (see Appendix I.13.2 for protocol). Sequences were then aligned using Autoassembler and combined to produce consensus sequences, which were then subjected to a BLAST search (BLASTN Version 2.2.1) (Altschul, *et al*., 1997) and verified as the correct products.

4.1.2.2 Cloning of AhR2 and ARNT2 fragments

Sequence verified products were ligated into prepared 'T' vector suitable for cloning PCR fragments as described below. 'T' vector (i.e. vector with a 'T' overhang) is suitable for direct cloning of adenylated PCR products which naturally occurs during PCR using certain polymerases (e.g. *Taq*) (e.g. Marchuk, Drumm, Saulino and Collins, 1991). T vector was produced by setting up the following reaction:

Component	**Volume**	**Working concentration / amount**
500ng/µl *Eco*RV cut pBSII KS-	1µl	[50ng/µl]
10× *Taq* polymerase buffer (Promega, UK)	1µl	[1×]
25mM $MgCl_2$	0.4µl	[1.0mM]
10mM dTTP	0.2µl	[200µM]
5U/µl T*aq* DNA polymerase (Promega, UK)	0.1µl	[0.05U/µl]
dH_2O	xµl	to 10µl final volume

The reaction was then incubated at 72°C for 15min, and placed on ice. Vector was then purified using a GFX gel band and DNA purification kit according to the manufacturer's protocol.

Ligation of fragments into vector

cDNA PCR products were ligated into the prepared vector according to the following procedure at an insert:vector ration of 2:1. Reactions were set up to contain:

Component	Volume	Working concentration / amount
5× T4 DNA Ligase Buffer	4µl	[1×]
Insert DNA	xµl	[xng]
50ng/µl T-Vector	1µl	[50ng]
High conc. T4 DNA Ligase	1µl	[20U]
dH_2O	yµl	to 20µl final volume

The reaction was incubated overnight at 15°C in a water bath, and products purified the following day using a QIAquick PCR clean up kit (QIAgen).

Transformation of 'Top 10' competent cells

The purified phagemid constructs (ligation reaction products of vector + insert) were then used to transform 'Top10' competent cells according to standard protocols (see Appendix I.9 for full protocol) in order to grow up plasmid stocks. Aliquots of the transformations were plated out according to standard protocols on LB agar (100µg/ml ampicillin) plates, utilising IPTG and X-Gal for blue/white colour selection (see Appendix I.10 for protocol). The remainder of the transformation was frozen down at –80°C in 15% glycerol as a stock.

Colony selection

The plates were transferred to a refrigerator after over-night growth to allow full development of colour (blue/white selection). White colonies (i.e. those containing plasmid with insert) were selected, and transferred to universals containing 5ml of

LB Broth (100µg/ml ampicillin). The broth preparations were incubated overnight at 37°C in a shaking incubator at 250RPM.

Plasmid preparation

Plasmid DNA was purified from the colony preparations using 'GFX column plasmid preparation kits' (Amersham Pharmacia, UK). Once plasmid constructs were purified, standard PCR reactions using vector specific T3 and T7 primers were carried out to check whether the correct size of inserts were present in each plasmid. Table 4.4 gives details of the three clones chosen as the most suitable candidates.

Table 4.4: Details of AhR2 and ARNT2 phagemid constructs – Clone name is given, and data for original target fragment, expected size of fragment (based on original clone) and actual size of fragment (cloned from *P. flesus*) are provided.

Clone Name	Original target fragment	Expected size of fragment (bp)	Actual size of fragment deduced from sequence
ARNT2 (3A)	*F. heteroclitus* ARNT2	550	510
AhR2 (1E)	*P. flesus* AhR2	550	550
AhR2 (2A)	*P. flesus* AhR2	269	269

All construct PCRs gave a product equivalent to the expected size of fragment. Primers were then tested using both plasmid and cDNA templates (Figure 4.1), AhR2 (2A) appeared to exhibit additional bands with amplification from cDNA indicating possible non-specificity of the primer set, and was therefore excluded from further analysis.

Those clones with the correct size of insert were then sequenced on an ABI PRISM 377™ Automated DNA Sequencer using the vector specific T3 and T7 primers (Appendix I.13.1). Figure 4.2 and Figure 4.3 below are diagrammatical

representations of the ARNT2 and AhR2 gene structures, respectively, showing primer bind regions for the successfully amplified fragments.

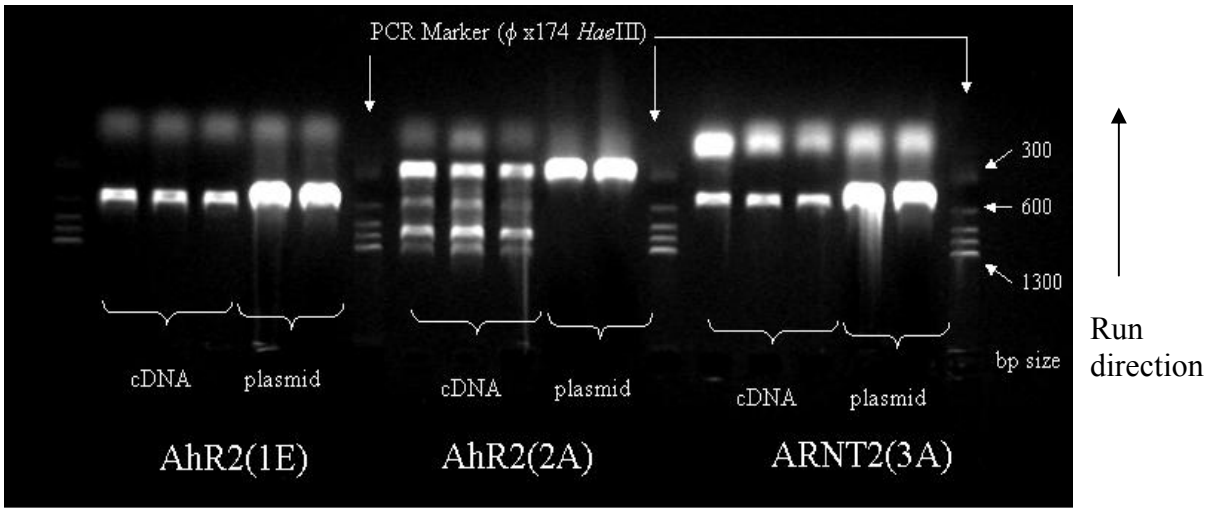

Figure 4.1 Agarose gel image of PCR products of primer sets using plasmid and cDNA templates. Names given below picture are primer set names which correspond to clone names. Note additional bands present using primer set AhR2(2A). PCR marker (ϕx174 *Hae*III) is included, and approximate sizes indicated on the right hand side.

ARNT2 sequence

The ARNT2 sequence was submitted to GenBank (Acc. No. AJ518832), and is presented below (Sequence 1). Note locations of Reverse primer and Forward primer (complement).

Sequence 1: *P. flesus* ARNT2 partial clone (5' to 3') – primer sequences are highlighted

TGTAGGAGGCCGGGTCAGTCTGGTAGTTGTGGCCAGAACCAATGCCAAATGGACTGGACTGGGCTTT
ACTGGACTGTCCCGAGAAGGGGGGTCTGTTGCCGGACCATGCAACCTGGCCTGGATTGAGCTGCCTA
GATATCTGAGCCAGATTCTGATTGGATGAACCTGATGGCTGGATATCATTCACACCAGGGACGTGGA
TCACAGATGAACTGAAGCTTTTGCCAGAGTGTCCTTGCTGGAAAGGCGAGCCCTGGGAGTAGAGCTG
CTGTCCTCCAGCTGTGGAGGAGGGAACCATCATTTTCTTATCTCCCGAGCCAGAGATGCCGGTGTAC
ATCTCAGCAAAGCGTGGGTCTCTCCCCTGGGAGAACAGAGCCTCCGTCTTGTCGATGTTCTTGCCTG
CCTCATGGACTCCACTGCTGACGCTGGCCACAGGAACCTGGGACAGATCGTAGGCAGTTAGTCCATC
TCTCTGATGAACCTCTAACTCTGCCTGCTGCTGT 510 bps

Homologies of the sequence to published data following a BLASTN (nucleotide-nucleotide query) and a BLASTX (nucleotide query translated and compared to protein database) search are presented in Table 4.5 and Table 4.6, respectively.

Table 4.5: Homology of *P. flesus* ARNT2 partial cDNA cloned in present study – results of BLASTN search for nucleotide homolgy. Species data including identities and probability values are provided.

Species	Identities	Probability
F. heteroclitus	87% (442/503)	1.7^{e}-82
D. rerio	76% (347/455)	9.4e-57
**Mus musculus*	69% (353/506)	
**Rattus norvegicus*	70% (358/586)	5.1^{e}-44
**Homo sapiens*	61% (254/411)	3.2^{e}12

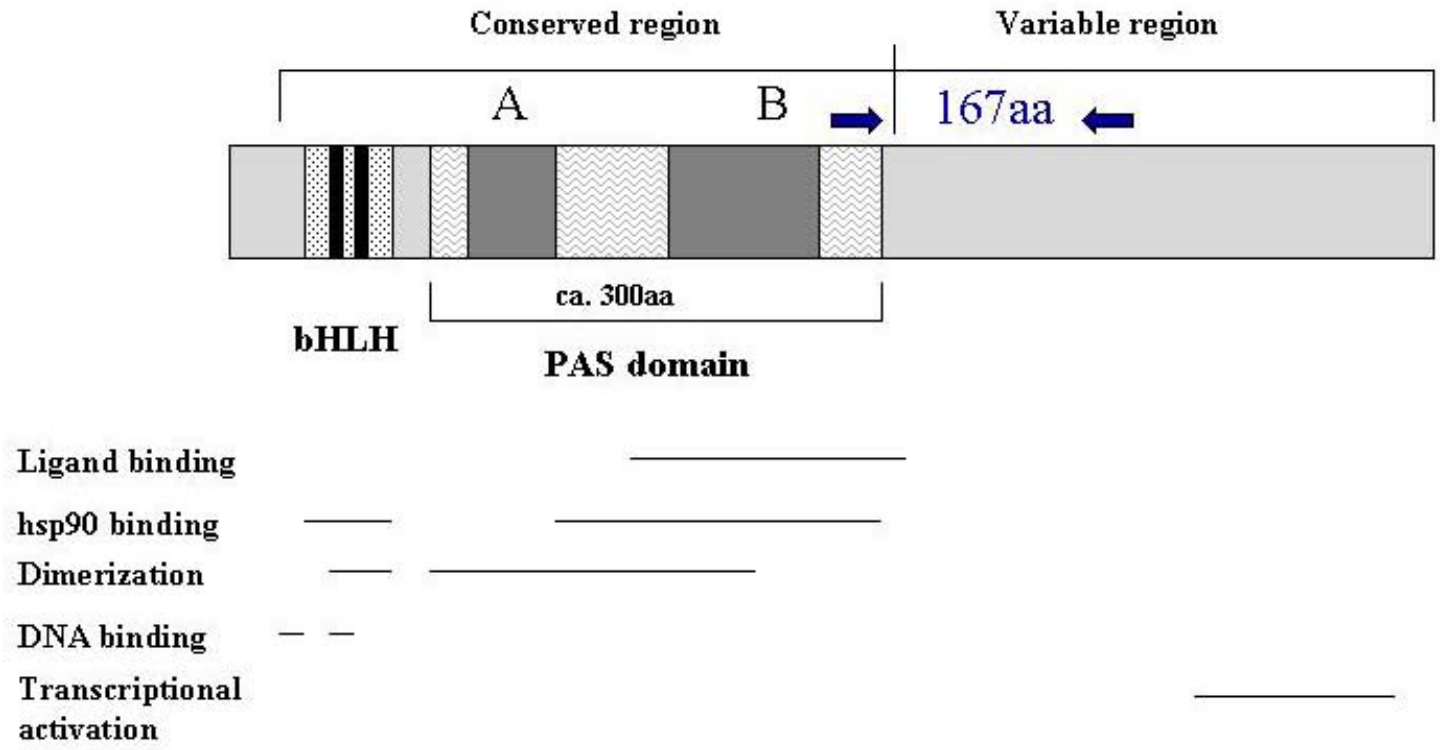

Figure 4.2: A diagram of the ARNT2 gene deduced from the *Fundulus heteroclitus* ARNT2 sequence (Powell, Karchner Bright and Hahn, 1999; Powell and Hahn, 2000. Basic-helix-loop-helix (bHLH), Per-ARNT-Sim (PAS) A and PAS B, and transcriptional activation domains are marked. Arrows denote forward and reverse primers respectively, and amplicon is area between arrows (equivalent to 167aa.s).

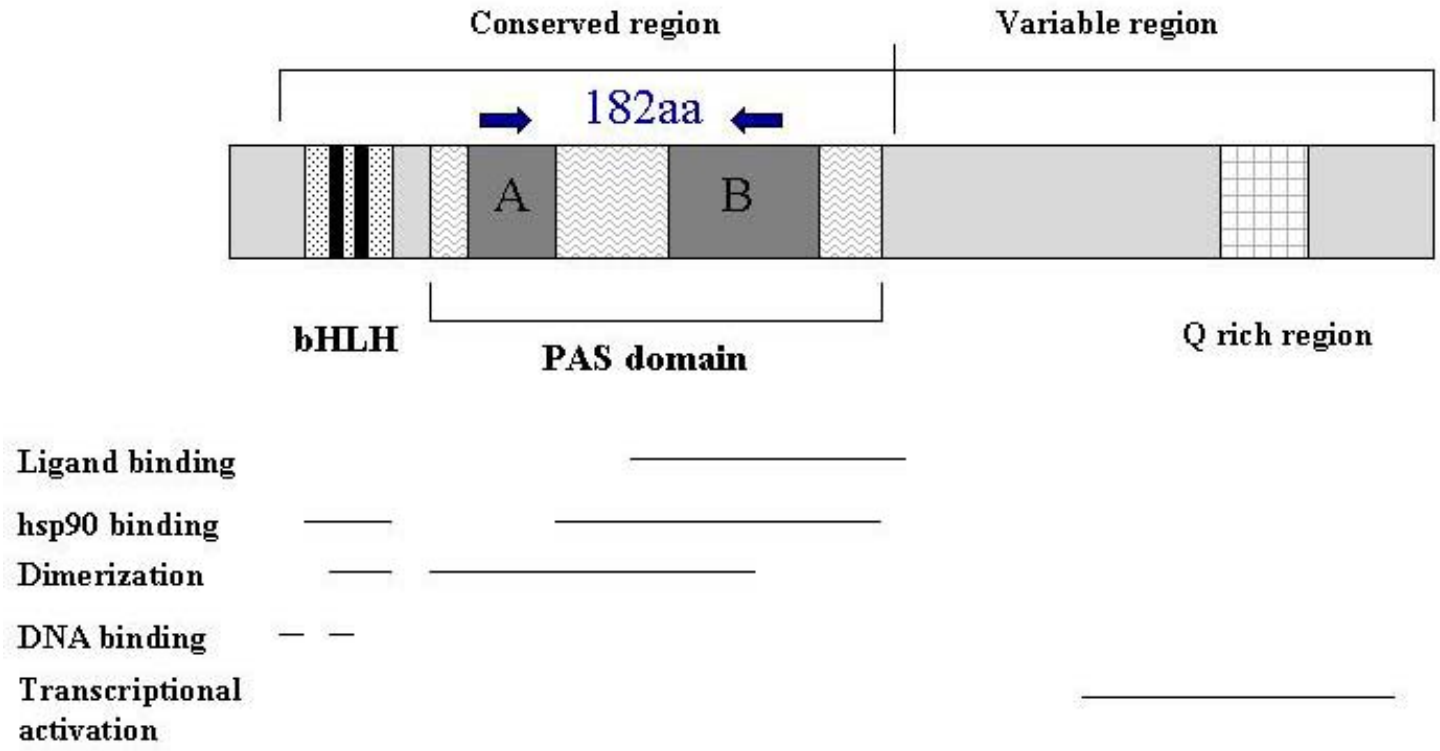

Figure 4.3: A diagram of the AhR2 gene based on the *Fundulus heteroclitus* AhR2 sequence and diagrams from Karchner, Kennedy, Trudeau and Hahn, (2000) and Kim and Hahn (20002). Basic-helix-loop-helix (bHLH), PAS A and PAS B, and transcriptional activation domains are marked. Arrows denote forward and reverse primers respectively, and amplicon is area between arrows (equivalent to 182aa.s).

Table 4.6: Homology of *P. flesus* ARNT2 partial cDNA cloned in present study – results of BLASTX search. Species data including identities and probability values are provided.

Species	Identities	Probability	Gaps
F. heteroclitus	91% (152/166)	3e-83	-
D. rerio	80% (139/172)	3e-73	3% (6/172)
**Mus musculus*	71% (119/166)	3e-65	-
**Rattus norvegicus*	71% (119/166)	7e-65	-
**Homo sapiens*	70% (120/171)	2e-64	2% (5/171)
O. mykiss isoform a	33% (60/181)	1e-08	13% (25/181)

Translation (3' to 5', frame 2 of above sequence)

A translated sequence was aligned to published sequences (obtained from BLASTX search) using ClustalX to allow homologies to be deduced (Figure 4.4). Conservation is demonstrated by the high degree of homology to rat and human ARNT2 sequences. Further phylogenetic analysis was carried out, and an unrooted phylogenetic tree derived by Saitou and Nei's (1987) genetic distance method using ClustalW (Thompson, Gibson, Plewniak, Jeanmougin, and Higgins, 1997) and plotted using the neighbour joining method (NJplot) is presented below (see Figure 4.5). The highest degree of homology was shown to the *Fundulus* ARNT2.

```
Flounder    QQQ-AELEVHQRDGLTAYDLSQVPVASVSSGVHEAGKNIDKTEALFSQGRDPRFAEMYTG 59
Fundulus    QQQ-AELEVHQRDGLTAYDLSQVPVAGVSSAVHDTGKTIDKTETLFSQERDQRFAEMYTS 59
zebrac      QQQQAELEVHQRDGLTAYDLSQVPVSGVSAGVHESGKSIDKTESLFSQERDPRFSDIYTG 60
zebrab      QQQQAELEVHQRDGLTAYDLSQVPVSGVSAGVHESGKTIDKTESLFSQERDPRFSDIYTG 60
mouse       QQQ-AELEVHQRDGLSSYDLSQVPVPNLPAGVHEAGKSVEKADAIFSQERDPRFAEMFAG 59
rat         QQQ-AELEVHQRDGLSSYDLSQVPVPNLPTGVHEAGKPVEKADAIFSQERDPRFAEMFAG 59
human       QQQ-AELEVHQRDGLSSYDLSQVPVPNLPAGVHEAGKSVEKADAIFSQERDPRFAEMFAG 59
            *** ***********::********..:.:.**::** ::*::::*** ** **::::::.

Flounder   ISGSGDKKMMVPSSTAGGQQLYSQGSPFQQGHSGKSFSSSVIHVPGVNDIQ-PSGSSNQN 118
Fundulus   ISGSGDKKMMVPSSTAGGQPLYTQSSPFQQGHSGKSFSSSVIHVPGVNDIQ-SSGSSNQN 118
zebrac     ISTS-EKKMMVPSSTSGGQQLYSQGSPFQPGHSGKSFSSSVIHVPGVNDIQSTAGSAGQN 119
zebrab     ISTS-EKKMMVPSSTSGGQQLYSQGSPFQPGHSGKSFSSSVIHVPGVNDIQSTAGSAGQN 119
mouse      ISAS-EKKMMSSASASGSQQIYSQGSPFPAGHSGKAFSSSVVHVPGVNDIQ-SSSSTGQN 117
rat        ISAS-EKKMMSSASASGSQQIYSQGSPFPAGHSGKAFSSSVVHVPGVNDIQ-SSSSTGQN 117
human      ISAS-EKKMMSSASAAGTQQIYSQGSPFPSGHSGKAFSSSVVHVPGVNDIQ-SSSSTGQN 117
           ** * :**** .:*::* * :*:*.***  *****:*****:********* .:.*:.**

Flounder    LAQISRQLNPGQVAWSGNRPPFSGQ-----SSKAQSSPFGIGSGHNYQTDPASY 167
Fundulus    LAQISRQLNPGQVAWSGNRPPFTGQ-----STKAQSSPFGIGSGHNYQTDPASY 167
zebrac      LSQISRQINTGQVSWSGNRPPFSGQQIPAQSNKAQSSPFGIGSSHSYQVDPSSY 173
zebrab      LSQISRQINTGQVSWSGNRPPFSGQQIPAQSNKAQSSPFGIGSSHSYQADPSSY 173
mouse       ISQISRQLNQGQVAWTGSRPPFPGQ-----PSKTQSSAFGIGSSHPYPADPSSY 166
rat         ISQISRQLNQGQVAWTGSRPPFPGQ-----PSKTQSSAFGIGSSHPYPADPSSY 166
human       MSQISRQLNQSQVAWTGSRPPFPGQQIPSQSSKTQSSPFGIGTSHTYPADPSSY 171
            ::*****:* .**:*:*.****.**     ..*:***.****:.* * .**:**
```

Figure 4.4: The translated flounder sequence isolated in the current study as part of a ClustalX alignment of six other ARNT2 protein sequences from the NCBI database including: fundulus, two separate zebrafish clones (a & b), mouse, rat and human.

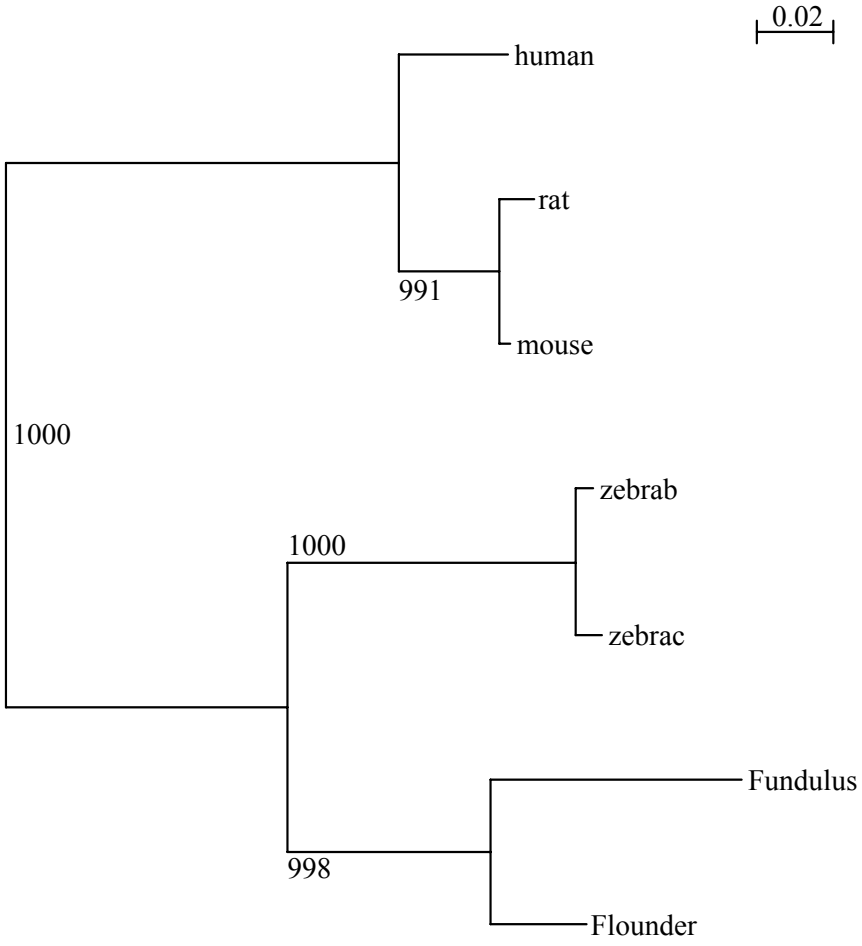

Figure 4.5: An unrooted phylogenetic tree of ARNT2 partial sequence derived by the distance method using ClustalW and plotted using the neighbour joining method (NJ plot), illustrating homology of the flounder and fundulus sequences. Partial nucleotide sequences were used, and the bootstrap values are based on 1000 re-samplings of the data and gives a measure of confidence for each branch position.

The flounder AhR2 sequence

The AhR2 sequence cloned is presented below (Sequence 2). Note locations of Forward primer and Reverse primer (complement). Homologies of the sequence to published data following a BLASTX search are presented in Table 4.7. (A BLASTX search compares a translated version of the nucleotide sequence with known proteins allowing gene sequences to be compared for functionality). Full phylogenetic analysis and alignment for the flounder AhR2 sequence is published by Besselink *et al*. (1998).

Sequence 2: *P. flesus* AhR2 partial clone (5' to 3') – primer sequences are highlighted

GTTTTATGCCTCTCCCACAATCAAGGAATACCTTGGCTTTCATCAGTCAGATGTGGTTCACCAGAGT
GTCTTCGATCTCATCCACACTGACGACCGAGACATGTTCAGACAGCAGCTGCACTTCGCCCTGAACC
CATCACCAATCAGCACGGCAGGAAATGGCTTGCAGAGCTCTGGTAACACAGAGAGTTACAGTCCTGA
GCACCTTCCTCCAGAGAACTCGTCCATCCTGGAGCGGAGCTTTGTGTGTCGCTTCCGCTGCCTCCTG
GACAACTCGTCCGGCTTTCTGGCGTTGAAGTTCCAGGGGCGACTGAAGTTCCTCCACGGTCAGAGTG
TTGTGAAGGACAACAGGACGTGCAACCACCCGCAGCTGGCTCTGTTCAGCATTGCAATGCCTGTTCA
GTCCCCGTCCATCGTGGAGATCCGAGCTAAGATGATTCTGTTTCAAACCAAACACCAGCTGGACTTC
ACGCCCATGGGCATTGACAGCAGGGGGAAAATAATCCTGGGCTACTCAGAGACTGAACTGTGTATGA
AAGGCTCCGGCTA 549 bps

Translation (5' to 3' Frame 2 of above sequence)

FYASPTIKEYLGFHQSDVVHQSVFDLIHTDDRDMFRQQLHFALNPSPISTAG
NGLQSSGNTESYSPEHLPPENSSILERSFVCRFRCLLDNSSGFLALKFQGRLK
FLHGQSVVKDNRTCNHPQLALFSIAMPVQSPSIVEIRAKMILFQTKHQLDFT
PMGIDSRGKIILGYSETELCMKGSG

Table 4.7: Homology of *P. flesus* AhR2 partial cDNA cloned in present study – results of BLASTX search. Species data including identities and probability values are provided.

Species	Identities	Probability	Gaps
P. flesus	100% (180/180)	e-101	-
F. heteroclitus	73% (133/180)	1e-74	-
Microgadus tomcod	72% (133/184)	8e-73	2% (4/184)
O. mykiss beta	70% (132/186)	2e-72	3% 6/186
D. rerio	70% (130/185)	8e-72	2% (5/185)
**H. sapiens*	57% (106/184)	6e-60	2% (4/184)
**Delphinapterus leucas*	59% (109/184)	6e-60	2% (4/184)
**Phoca vitulina*	58% (108/184)	1e-59	2% (4/184)

*Mammalian examples are included to emphasise the high degree of conservation of this region between vertebrates.

Those plasmids identified as containing the correct product were then re-transformed, plated out and grown up overnight to make large volume stock preparations. The following day, single colonies were selected, and two from each clone were grown up in 5ml LB (100μg/ml ampicillin) broth. The phagemid constructs were then re-purified using ultra-pure 'QIAquick' plasmid preps (QIAgen, UK), and the DNA quantified spectrophotometrically. Copy number was deduced using equation one (Appendix IV), and appropriate dilutions were made up using 'Tris TE λ' buffer as recommended for plasmid dilutions in quantitative PCR assays (see Appendix IV for recipes).

Gene	Plasmid	GenBank accession (original sequence)	Reference
AhR2	PflAhR2	AF034412	Besselink *et al*. (1998)
ARNT2	PflARNT2	AJ518832	(Current study)

Probes for blotting studies were produced by restriction digest and purification of the appropriate insert as in section 4.1.1. below which gives details of restriction enzymes used to isolate probe fragments.

Table 4.8: Conditions specific to each probe of the CYP1A transcription pathway including restriction enzymes used to isolate probe fragments, and hybridisation temperatures used for probe hybridisation.

Probe	Plasmid	Restriction enzyme	Hybridisation temperature
AhR2	PflAhR2	*Eco*RV / *Hind*III	67°C
ARNT2	PflARNT2	*Eco*RI / *Hinc*III	67°C

Summary

Phagemid constructs were available within our laboratory for genes involved in the PAH metabolic pathway, and were used as standards for generating standard curves for conventional RT-PCR and Real-time RT-PCR reactions, and restriction digested probe fragments were isolated for use in blot hybridisations. In addition, this study has provided sequence data for the previously unreported *P. flesus* ARNT2, and cloned a probe fragment from the flounder AhR2 by cloning via an RT-PCR based protocol. The two primer sets were chosen above others as they all produced a single clean band from cDNA on agarose in a PCR reaction, whereas the other primer sets did not. One set of primers designed from the *F. heteroclitus* ARNT2 sequence produced an RT-PCR product exhibiting a single band with flounder cDNA. This band was sequenced, and BLAST alignment showed 91% homology with the corresponding domain of *F. heteroclitus* ARNT2, and was deduced to be a

flounder ARNT2. The high level of homology is interesting since Pollenz and Hahn (2000) have shown high homology between PAS domains, but not other regions of the ARNT2 sequence. There are also high homologies with rat (71%) and human (69%) sequences. The differentiation between the trout (*O. mykiss*) ARNT isoforms and other vertebrate isoforms (as described by Pollenz and Hahn (2000) for the PAS domain) is also highlighted in this alignment (30% homology between flounder sequence and trout isoform a). The AhR2 and ARNT2 fragments were successfully cloned into vector to produce a phagemid for use later in the study as standards for quantitative RT-PCR, and probes for hybridisation analyses.

4.2 Quantitative procedures for genes involved in the *Ah* pathway

4.2.1 Real-time PCR

There are five main chemistries currently in use for tracking amplicon production in real-time PCR, and these fall into two categories: sequence specific and non-specific (MacKay, Arden and Nitsche, 2000). The method employed in this study was the non-sequence specific intercalating dye, SYBR® Green 1 (Molecular Probes Inc.) which fluoresces when it becomes incorporated into double stranded (ds)DNA (it binds to the minor groove in dsDNA) but fluorescence is minimal when in solution (Bell and Randford-Cartwright, 2002). The amount of fluorescence is directly proportional to the amount of amplification of dsDNA product (Bustin, 2000). SYBR® Green 1 was chosen over sequence specific probes for several reasons in this study: first and foremost, the system can be used with relative ease for many different applications, with any set of amplification primers. As it is a non-

specific dye system, in order to look at any particular region of interest, standard PCR primers specific to that region are designed and SYBR® Green 1 included in the reaction. Thus it is relatively simple and inexpensive when compared to the employment of specific fluorogenic probes (Yin et al 2001), which when investigating several different systems makes it the method of choice.

4.2.2 Design and optimisation of real-time PCR primers - CYP1A locus

PCR primers were designed to correspond to conserved regions of the flounder (Williams *et al.,* 2000) (EMBL Acc. No. AJ132353) and plaice (to correspond to the plasmid construct held in our laboratories which was used as a standard) CYP1A sequences (Leaver, Pirrit and George, 1993)(EMBL Acc. No. X73631). To minimise the chance of amplification of any contaminant genomic DNA in the reaction, primers were designed to span several exons, and the forward primer spanned an exon/exon join. A primer set was designed using PrimerSelect (DNAStar Inc.) according to guidelines issued by the supplier of the Rotorgene (Biogene, UK) (i.e. minimal primer-dimer formation, and optimal amplification size of 200-600bp, spanning multiple exons) and avoiding the haem binding site (common to all cytochromes (Stegeman, 1989)). Primers were synthesised by MWG Biotech (Germany). The 326bp amplification product (amplicon) spans exons 3 to 7 of the flounder CYP1A cDNA (the forward primer spans exons 3 and 4). Primer sequences are detailed below (Table 4.9) (the full annotated sequence showing the primer binding regions and exon joins is provided in Appendix III). Optimal annealing temperature was deduced as 65^{o}C after several tests on both agarose and the Rotorgene real-time thermocycler.

Table 4.9: CYP1A real-time PCR primers detailing primer name, sequence (5' to 3'), location relative to the putative transcriptional start site of the flounder CYP1A gene (Williams *et al.*, 2000) and optimised T_a

Primer	Sequence 5' to 3'	Location	Optimised Ta
PfCYP1A Forward	GTT TCG ATA CCG TCT CTA CTG C	1107-1128	65°C
PfCYP1A Reverse	AGG AAG CGA TCT GGG TTG AAG	1452-1432	

T – In published flounder sequence, 'T' is replaced with 'A'

Preparation of plasmid constructs for use as standards

Fresh transformations were prepared from stock plasmids held within our laboratories, and plated out on LB agar plates (Appendices I.9 and I.10). A single colony was picked from each plate, and grown up overnight in LB (100µg/ml ampicillin) broth at 37°C. Plasmid constructs were purified from cell cultures using ultrapure plasmid preps (QIAgen, UK) according to the manufacturer's protocol, and eluted in dH_2O (BDH, UK).

Linearisation of plasmid construct and creation of dilution series

Plasmid containing the target sequence insert was linearised as below by digesting with the appropriate restriction enzyme (RE) (i.e. containing only one site in the construct). To a 200µl PCR tube, the following was added:

Component	Amount
Plasmid construct	2-4µg
Restriction enzyme	15 Units
10× Reaction buffer (Promega, UK)	1µl
dH_2O	Xµl
Total	10µl

The reaction was incubated for 2 hrs at 37°C, then the RE heat inactivated by incubation at 85°C for 10 min. Plasmid was then quantified spectrophotometrically, and copy number deduced according to equation one (Appendix IV) based on Avogadro's Law, and a dilution series was created. The original sample was initially diluted to 10^8 copies per 5µl in modified TE λ buffer (Appendix IV), and further diluted in a tenfold series from 10^8 to 10 copies per 5µl. (λ *Hind*III was added to the buffer at 50pg/µl as a carrier to prevent loss of low copy number standards by adhesion to PCR tubes and pipette tips). The linearised plasmid of known concentration was then used as template in the PCR reaction to generate a standard curve, as described below.

Optimisation of PCR conditions for the CYP1A primer set was carried out on linearised BNF2PpP450 (Leaver *et al.,* 1993) plasmid (containing the full length pleuronectid CYP1A cDNA) in a standard PCR reaction as follows:

Component	Volume	Working concentration / amount
template DNA	1µl	[50ng]
10× *Taq* polymerase buffer (Promega, UK)	1µl	[1×]
25mM $MgCl_2$	0.8µl	[2.0mM]
5mM dNTPs	0.26µl	[130µM]
10µM forward primer	0.75µl	[0.75 µM]
10µM reverse primer	0.75µl	[0.75 µM]
5U/µl *Taq* DNA polymerase (Promega, UK)	0.1µl	[0.5 U]
dH_2O	Xµl	to a total 10µl volume

Ten microlitre reactions were run on a Biometra Tgrad gradient thermocycler at increments of ≈2°C, and products were resolved on 1.0% agarose (0.7× TAE) gels (0.5mg/ml EtBr). These optimised conditions were used as a guide to work out the

optimum temperatures to use for later real-time PCR reactions. Conditions were then tested on the Rotorgene real-time PCR machine and optimised further until a single product was produced (as indicated by melt curve analysis). Reaction conditions for the Rotorgene involved the use of a commercial master mix (ABgene, UK) to ensure consistency. The commercial master mix contained (per reaction): 1x Thermo-Start® QPCR Buffer, 500µM dNTP, 5.5mM $MgCl_2$, 2.5U/100µl Thermo-Start® enzyme. Master mixes were set up containing all components minus template. Each reaction contained the following components:

Component	Volume	Working concentration
2× commercial master mix	10µl	[as above]
template	5µl	[variable plasmid standard]
10µM forward primer	0.5µl	[0.25 µM]
10µM reverse primer	0.5µl	[0.25 µM]
SYBR® Green 1	0.1µl	[as supplied]
dH_2O	3.9µl	to a total 20µl volume

All reactions were prepared in a 'clean room' to reduce the likelihood of any contamination of samples by plasmid constructs. After aliquoting 15µl of reaction mix into separate 0.2ml PCR tubes, reactions were transferred to a separate preparation area and template added, from lowest copy number first to highest copy number last (to minimise contamination). Due to the sensitivity of the real-time PCR process, and the new arrival of the machine, extensive optimisation of the reaction conditions was carried out to check for machine and user error. These checks were carried out by testing variability both between and within runs.

Preparation of samples – first strand cDNA Synthesis

In order to measure expression levels at the mRNA level, 1st strand cDNA was first prepared from total RNA preparations using MMLV Reverse Transcriptase (Promega, UK) and poly T primers (to amplify all mRNA) according to standard protocols (Promega technical data and Sambrook *et al*., 1996). See Appendix I.3 for details. This cDNA was then used in the amplification reactions as template.

Purification of cDNA

Initial preparations of first strand cDNA were found to be inhibitory in the real-time PCR reaction, and gave very little amplification for several different samples using the CYP1A system. This problem was overcome by including a purification step to remove components of the reverse transcription (RT) step. Purification of cDNAs was carried out using QIAquick PCR purification kits (Qiagen, UK). Ten microlitres of each RT reaction was purified according to the manufacturer's protocol, and eluted in 100µl of modified TE λ buffer (see recipes, Appendix IV). The resultant solution was therefore a 1 in 10 dilution of the original sample, which allowed detection at a satisfactory level, without any inhibition by salts or enzyme carried over from the RT reaction. Samples for comparison were all prepared simultaneously, using reagents from the same batch to ensure consistency. For full protocol see Appendix I.4.

Generation of standard curve

Prior to quantification of any samples, a reproducible standard curve was required. Standard curves were generated by running triplicates of each linearised plasmid standard within the dilution series. In addition to standards from 10-10^8 copies, a no template (water) control (NTC) was included in each run to check for possible

amplification as a result of aerosol contamination of the reaction. Reactions were set up as follows:

Component	Volume	Working concentration
2× real-time PCR Master Mix	10µl	[1×]
10µM forward primer	0.5µl	[0.25µM]
10µM reverse primer	0.5µl	[0.25µM]
SYBR® Green 1	0.1µl	[as supplied]
5ng/µl template	5µl	see note
dH_2O	3.9µl	to a 20µl final volume

Template was produced from a 500ng total RNA RT reaction eluted in a final volume of 100µl, therefore 5µl prod = 25ng total RNA equivalent. The reaction was then run on the Rotorgene thermal cycler as follows: 10min initial denaturation (and enzyme-start) at 95°C, followed by 45 cycles of denaturation at 95°C for 30s, annealing at 65°C for 20s, and extension at 72°C for 25s, followed by a melt at 2°C increments for 10min (A 10 minute melt from 65–100°C is included in order to verify specificity of the product produced in the reaction).

Reproducibility – Inter and Intra-run variability

Once a standard curve was successfully generated, trials were carried out to ensure replication of results was possible (i.e. to assess experimental error). In addition to standards from 10-10^8 copies and a no template (water) control (NTC), several cDNA samples were included in each run, as ultimately it is cDNA amplicon variation which was of interest. Components were identical for those described above, and cDNA template was produced from a 500ng total RNA RT reaction eluted in a final volume of 100µl, therefore 5µl prod = 25ng total RNA equivalent.

The reaction was then run on the Rotorgene thermal cycler as described above. A standard Rotorgene run (using a 36 place carousel) included the following reactions:

2 × NTC

16 × standard (linearised plasmid construct) samples (8 × individual standards from 10-10^8 copies, in duplicate)

18 × cDNA samples (6 × individual cDNA samples in triplicate)

This format was applied to all further runs.

Generation of standard curve for CYP1A

Standard curves were generated for CYP1A from 10-10^8 copies with linearised plasmid. High R^2 values (consistently around 0.98 ($P < 0.01$)) were obtained when theoretical concentration of standards was plotted against C_T (the time at which the product production crossed the threshold value)(using the least squares method in Minitab V.10 for windows). Figure 4.6 is an example of a reaction report generated using CYP1A cDNA and plasmid standards: the top panel shows a graph displaying reaction progress (cycle versus product production), the lower panel shows copy number (concentration) versus C_T (standard curve) and includes a regression analysis of the standards. Figure 4.7 shows melt curve analysis of the reaction to confirm the amplification of a single product (shown by a single melting temperature for all product).

Inter and Intra-run variability

Inter-run variability of standards

Variability was investigated between runs by comparing C_T values with copy number for samples of known quantity for three separate runs (i.e. linearised

plasmid construct standards). See Figure 4.8 below for comparison using the CYP1A probe. No difference was observed between the three runs, and therefore it was assumed that the system is robust and repeatable.

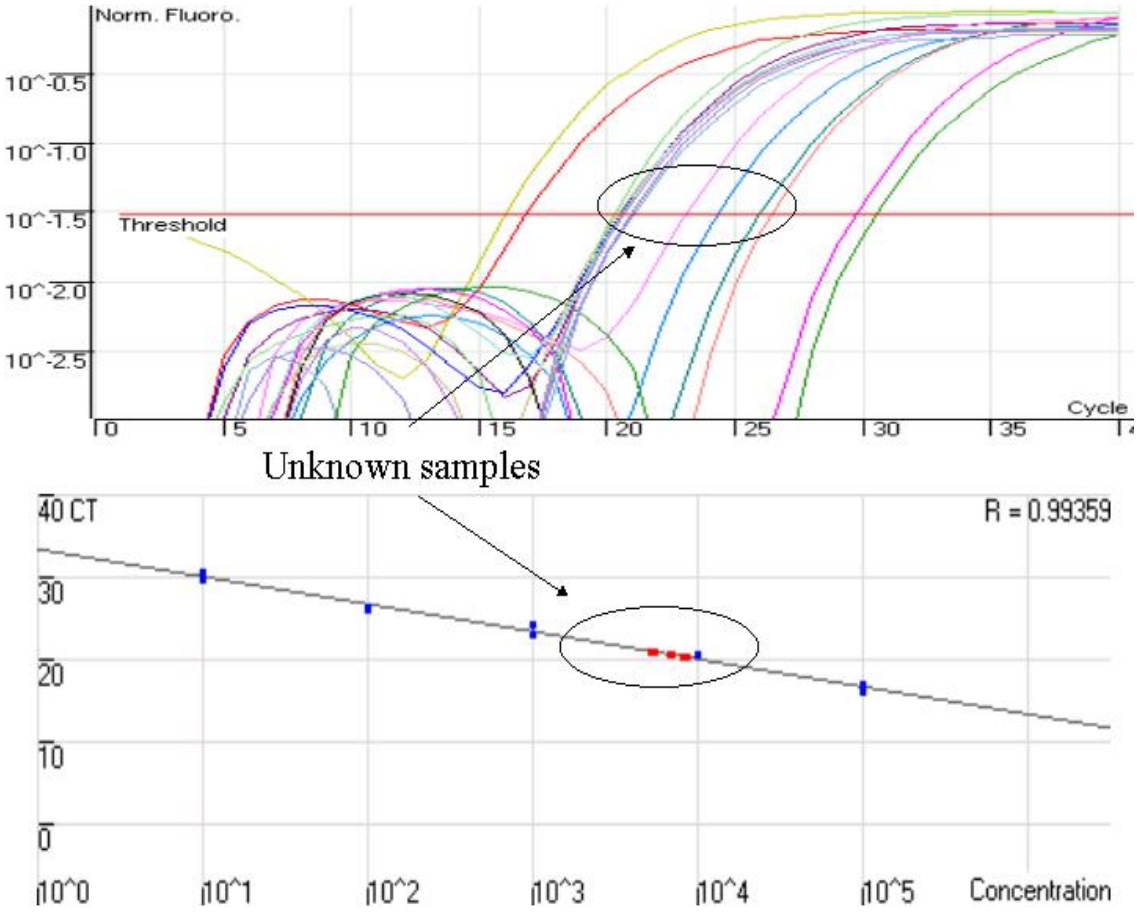

Figure 4.6: Experimental real-time PCR graph for CYP1A including: Top panel - reaction graph plotting fluorescence versus cycle number; Lower panel - standard curve illustrating sample concentration versus cycle number. Unknown samples are highlighted by a black circle.

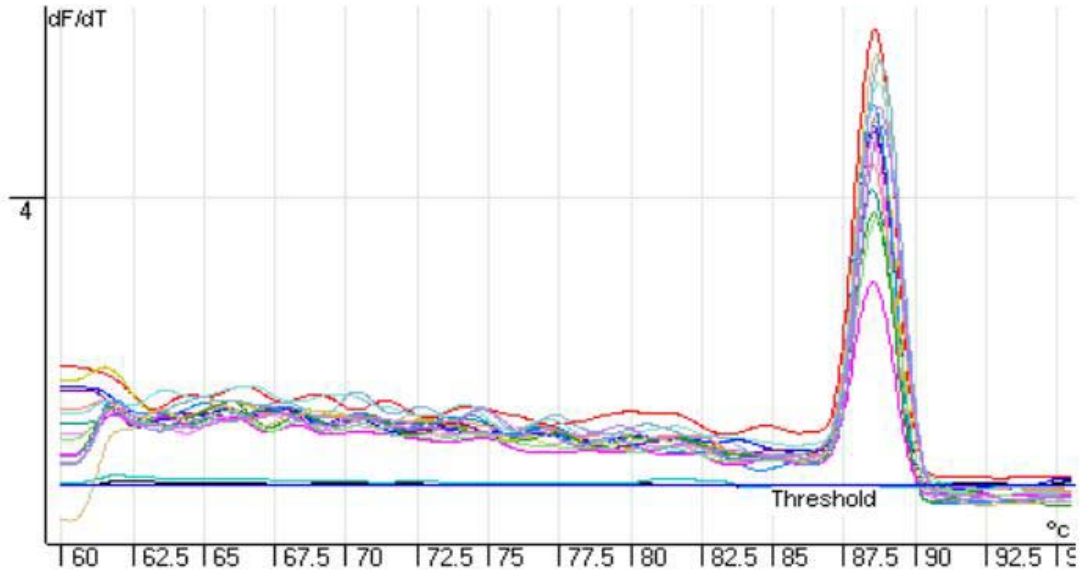

Figure 4.7: Melt curve analysis to confirm product specificity plotting fluorescence versus temperature. A single peak of expected melting temperature indicates that all product is of desired sequence as deduced from melting point analysis.

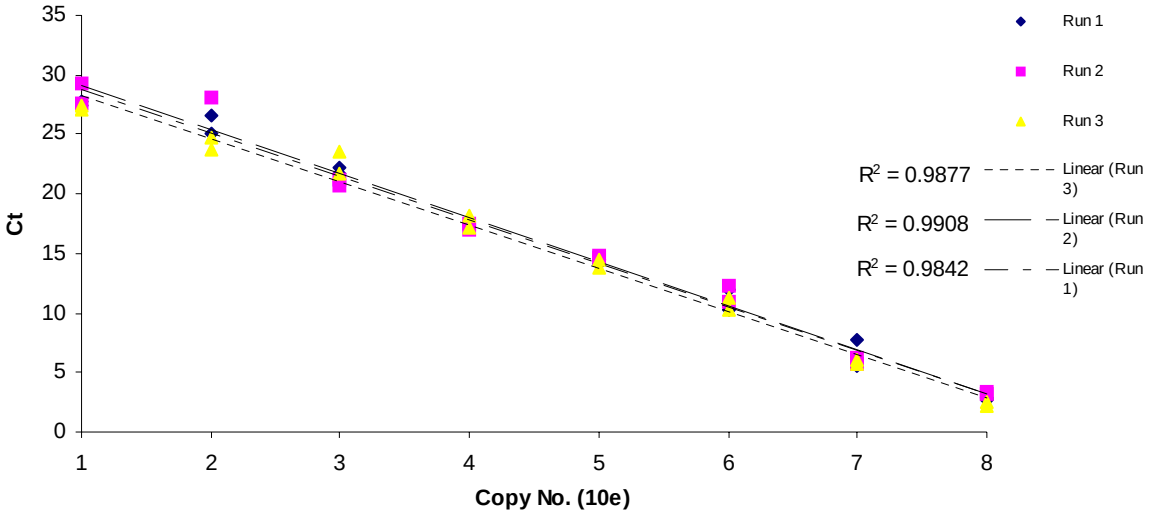

Figure 4.8: Data illustrating inter-run variability of three separate runs: Copy number versus cycle number. R^2 values are included in the legend.

Intra-run variability

Variability within runs was investigated by running all samples in triplicate. Regression analysis was then carried out on these triplicate samples, by comparing replicates following methods previously employed in published studies (described by Yin *et al*., (2001)) using regression analysis. Linear regression analysis of three separate PCR reactions amplified from aliquots of the same cDNA synthesis is shown below (see Figure 4.9, 4.10 and 4.11 showing variation between replicate one vs replicate two; replicate two vs replicate three; and replicate one vs replicate three respectively (data in Table 4.10)). Samples were run on four separate runs, but all replicates were carried out during the same run.

Table 4.10: Regression analysis of intra-run variation (replicates within run, n=53) – summary table of figures above. Replicates compared, R^2 and probability values are presented.

Replicates compared	R^2	Probability
1 vs. 2	0.951	P<0.001
2 vs. 3	0.868	P<0.001
1 vs. 3	0.887	P<0.001

All replicates above show that the method is reproducible, and there is no statistically significant difference between analysis of samples both within a run, and between runs using the CYP1A system developed here.

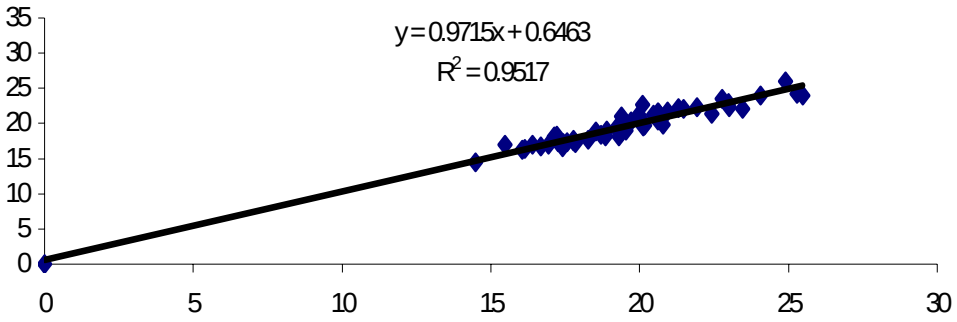

Figure 4.9: Replicate one plotted against replicate two

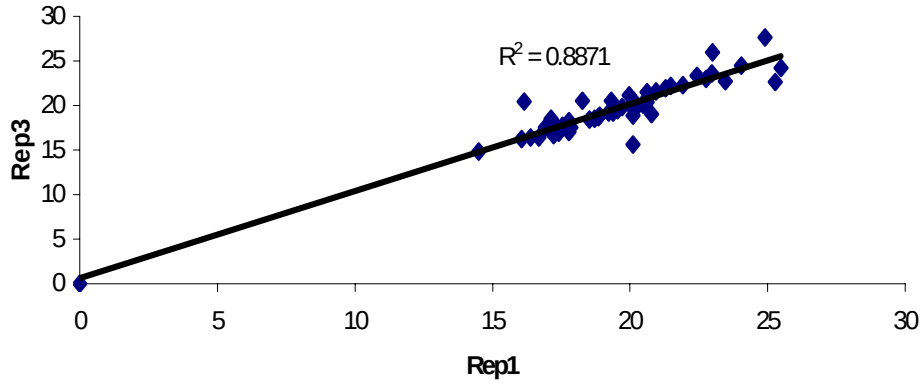

Figure 4.10: Replicate one plotted against replicate three

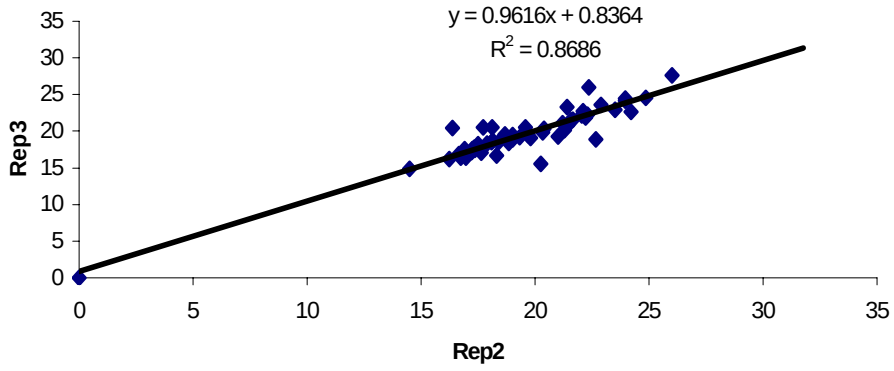

Figure 4.11: Replicate two plotted against replicate three

UGT1B1

Primers were designed for the phase II detoxification enzyme, UDP glucuronosyl-transferase 1B1 (UGT1B1) for use in real-time PCR from the published plaice sequence (EMBL Acc. No. AF AJ249081) according to the guidelines followed for CYP1A (current chapter). Primer sequences are as follows:

Primer sequence (5' > 3')	Primer Name
ATG CTG ATG TTC CCG CTG TTT G	PpUGT1B1F
GAT TTG GTC TTT GCC GTC CCT CTC	PpUGT1B1R

Standard curves were successfully produced for UGT1B1 using a dilution series of phagemid construct PpUGT1B1 (held in our laboratories). Unfortunately due to technical problems with the Rotorgene it was only possible to assay 18 cDNA samples. These samples displayed levels outside of the dynamic range of the SYBR® Green 1 system (i.e. <1000 copies per sample), and no analysis of the data was possible. Figure 4.12 below is an example of a standard curve (including cDNA samples) from the UGT1B1 initial trial. The initial trial of the UGT1B1 locus was successful, a standard curve was produced, and a single product was produced as highlighted in the melt curve analysis. Data is provided for possible utilisation in further trials.

4.2.3 Quantitative real-time PCR of genes of the CYP1A transcription pathway

AhR2 and ARNT2

An assay method using real-time PCR was attempted for the AhR2 and ARNT2 gene transcript expression levels, as described in section 4.2 above for the PAH

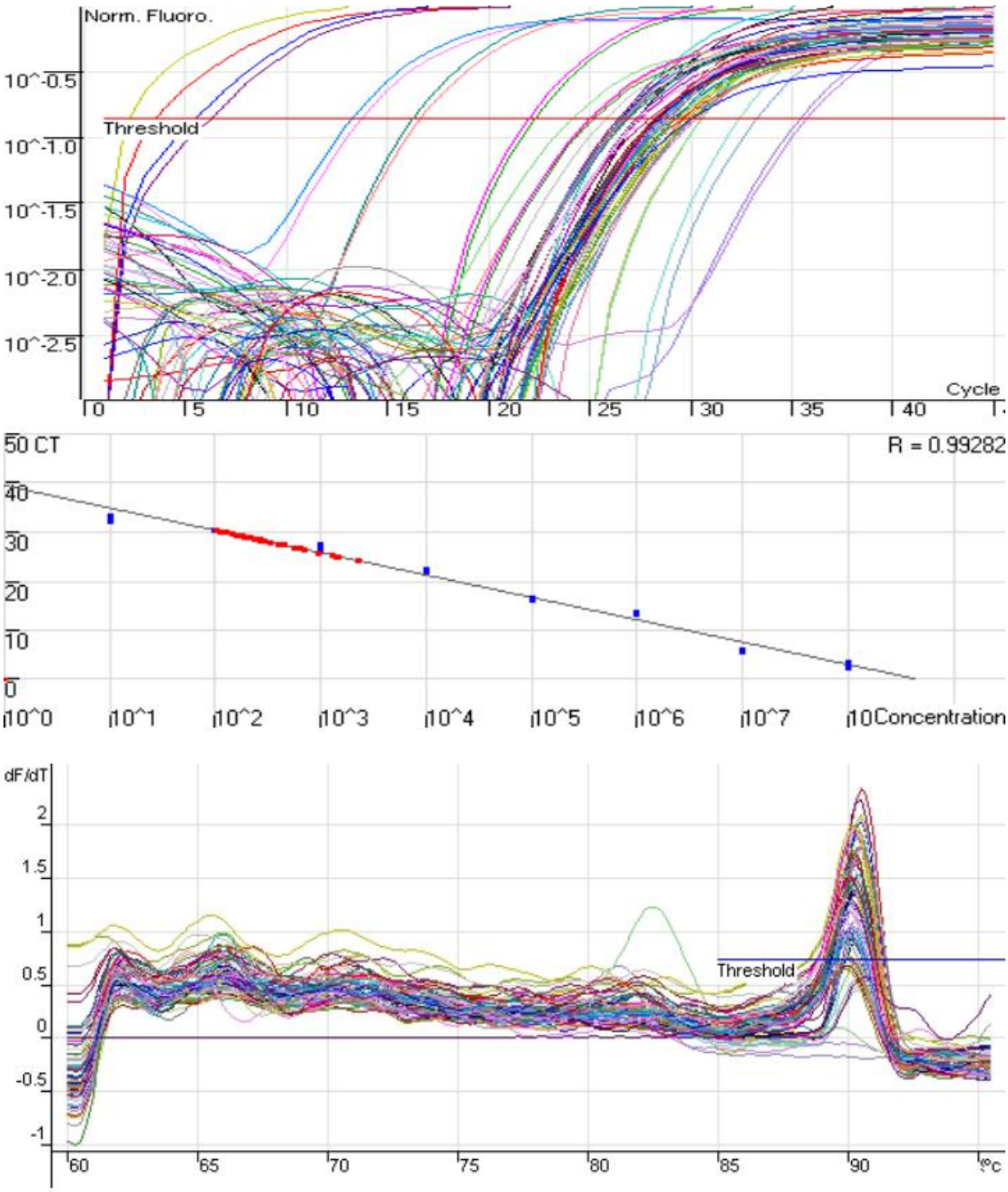

Figure 4.12: Experimental real-time PCR analysis of UGT1B1. Upper panel shows reaction graph plotting fluorescence versus cycle number; centre panel shows standard curve illustrating sample concentration versus cycle number; and lower panel shows melt curve analysis to confirm product specificity plotting fluorescence versus temperature. A single peak of expected melting temperature indicates that all product is of desired sequence.

responsive genes CYP1A and UGT1B1. Primer sets for AhR2 and ARNT2 were designed in section 4.1, and applied to this study, and standard curves were successfully generated for AhR2 and ARNT2 (Appendix V) using the techniques described for CYP1A. T_a°C for each primer set was deduced as 60°C. Analysis of several cDNA samples confirmed that quantification of these transcription factors was below the dynamic range attainable via real-time PCR using the SYBR® Green 1 system, and not pursued further.

Although successful standard curves were generated for both AhR2 and ARNT2, real-time PCR via a SYBR® Green I method was found to be unsuitable for quantification of the transcription factors. An alternative approach to quantitation using Northern / Southern blotting techniques was adopted, as described later in this chapter.

4.3 Blot studies

4.3.1 Northern blot studies

Probes developed in section 4.1 (current chapter) were used in blot studies for the PAH metabolising enzymes CYP1A, UGT1B1 and GSTA, and the CYP1A transcription pathway genes (AhR2 and ARNT2). Northern blot studies have been extensively optimised within our laboratories, and protocols were based on these methods and standard protocols (Sambrook *et al.*, 1996) as described below. For Northern blot analysis, total RNA was glyoxalated and size separated on an agarose gel. Transfer membranes of gels were prepared and hybridised with various probes. Northern blot analysis was carried out to measure CYP1A, UGT1B1, GSTA1, AhR2 and ARNT2 gene (mRNA) expression levels, as follows.

Glyoxalation of RNA

RNA denaturation solution was prepared by addition of 125µl de-ionised glyoxal, 190µl di-methyl sulphoxide (DMSO) (Sigma, UK) and 13µl 25× MOPS (see Appendix IV for recipes), followed by thorough mixing. Two volumes of the solution (i.e. 20µl) were added to ten micrograms of total RNA (10µl) in a 200µl microfuge tube (or 96-well PCR plate). Samples were then incubated at 55^{o}C for 60 minutes to denature, then transferred immediately to ice to minimise degradation. Whilst on ice, 1/20th volume (1.5µl) of loading dye (40% Ficoll 400, 0.4% Bromophenol blue, 0.4% Xylene cyanol) was added to each sample to permit loading.

Agarose gel electrophoresis of RNA

A 1% agarose (1× MOPS) gel (0.5mg/ml EtBr) was prepared according to standard protocols (see Appendix I.5). Glyoxalated samples were then loaded (31.5µl per well) into the prepared gel. Samples were electrophoresed in 1× MOPS run buffer at 4V/cm for approximately 40 minutes until the bromophenol blue dye had migrated 4-6cm. Following electrophoresis the gel was rinsed in dH_2O, and transferred to a UV transilluminator, and the image captured and digitised (Gelworks). A Windows® bitmap image of each gel was stored to allow normalisation of gels against total RNA by densitometry of 18s and 28s RNA bands.

Northern Blot preparation

Electrophoresed RNA samples were transferred to membrane (Hybond 'N', Amersham Pharmacia, UK) by standard capillary blot techniques according to standard protocols as described in Appendix I.6. The blots were left for 18 hours at

room temperature by which time the gels had compressed to approximately ¼ of their original thickness. The membranes were then removed and gently rinsed in 6×SSC to remove any agarose debris. Gels were viewed under UV to ensure complete transfer of RNA indicated by absence of RNA on gel after blotting. The membrane was then baked at 80^{o}C for one hour to fix the RNA. The membranes were then stored dry between sheets of Whatman 3MM paper in sealed polythene bags at room temperature until required.

Probe hybridisation

Probing of filters was carried out using cDNA probes derived from plasmids as described in section 4.1. 20ng of the probe fragment was labelled with α ^{32}P dCTP (4000 Ci / mmol; 10μCi/μl) by random priming using the 'Random primers DNA labelling' kit (Gibco BRL), according to the manufacturer's instructions. The probe was then purified using a Probe Quant G50 micro-column (Amersham Pharmacia, UK) following the manufacturer's instructions, denatured at 95°C for 5min, then placed on ice. The labelled probe was then hybridised with the transfer membrane in a rotisserie style hybridisation oven (Techne), as follows: Pre-hybridisation was carried out at 65^{o}C for three hours in Church buffer (Appendix IV). Hybridisation was carried out with overnight incubation with isotopically labelled probe at the appropriate temperature (Table 4.11).

Table 4.11: Conditions specific to each probe used in Northern blot. Restriction enzymes used to isolate probe fragments, plasmid from which each probe was isolated and appropriate restriction enzyme, and hybridisation temperature are presented.

Probe	Plasmid	Restriction enzyme	Hybridisation temperature
AhR2	PflAhR2	*Eco*RV / *Hind*III	67°C
ARNT2	PflARNT2	*Eco*RI / *Hinc*III	67°C
GSTA	PpGSTA	*Eco*RI / *Hind*III	65°C
UGT1B1	PpUGT1B1	*Eco*RI / *Nco*I	65°C
CYP1A	PpP450BNF2	*Eco*RV / *Hind*III	67°C

Following hybridisation, washes were carried out to remove excess unbound probe. Washes were: 2 × 20 min at 2×SSC, 0.1% SDS; 2 × 20 min at 1×SSC, 0.1% SDS, with a final wash for 20min at 0.5×SSC, 0.1%SDS, all at 65°C (NB: AhR2 probe was washed further to 2× 20 min at 0.1× SSC, 0.1%SDS due to excessive counts remaining). Unfortunately the UGT1B1 probe returned no signal after repeated attempts at blot studies, and was excluded from further analysis in this study.

4.3.1.1 Densitometry of blots

Autoradiographs were digitised on a flatbed scanner (Epson GT2000) and stored as 'tagged image file format' (TIFF) files. Radioactive spots and 18s and 28s RNA bands (from agarose gel images) were quantified by using the 'gel capture' macros of Scion Image version Beta 4.0.2 (Scion Corporation, Frederick, MD, based on NIH image, Bethesda, MD, USA), and intensities recorded. Each lane on the gel was standardised by inclusion of 2 standard RNA samples, induced (+ve) and control (-ve) standards (standards were RNA samples obtained from previous IP

injected Aroclor 1254 induction (50mg Aroclor 1254 / kg body weight) trials and control samples, respectively, from within our laboratory. 10μg total RNA / lane were included). These values were then used to calculate semi-quantitative expression values (relative to standards on each gel, to allow comparison of expression) following methods described by e.g. (Leaver *et al.,* 1993; Courtenay *et al*., 1999; Vasiliou, Buetler, Eaton and Nebert, 2000). Normalisation was carried out to allow standardisation between gels using the following equation:

$$\frac{\text{Sample intensity}}{\text{Mean standard intensity}} = \text{Normalised intensity}$$

4.3.2 Summary of Northern blot studies

Northern blots were successfully used to quantitate mRNA from flounder for CYP1A and GSTA. No detectable signal was attained from UGT1B1 (possibly due to probe degradation or unsuitable blot conditions) AhR2 and ARNT2 analyses.

4.3.3 RT-PCR and Southern Blot analysis

Due to absence of detectable signal via real-time PCR and Northern blot analysis (current chapter) an alternative method of quantitation was developed for AhR2 and ARNT2. Two step RT-PCR followed by Southern blot analysis was carried out following the methods developed and optimised as follows. The plasmids developed in section 4.1 were used as standards for RT-PCR reactions.

4.3.4 Quantitative RT-PCR as a tool to investigate expression levels of AhR2 and ARNT2

First strand cDNA was synthesised by reverse transcription of flounder hepatic total RNA (see Appendix I.3), and purified using QIAquick PCR purification columns

(QIAgen, UK). The purified first strand cDNA was then used as template in the PCR reactions. PCR reactions were set up based on modified methods described by (Sojka and Pollenz, 2001) for investigating ARNT expression in the rainbow trout, as described below.

4.3.4.1 Optimisation of PCR as a quantitative assay

Initial amplifications and product specificity checks were carried out by PCR using the reaction compoments given below. PCR cycling conditions were: an initial denaturation at 95°C for 3min, followed by 32 cycles of 95°C for 50s, annealing at 60°C for 50s, then extension at 72°C for 2min, followed by a hold at 4°C. Products were then mixed with the appropriate amount of 2× loading dye, and 8µl (half) of each reaction was electrophoresed on a 1.2% agarose (1×TAE) gel. Plasmid standards (generated above) were included alongside cDNA from three individuals to confirm amplification of correct size of product.

Component	Volume	Working concentration / amount
10× *Taq* polymerase buffer (Promega, UK)	2µl	[1×]
10mM dNTPs	1µl	[0.5mM]
25mM $MgCl_2$	1.6µl	[2mM]
10pm/µl forward primer	2µl	[1pmol/µl]
10pm/µl reverse primer	2µl	[1pmol/µl]
5U/ µl *Taq* DNA polymerase (Promega, UK)	0.5	[0.125 U/µl]
template	2µl	[cDNA derived from 10ng total RNA]
dH_2O	xµl	to final 20µl

Defining optimum cycle number in RT-PCR

Optimum cycle numbers to use in the PCR reaction were deduced to allow quantitation of a range of cDNAs (i.e. to allow quantitation of the reaction whilst in exponential phase of the reaction). A series of reactions were set up for the two best primer sets (i.e. those which yielded a single product of correct size), one primer set for each of the targeted genes. 120µl PCR reactions were set up as described in section 4.3.4.1 above, and 12 × 10µl aliquots of the reaction were placed into 12 separate tubes to allow removal of aliquots at particular cycles in the reaction, and the reaction was run on a Biometra T-Grad thermal cycler using the cycling conditions above (section 4.3.4.1). A tube was removed at 2 cycle intervals from cycles 8-30 inclusive.

6µl of 2× loading dye was added to each reaction to provide a convenient volume for loading onto the gel, and half of the total reaction (i.e. 8µl) was then electrophoresed on non-denaturing 1% agarose, 1×TAE gels for 30 min at 2 V/cm. Gels were then incubated in denaturation solution (1.5M NaCl, 0.5 M NaOH) in a glass tray for 45 minutes at room temperature with gentle agitation on an orbital mixer. After denaturation, gels were rinsed in dH_2O, transferred to neutralising solution (1M Tris, pH 7.4, 1.5M NaCl) and returned to gentle agitation on an orbital mixer. After 30min the neutralising solution was replenished, and incubation continued for a further 15min. Samples were blotted via capillary transfer in 20× SSC over-night (see Appendix I.6 and Sambook *et al*., 1996) onto Hybond 'N' membrane (Amersham Pharmacia, UK). After overnight blotting, membranes were rinsed in 2× SSC to remove any excess agarose, and baked for one hour in an 80°C oven to fix the cDNA PCR products to the membrane.

Probing of Southern blots

Probe labelling

Probe fragments were (see 4.1) were labelled with α ^{32}P-dCTP (4000 Ci / mmol; 10μCi/μl) by random priming using the 'Random Primers DNA labelling kit' (GIBCO BRL) according to the manufacturer's protocol. The labelled probes were then purified using ProbeQuant™ G-50 Micro Columns (Amersham Pharmacia, UK) according to the manufacturer's instructions, dentured at 95ºC for 5min, and kept on ice.

Probing of blots

Pre-hybridisation / hybridisation solution ('Church' buffer - see Appendix IV, recipes) was prepared, and pre-hybridisation was carried out for three hours in 25ml of solution in a rotisserie style hybridisation oven (Techne) (see Appendix I.7). Pre-hybridisation solution was discarded, and the freshly denatured labelled probe fragments were then added to 25ml fresh hybridisation solution, and the complete solution was added to the appropriate (i.e. AhR2 probe to AhR2 product filter / ARNT2 probe to ARNT2 product filter) membranes. Hybridisation was then carried out overnight at 67°C.

Post-hybridisation washes were carried out at 65°C to a stringency of two × 20 min at 2×SSC, 0.1% SDS, two × 20 min at 1×SSC, 0.1% SDS, one × 20 min at 0.5×SSC, 0.1% SDS, with a final 20 min at 0.1×SSC / 0.1%SDS to ensure only specific products were bound by probe. Membranes were then placed on Whatman 3MM paper to remove excess moisture, wrapped in Saranwrap™, and loaded into autoradiography cassettes with X-ray film (Blue sensitive X-Ray film, GRI, UK).

Optimal exposure time was established to allow all fragments to give a signal, without saturating the film.

Image analysis of blots

Autoradiographic images were digitised, and densitometry of blots was carried out as for Northern blots. Values for samples were normalised against standards included on each gel, which allowed inter-gel comparison to be made. Optimum PCR cycle number was chosen according to values returned from running a serial dilution of known standard (10-10^4 copies) on a trial gel.

Agarose gel analysis

PCR was carried out using AhR2 primers for a variable number of cycles to test optimal cycling conditions for quantitation. Figure 4.13 below is an example of products following electrophoresis and visualisation under UV. An aliquot of AhR2 fragment obtained from restriction digest of the phagemid construct was included to ensure products were of the correct size, and prove that correct binding of the probe was taking place.

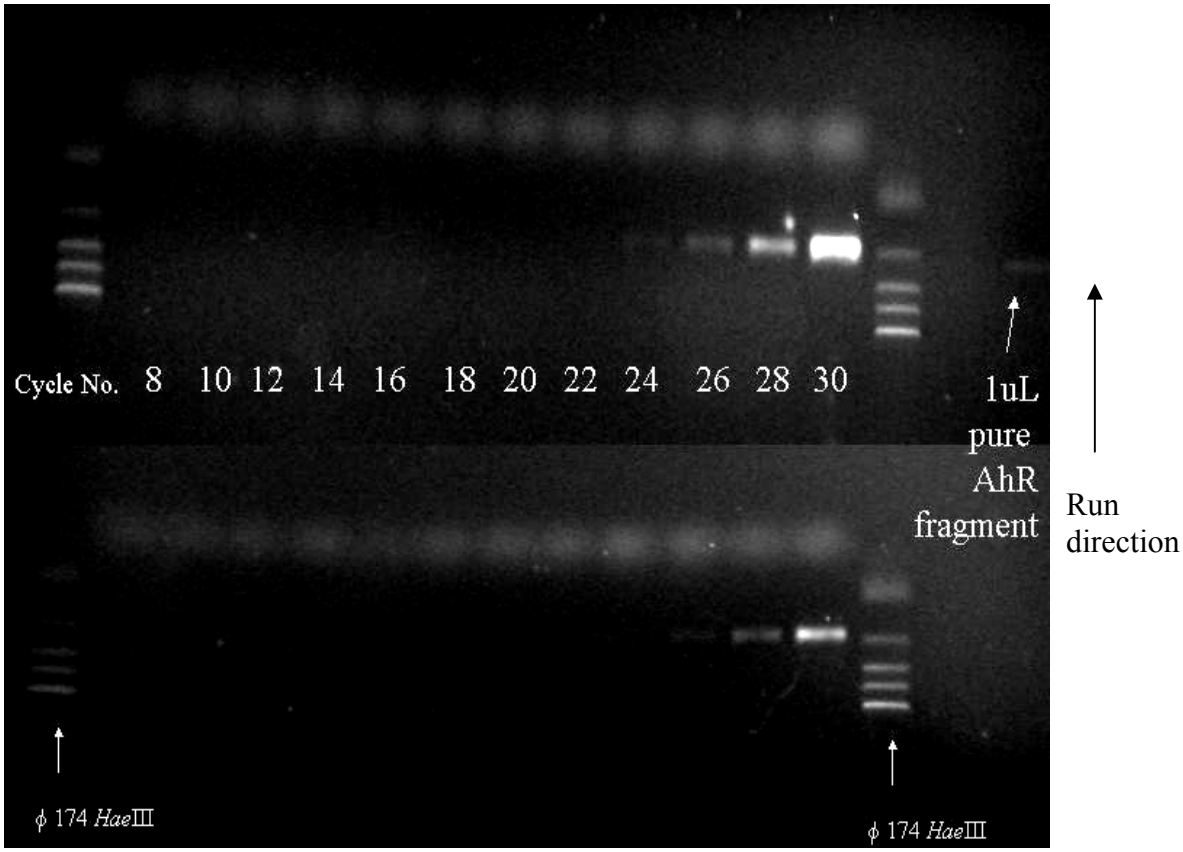

Figure 4.13: Agarose gel image showing effect of variable PCR cycle number on amplification product for AhR2 using primers developed in the current study – Upper panel is an aliquot of a PCR of cDNA of unknown quantity, and lower panel is an aliquot of PCR product from linearised plasmid as described above. 1µl (5ng) of AhR2 fragment was run on gel to allow sizing of product compared to probe.

Southern blot analysis and densitometry

Results of the agarose gel (Figure 4.13) shown above following Southern blotting, and probing with the ^{32}P labelled AhR2 cDNA probe, are presented below (Figure 4.14).

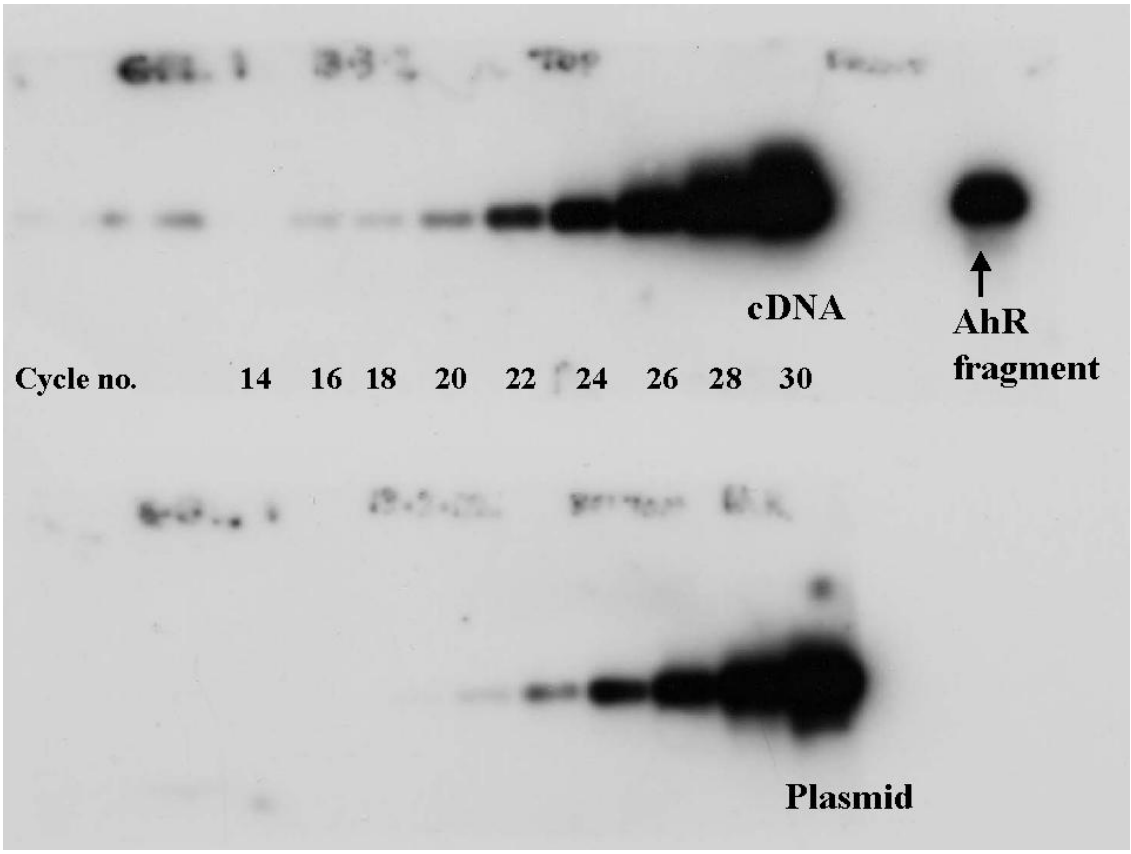

Figure 4.14: An autoradiograph of a Southern blot of the agarose gel shown in figure 4.13 above, probed with ^{32}P labelled AhR2 cDNA fragment. Upper panel is an aliquot of a PCR of cDNA of unknown quantity, and lower panel is an aliquot of PCR product from linearised plasmid as described above

Densitometry readings from Scion Image (Frederick, MD, USA) analysis for the blot above are provided in Table 4.12 below. These readings were then plotted, and regression analysis carried out (Figure 4.15).

Table 4.12: Densitometry readings of effect of increasing cycle number on amplification production deduced from image analysis of the AhR2 Southern blot presented in Figure 4.14.

Cycle Number	22	24	26	28	30
cDNA area	470	2230	5200	8440	14020
Plasmid area	0	770	2800	6180	11610

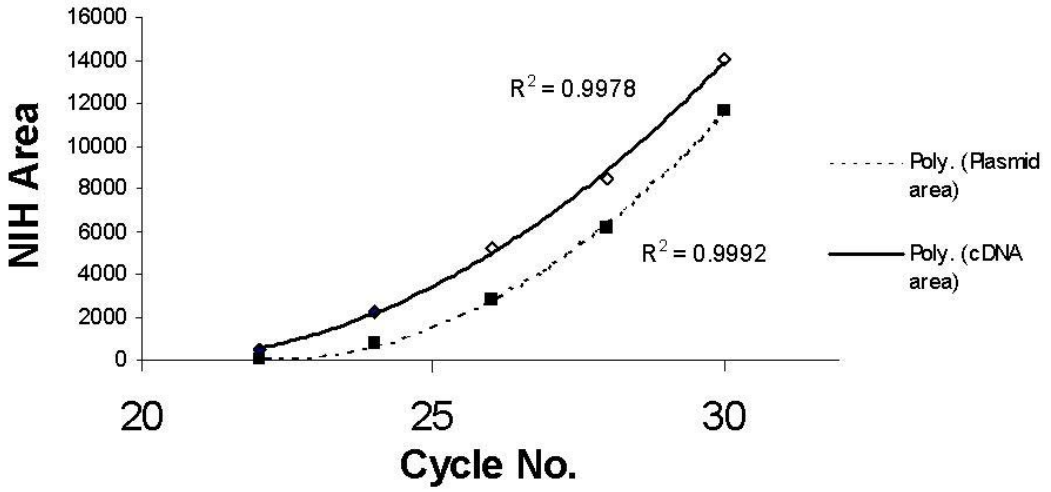

Figure 4.15: A plot of polynomial regression analysis of data from AhR2 product production vs cycle number from Table 4.12 above.

All correlation coefficients were found to be highly significant ($P<0.001$) indicating that it was possible to quantitate levels of AhR2 accurately from cycle 22 to cycle 30 at templates of the chosen concentration. Cycle 25 was however selected as an optimum cycle number to use, as detection was possible before reaching saturation of signal. Although no saturation was observed at the levels of expression plotted above, cycle number was chosen at a lower value to prevent saturation of result when testing the unknown samples. 25 cycles were used for further analysis of replication of results (see below).

Replication of results

Plasmid standards and cDNA samples were amplified for 25 cycles in a PCR reaction. Replication was tested by running PCR reactions in duplicate, and blotting as described above. An example blot of the replication is presented in Figure 4.16 below.

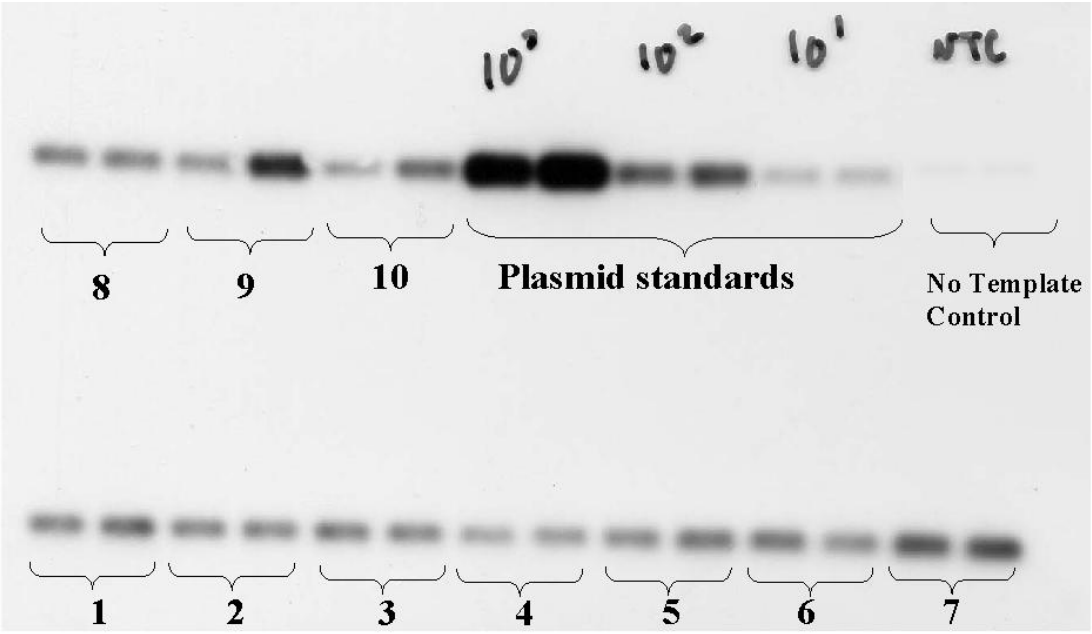

Figure 4.16: An autoradiograph of a Southern blot of the agarose gel of replicated samples probed with ^{32}P labelled AhR2 cDNA fragment. Each sample is a PCR of a cDNA sample of AhR2 of unknown quantity, and are duplicate reactions. Linearised plasmid standards of known copy number (illustrated above each sample) are included.

Results were plotted, and regression analysis was carried out following methods in Yin et al., 2001. Results of the densitometry are presented below Figure 4.17). Although 2 samples displayed a slight deviation from the trend, all regressions were found to be statistically highly significant ($P<0.001$), and the technique found to be consistent for this range of expression at 25 cycles.

4.3.4.2 ARNT2 analysis

The analysis from ARNT2 is identical for that described for AhR2 above. Results are presented below for agarose gel electrophoresis of ARNT2 PCR products (see Figure 4.18).

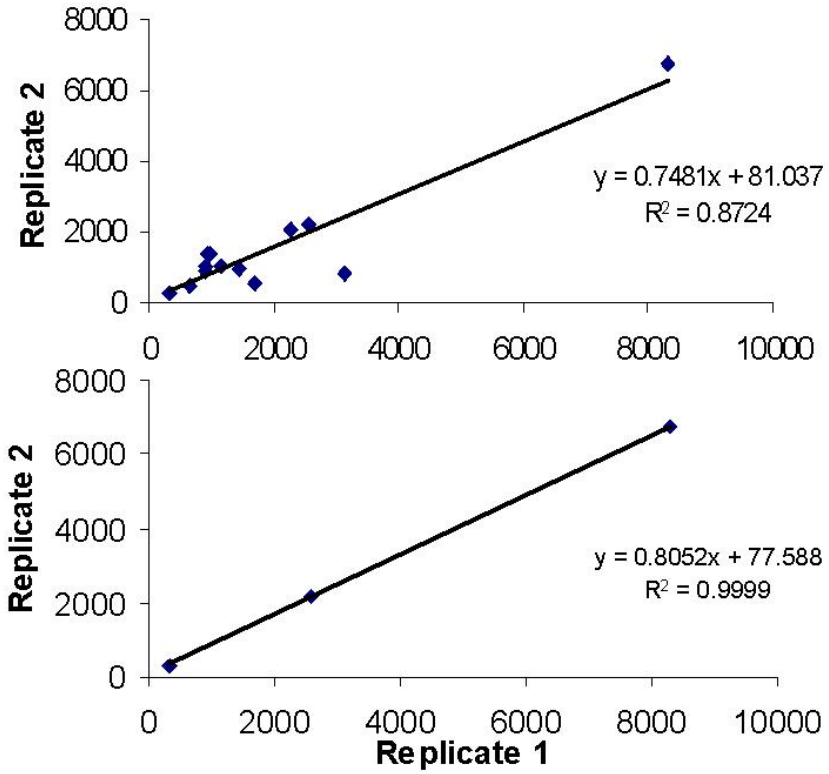

Figure 4.17: Regression analysis of AhR2 replication – Upper panel is cDNA analysis, lower panel is plasmid standard.

Southern blot

Southern blotting was carried out of the above gel, and results are presented below (Figure 4.19).

Densitometry of the above blot was carried out, and results presented in Table 4.13 and Figure 4.20, below.

Table 4.13: Densitometry readings of effect of increasing cycle number on amplification production – ARNT2

Cycle Number	**22**	**24**	**26**	**28**	**30**
cDNA area	680	2410	4820	7370	12210
Plasmid area	0	80	840	2960	5730

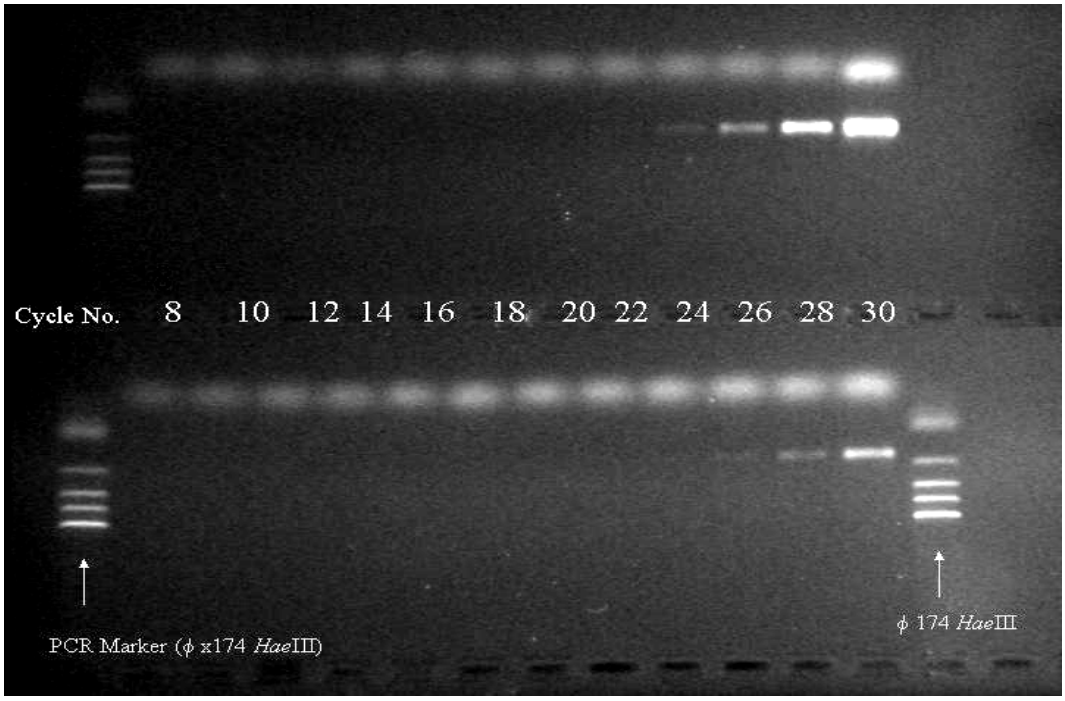

Figure 4.18: Agarose gel image showing effect of variable PCR cycle number on amplification product for ARNT2 using primers developed in the current study: Upper panel is an aliquot of a PCR of cDNA of unknown quantity, the lower panel is an aliquot of PCR product from linearised plasmid as described above.

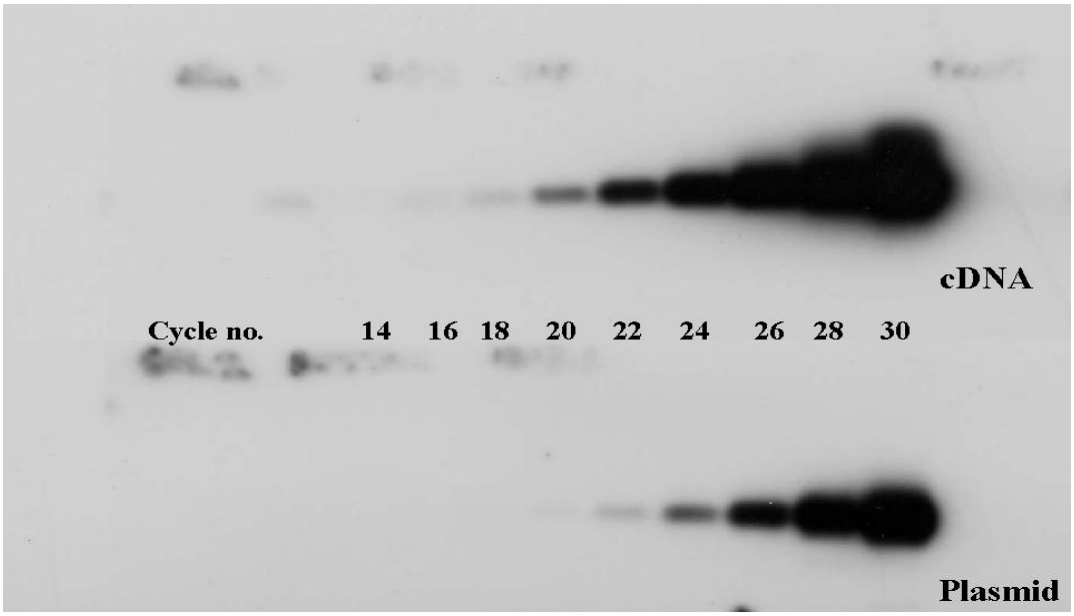

Figure 4.19: An autoradiograph of a Southern blot of the agarose gel shown in fig 4.18 above, probed with ^{32}P labelled ARNT2 cDNA fragment. Upper panel is an aliquot of a PCR of cDNA of unknown quantity, and lower panel is an aliquot of PCR product from linearised plasmid as described above.

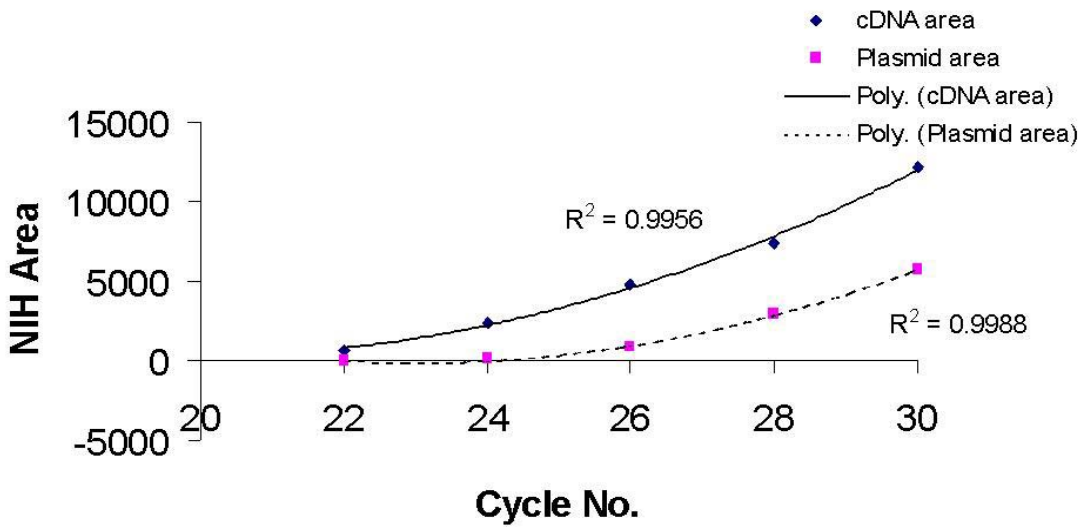

Figure 4.20: Polynomial regression analysis of ARNT2 cycle number vs product production to determine optimal cycle number for analysis of samples. Data were collected from image analysis of the Southern blot presented in Figure 4.19 above.

Regression analysis of densitometry results showed a highly significant correlation ($P<0.001$) indicating that it was possible to quantitate levels of ARNT2 consistently from cycle 22-30. Cycle 25 was however selected as an optimum cycle number to use, as detection was possible without saturation of signal, and the reaction had not reached plateau at this stage.

Replication of results

Replication of results was tested by running PCR reactions in duplicate as described for AhR2 above, and an example blot is presented below (Figure 4.21). Results of the densitometry are presented below (Figure 4.22).

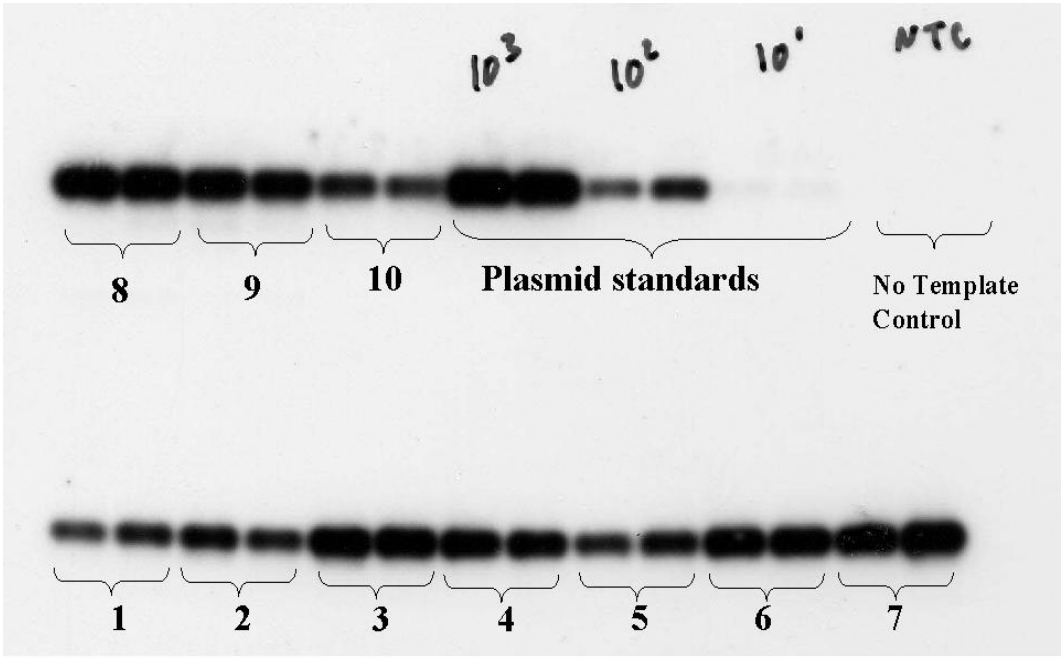

Figure 4.21: An autoradiograph of a Southern blot of the agarose gel of replicated samples probed with ^{32}P labelled ARNT2 cDNA fragment. Each sample is a PCR of a cDNA sample of ARNT2 of unknown quantity, and are duplicate reactions. Linearised plasmid standards of known copy number (illustrated above each sample) are included

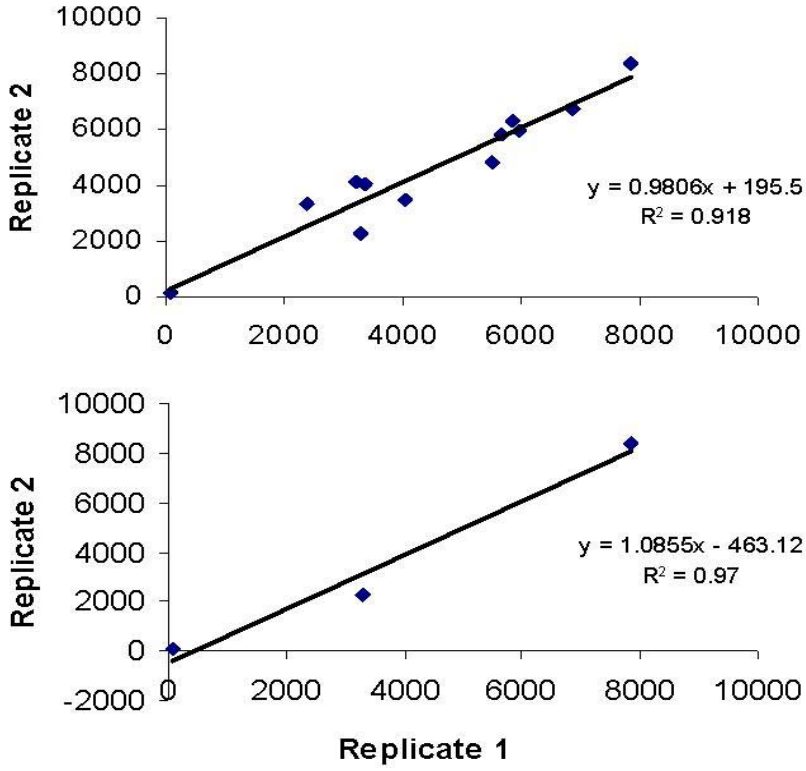

Figure 4.22 Regression analysis of ARNT2 replication, replicate 1 versus replicate 2 – Upper panel is cDNA analysis, lower panel is plasmid standard.

Regressions for cDNA ($R^2 = 0.918$) and plasmid standard ($R^2 = 0.97$) were found to be statistically significant ($P<0.001$ for cDNA, and $P<0.05$ for plasmid), and therefore the technique was accepted as consistent in ARNT2 quantification over a range of quantities.

4.4 Discussion

Several probes were already available within our laboratories, and quantitative assays developed previously were found to work satisfactorily for use in this study. Further assays were developed for quantification of CYP1A using real-time PCR techniques, and these are discussed below. UGT1B1 analysis was only possible for a few samples using this technique due to technical problems encountered with the

Rotorgene machine, and was excluded from further analyses. Results which were returned, however, indicated that the primers developed have potential for future use, and are thus included in this study.

Quantitation of AhR2 and ARNT2 expression

Two sets of primers were selected for AhR2 amplification, one of which was used in preference due to specificity of product production. A probing system has been developed which will be of use to future studies, and is employed later in this study (Chapter 5). This study has demonstrated the potential usefulness of RT-PCR and Southern blot analysis as tools in investigating expression levels of AhR2 and ARNT2 gene expression in the European flounder. Densitometry of Southern blot analysis confirmed that it was possible to analyse samples during the linear phase of amplification for both AhR2 and ARNT2, and that both products remained in linear phase at 25 cycles of PCR, which followed results described by (Karchner, 1996). It has also developed a probe system for use in investigation of relative expression levels of AhR2 and ARNT2 at the molecular level in the European flounder. The use of these techniques with application to measuring levels of AhR2 and ARNT2 after toxicity trials is reported later in the study, along with correlation to other detoxification enzyme studies (see Chapter 5). As well as their usefulness in the current study, these methods of quantification of the AhR2 and ARNT2 system presented above have potential use for many different species, with application to a wide variety of problems.

4.4.1 Real-time PCR

This study has further confirmed the usefulness of real-time PCR as a tool in quantification of gene expression in biomonitoring studies as highlighted by the quantification of CYP1A. The method is reproducible as deduced by regression analysis following guidelines of published work (Yin *et al*., 2001). Primers were also designed and optimised for use in UGT1B1, AhR2 and ARNT2, and reproducible standard curves were produced. Unfortunately due to technical problems with the machine during the course of the study it was not possible to further optimise these systems for quantification of transcripts. Although preliminary results were produced for AhR and ARNT analysis, transcripts were at the lower levels of the detection limit of the SYBR® Green 1 system (Yin et al., 2001) and accurate quantification was not attainable. Results of CYP1A transcript analysis using the real-time PCR system developed here are presented in chapter 8 of this study for Aroclor 1254 treated flounder.

Real-time PCR determination of CYP1A

The development of PCR methodology has permitted both qualitative and quantitative analysis of DNA and RNA sequences from extremely small numbers of template molecules (Kaplan, Cleef, Wirgin and Crivello, 1995). Real-time PCR potentially allows an equally sensitive level of detection possible with other methods of RT-PCR based analysis (e.g. Southern blot) due to the sensitivity levels possible using a fluorescence based system, without having to use isotope. However, due to the sensitivity of the reaction process, careful optimisations are essential prior to any sample runs. Factors such as temperature gradients across machines and the importance of reaction inhibition have been highlighted (Wilhelm,

Hahan and Pingoud, 2000). Although SYBR® Green I appears to offer sensitive detection, at low copy number transcripts this study has confirmed difficulties in obtaining reproducibility at low copy number. SYBR® Green I detection was compared with that attainable using fluorescent probes for quantitation of cytokine growth factor (Yin, Shackel, Zekry, McGuinness, Richards, Van der Putten, McCaughan, Eris, and Bishop, 2001) and it was found that SYBR® Green I gave reproducible quantification when the target gene was expressed at moderate to high levels (≥1000 copies/reaction), but did not give consistently reproducible quantification when the target gene was expressed at low levels (<1000 copies). Although optimisation of melting temperature improved the specificity of SYBR® Green I detection, it did not equal the reproducible sensitivity and specificity of fluorogenic probes (Yin *et al*, 2001). However, fluorogenic probes are specific to the product for which they are designed, and relatively expensive to synthesise, and therefore SYBR® Green I confers advantages to the user who may look at many different systems.

Real-time PCR as a tool to quantify transcription factors of CYP1A: AhR and ARNT

In this study real-time PCR using the SYBR® Green system was not sensitive enough to detect (with any accuracy or reproducibility) the expression of AhR or ARNT in the European flounder. Accurate standard curves were generated using the partial clones developed in Chapter 4, but it was concluded that levels present in mRNA were below the sensitivity of the SYBR® Green I system. Although detection was possible, it was at such a low level that no differentiation could be detected between samples. Concentration of the samples to be assayed would

require a factor of at least two magnitudes, and as samples were at 100µl volumes they would have to be concentrated to 1µl, which would not allow enough mix to do one run. Differences were however measurable and reproducible using a more traditional RT-PCR system (followed by Southern blotting and hybridisation analysis) which was further developed in this study. This study has provided useful data with regard to quantification of these transcription factors using the real-time PCR technique, and can provide further suggestions for future work. However, further work is required on developing the method for quantification of AhR2 and ARNT2. Work is being carried out in our laboratory using the more sensitive molecular beacons as probes (Dr W. Starky pers comm.). From the present study therefore we can suggest the use of molecular beacons for AhR and ARNT quantification as they would probably offer a more accurate method of quantification for low transcript detection.

Standardisation between reactions

One of the major drawbacks in detecting gene transcript levels using any (molecular) system is the standardisation of reactions. This becomes particularly relevant when investigating factors which up- or down-regulate an unknown number of different metabolic pathways. As the effects of polluting chemicals have the ability to affect many different bodily systems in the flounder and disrupt numerous enzyme pathways, (many of which are unknown but currently the subject of investigation in the GENIPOL project), it was decided not to use any housekeeping genes for this study. Samples were all quantified at the input stage, and precisely the same amount of template was used in the start point of each reaction, both for real-time PCR, RT-PCR studies, and northern blotting. This

approach has been found to be an acceptable method of target quantification (Tricarico, Pinzani, Bianchi, Palierani, Distante, Pazzagli, Bustin and Orlando, 2002), however none of the methods used for standardisation between samples is completely satisfactory, and individual experimental conditions will dictate what the most appropriate normaliser should be (Bustin, 2000). Single housekeeping gene levels should not be used to normalize RNA levels (Tricarico *et al*., 2002), and therefore the ability to achieve absolute quantification of target nucleic acids makes the search for a relevant and universal means of comparing and normalising between samples very urgent (Bustin, 2000). An ideal situation would involve isolating a panel of several genes (including, but not limited to: tubulin, β-actin, ribosomal RNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)) which are shown to be non-differentially expressed (or as close as practically possible to zero differtiation) following the treatments used in experimentation, and each of these panel included in the analysis.

Future applications of real-time PCR to toxicological / biomonitoring studies

This study has highlighted many of the benefits to the application of real-time PCR to biomonitoring studies, and investigations of gene transcript levels, including high sample throughput, sensitive detection (achievable without the use of isotope), and ease of data collection (no further image analysis is necessary such as with RT-PCR and Northern blot methods) (see also Dixon *et al*, 2002). However, it has also illustrated the stringent optimisation tests which must first be carried out in order to produce a robust and reproducible assay. Future suggestions for continuation of this study would be the use of a master mix which contains SYBR® Green I dye to ensure reproducibility. One major concern encountered in this study is the need for

'cleanliness' when setting up assays, and the ease of cross-contamination, as was found during initial trials of the machine. This was overcome by using filter tips, and multiple sets of 'clean' pipettes.

Summary

Successful isolation of partial sequences for flounder AhR2 and the previously unreported flounder ARNT2 was carried out, and allowed development of a quantitative PCR assay which would potentially allow quantitation of the transcription factor gene expressions. Quantitative RT-PCR has been used to demonstrate differential expression of CYP1A mRNA induction in previous toxicological studies in Atlantic salmon (*Salmo salar*)(Rees, McCormick, Vanden Heuve and Li, 2002) and success was achieved in this study. Real-time PCR using a single intercalated dye procedure is an effective, rapid, sensitive and simple method for quantitative determination of CYP1A mRNA transcript abundance in flounder tissues over a wide dynamic range, which easily covers those found in both uninduced and induced animals (Dixon *et al.*, 2002 and present study). For future studies on low transcript detection (e.g. AhR2 and ARNT2) the use of molecular beacons or similar would be recommended.

Chapter 5 *Ah* gene battery responses in the European flounder, *Platichthys flesus* (L.)

5.1 Introduction

Uptake, metabolism, pathological effects and regulation of genes responsible for poly-aromatic hydrocarbon (PAH) and poly-chlorinated biphenyl (PCB) metabolism (the *Ah* gene battery) have been extensively studied in benthic flatfish species including the European flounder and plaice, both in the lab (Alkidini, Brown, Waring and Collins, 1996; Eggens *et al.*, 1996) and in the field (Sulaiman, George and Burke, 1991; Bogovski, Sergeyev, Muzyka and Karlova, 1998; Bogovski, Sergeyev, Muzyka and Karlova, 1999) as discussed in the general introduction. The inductive response of the Ah gene battery to planar PAHs and PCBs is of particular interest to biological effects monitoring for environmental impact assessment. However, the study conducted by Eggens *et al*. (1996) has questioned the validity of these measurements in severely PCB polluted environments since a non-responsiveness to exposure was documented in the flounder. The variation in transcriptional responses of the genes of the PAH / PCB metabolic pathway (CYP1A and GSTA) and the transcription factors (AhR and ARNT) involved in the inductive response were studied in artificially reared juvenile flounder.

Initially studies were therefore carried out to identify a suitable chemical which induced levels of CYP1A mRNA in our experimental group of fish, and could be used to investigate inter-animal variation in expression levels. Several studies have utilised the PAH, BaP which is known to produce an inductive response in many species including the European flounder (Eggens *et al.,* 1996; Bogovski, *et al.,*

1999). In the current study post metamorphosed flounder (*Platichthys flesus*) were challenged with three PAHs, benzo(a)pyrene (BaP), β-naptho-flavone (BNF), and 3-methylcholanthrene (3MC), and the commercial PCB mix Aroclor 1254. Juvenile flounder (6-12 months of age, 1-7g in weight, 2-7cm in length) were used to minimise sex-specific differential CYP1A induction (observed naturally: George, Young, Leaver and Clarke, 1990; The and Hinton, 1998; Winzer, Cornelis, Van Noorden and Kohler, 2002). A variety of exposure times and xenobiotic doses were used in order to establish optimal exposure conditions. Once the initial trials had been carried out, a large-scale trial was conducted using the commercial PCB mix, Aroclor 1254.

In this chapter, transcriptional responses of the Ah battery gene pathway were measured by determining RNA levels using Northern blot, and RT-PCR followed by Southern blot analysis. The focus of the study was CYP1A and associated transcription factors (AhR2 and ARNT2). The liver is the main site of detoxification in the body of most animals, and several studies have demonstrated elevated levels of CYP1A in the livers of flounders which are directly correlated with pollution gradients of sedimentary pollutants (Sulaiman *et al.*, 1991; Beyer *et al.*, 1997), therefore this study focused on hepatic RNA. CYP1A was further investigated by real-time PCR techniques (as developed in Chapter 4.2). The phase II enzyme Glutathione-*S*-transferase A (GSTA), mRNA expression (which is also potentially inducible by PAHs) was also investigated using Northern blot techniques. Results of the above are presented for control fish (baseline data), and PAH / PCB treated fish (section 5.2). Expression of detoxification genes (CYP1A, GSTA, AhR2 and ARNT2) was further correlated with family history in order to identify any possible patterns, and results are presented below (section 5.3).

5.2 Inter-animal variation in levels of *Ah* battery gene transcript expression in the flounder

In order to assess levels of inter-individual variation in expression of Ah battery gene expression following xenobiotic exposure, trials were carried out with several potential inducing chemicals, as described below. Variation in basal levels of expression was also investigated using control fish for each trial. Initial trials were carried out using the known *Ah* inducer, benzo(a)pyrene (BaP).

5.2.1 Exposure of flounder to benzo(a)pyrene

BaP has been demonstrated in causing an inductive effect of CYP1A in fish when administered as an aqueous dose at environmentally relevant concentrations (e.g. 10µg/l in killifish (Van Veld *et al.,* 1997)), and therefore this method was attempted first in flounder. All fish used were artificially reared flounder from lab crosses. Fish were between 6 months to 1 year old, weighing 1-7g, and 1-7cm in length. Exposures were carried out in 6 l plastic tanks, containing aerated 32ppt ±1 seawater at a constant 8^{o}C, in darkness. Control fish were also kept in separate 6 l tanks under identical conditions. Animals were transferred from the communal aquarium to experimental tanks one week prior to exposure to allow them to acclimatise. All fish were starved during treatment, as starvation standardises conditions further by negating any possible metabolic effects. BaP was dissolved in appropriate volumes of DMSO vehicle with vigorous stirring to attain the required concentrations (from 25-500µg/l). The appropriate amount was aliquoted into each tank, and once the tank was dosed, the water was stirred to ensure thorough mixing of the BaP / DMSO cocktail (however a visible cloudiness was observed which may indicate insolubility of the BaP, this is discussed further in section 5.5). Control

tanks were dosed with the corresponding amount of DMSO vehicle. Studies in the flatfish dab (*Limanda limanda*), and previous trials in our laboratories with flounder, have shown that intraperitoneal administration of BaP at a dose of 50mg/kg causes a significant induction of CYP1A enzyme activity (EROD), and trials were also carried out with flounder at doses of 20-100mg BaP /kg body weight (see Appendix ##).

Post exposure – tissue sampling

Flounder were sacrificed 72-192 hrs after treatment with a single cephalic blow. They were measured, weighed and the livers were excised. Care was taken to minimise time between sacrifice of the fish and dissection of livers due to the rapid degradation rate of mRNA. *Ca*. 100mg of the excised liver was then transferred immediately to 1ml Tri Reagent (Sigma, UK), and homogenised for thirty seconds with a hand-held Polytron homogeniser (T25). Homogenate in Tri reagent was stored at -40^{o}C for subsequent RNA extraction. A tissue sample (fin clip) was removed from each fish, and stored in absolute ethanol for subsequent DNA extraction to allow family assignment via microsatellite analysis.

Hepatic RNA preparation

Samples in Tri reagent were allowed to defrost fully at room temperature (5-10min) before RNA extraction. Total hepatic RNA was extracted from the Tri Reagent homogenate according to the manufacturer's protocol. Aliquots of the RNA samples were quantified spectrophotometrically (see Appendix I.2), and all samples diluted to 1mg/µl in molecular grade dH_2O (BDH, UK) to simplify assay. Samples were then stored at –80^{o}C until required.

Blot preparation

Northern blots were prepared for all samples and probed with the CYP1A cDNA probe as described in Chapter 4.2, and an example blot is presented in Figure 5.1 below.

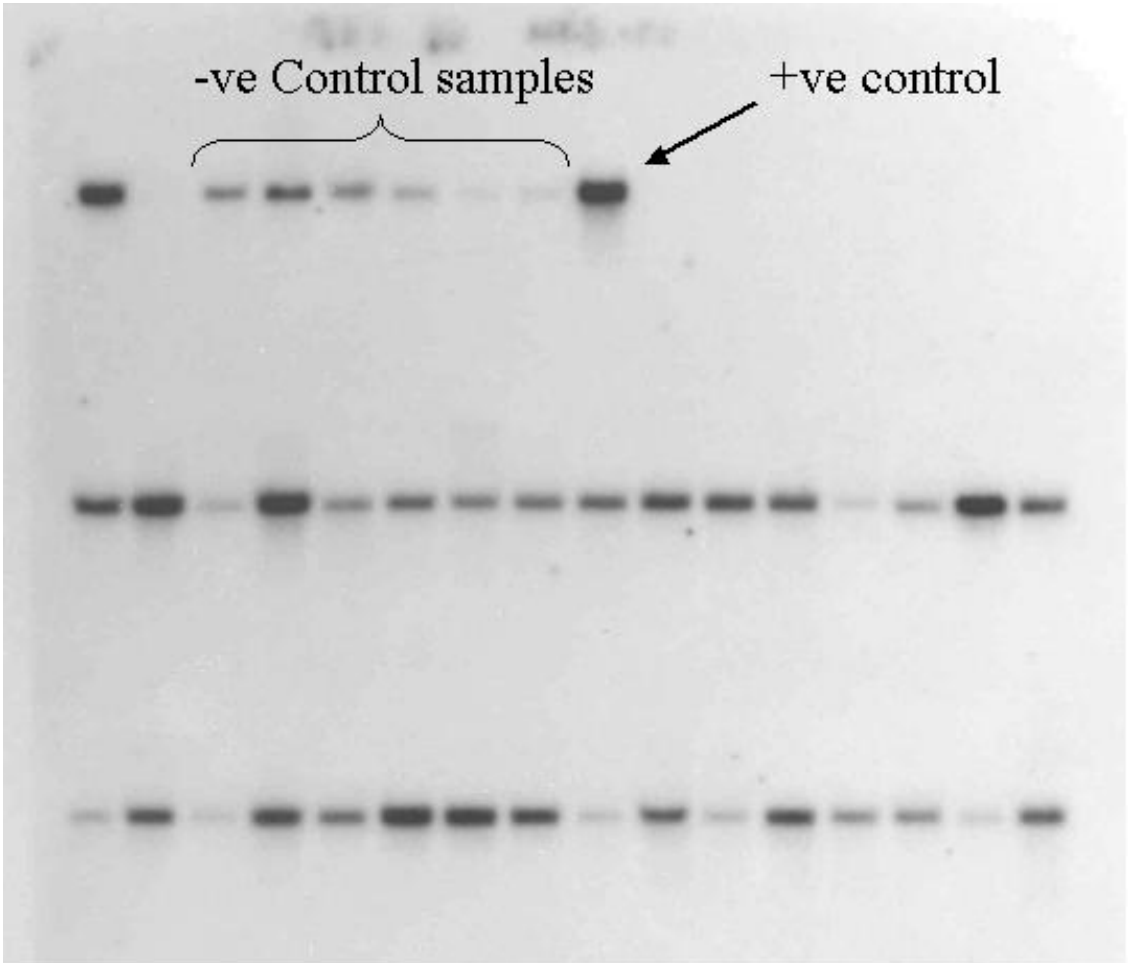

Figure 5.1: An example of an autoradiograph of a Northern blot of flounder total RNA (10µg total RNA / track) probed with ^{32}P labelled full length CYP1A cDNA probe. Each lane is a separate flounder sample, each following treatment with sea-water dosed to 30ppm BaP for 72 hrs. Positive and negative control samples are as previously described, are included for comparison purposes, and are indicated above.

The results showed that even in control flounder there was a large inter-animal variation in CYP1A mRNA expression. A proportion of the treated fish appeared to display elevated levels of CYP1A mRNA levels. However, following semi-quantitation via densitometry analysis of the autoradiographs and subsequent t-test analysis, no significant induction of CYP1A was found (P = 0.77). An F-test showed that no significant difference in variation of CYP1A mRNA expression was found (P = 0.3). This was apparent for the seawater dosed and IP injected BaP trials.

5.2.2 Comparison of potential inducers – Exposure to 3MC, BNF, BaP and Aroclor 1254

Following low levels of expression of CYP1A mRNA following exposure to BaP, a comparison of inducing chemicals was carried out. Large male fish (>100g, 2 yrs old) were exposed to four different chemicals to assess effects and inducer potential. Large fish were chosen for these trials to test possible intraperitoneal injection. The four chemicals used were BNF, BaP, 3MC and the commercial PCB mix, Aroclor 1254.

Table 5.1 Exposure of flounder to four inducing chemicals. Inducer type, dose, number of fish per trial and exposure time details are described.

Exposure type / inducer	Dose	No. Fish	Exposure time (hrs)
IP / BNF	25µg/g	3	72
IP / BaP	25µg/g	3	72
IP / 3MC	20µg/g	3	72
IP/ Aroclor 1254	100µg/g	3	72

Following exposure trials, fish were sacrificed, and hepatic RNA was extracted as described in section 5.2.1. A Northern blot of the different potential inducers was prepared, and is presented below (Figure 5.2):

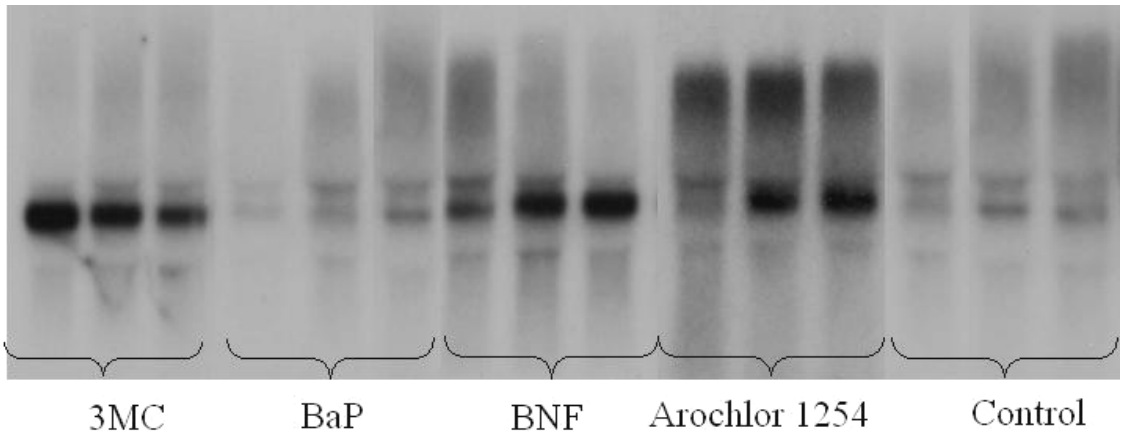

Figure 5.2: An example of an autoradiograph of a Northern blot of flounder total RNA (10µg total RNA / track) probed with ^{32}P labelled CYP1A cDNA full length probe. Each lane represents a separate individual, and each treatment includes three individuals. Groups were treated with 3MC, BNF, BaP and Aroclor 1254 (from left to right). Control (untreated) samples are included for comparison purposes.

Data analysis

Blots were analysed as described previously (Chapter 4.3), and results of the above blot are presented in Figure 5.3 below.

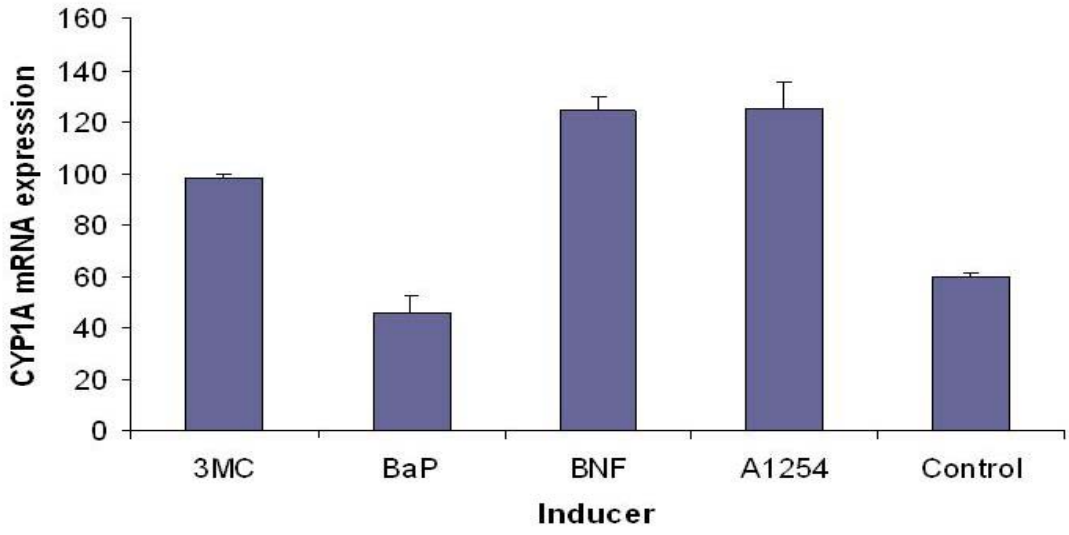

Figure 5.3: Mean CYP1A mRNA induction as deduced from image analysis of a Northern blot (Figure 5.2) following treatment of fish with different inducers. Error bars are standard errors of the means.

Normalised data was analysed for mean, median, standard deviation (SD), sample variance and skewness. These data are presented in Table 5.4 below, along with minimum and maximum for each. A one way ANOVA showed highly significant differences between the treatments ($P < 0.001$, $F = 15.14$).

Table 5.2: Expression values of CYP1A following treatment with various suspected inducers. Values for mean, minimum, maximum, median, standard deviation of the mean, sample variance and skewness of CYP1A are presented for each different inducer, including control samples.

Inducer	Mean	Min	Max	Median	SD	Variance	Skewness
3MC	98	92	101	100	4.6	21	-1.68
Benzo(a)pyrene	46	19.3	63.2	55	23.3	543	-1.49
BNF	124	105	137.3	131	16.9	286	-1.44
Aroclor 1254	125	89	159	127	34.8	1214	-0.21
Control	59	54.8	66.5	57	6.2	38.9	1.5

Fish treated with benzo(a)pyrene (BaP) showed no clear patterns of induction, and no significant elevation of levels of CYP1A, as has been found in previous trials in our laboratory (M. Leaver pers. comm.). The dose / time trials which were carried out using BaP returned a similar result, with no clear patterns of induction of CYP1A being shown, and no correlation was apparent between levels of exposure to chemicals and induction of CYP1A. This may however be partially attributable to a lack of solubility of the BaP in the vehicle used for the water borne exposure. However, the IP trial did not yield any significant induction of CYP1A at a dose of 25mg BaP / Kg body weight. Initial trials of 3-MC and Aroclor 1254 displayed a clear inductive effect (see below). As we were particularly interested in PCB exposure due to the prior history (Liverpool bay study, Leah, 1999), Aroclor 1254 was selected as an inducing chemical, and further trials were carried out as detailed below.

Exposure of flounder to Aroclor 1254

The above trial demonstrated that flounder showed elevated levels of CYP1A (i.e. induced) upon exposure to the commercial PCB mixture, Aroclor 1254, and the remainder of this study focuses on the use of Aroclor 1254 as an inducer. A large scale trial was set up in order to follow effects of Aroclor 1254 upon PAH / PCB metabolising enzymes CYP1A and GSTA, and the CYP1A transcription pathway AhR2 and ARNT2 levels. One hundred fish (derived from 8 families) were selected at random from the holding tanks of flounder which were artificially reared for this study (Chapter 2), and transferred to 70 l tanks containing 32ppt ±1 aerated seawater at a constant 8°C in darkness, and held for one week prior to exposure to allow them to acclimatise. Fish were anaesthetised by immersion for five minutes in

a seawater (32±1ppt) solution containing 1ml/l phenoxyethanol, weighed, and then given an I.P. injection of Aroclor 1254 (dissolved in olive oil (100mg/ml) as carrier) using a micro-syringe (Hamilton) at a dose of 100mg Aroclor 1254/kg body weight. After recovery following submersion in highly aerated seawater for 30min, fish were transferred back to the 70 l experimental tanks for 72 hours until sacrifice. Eighty fish were selected at random from holding tanks, and transferred to experimental tanks to provide control (baseline) data for the exposure trial. The control fish were anaesthetised as above, and injected with olive oil vehicle alone.

Northern blot analysis

Flounder were sacrificed, and livers dissected as described previously. Northern blots were prepared for all treated and control group fish following methods described in section 4.2, and probed with the pleuronectid CYP1A and GSTA cDNA probes. Figure 5.4 below shows an example of an autoradiograph of a Northern blot of total RNA from Aroclor 1254 treated and control fish probed with the ^{32}P labelled CYP1A cDNA probe. Figure 5.5 is an example of the corresponding agarose gel image of the total RNA prior to blotting, and was used to normalise results against. An example of the densitometry plot for the corresponding blot is also shown (Figure 5.6).

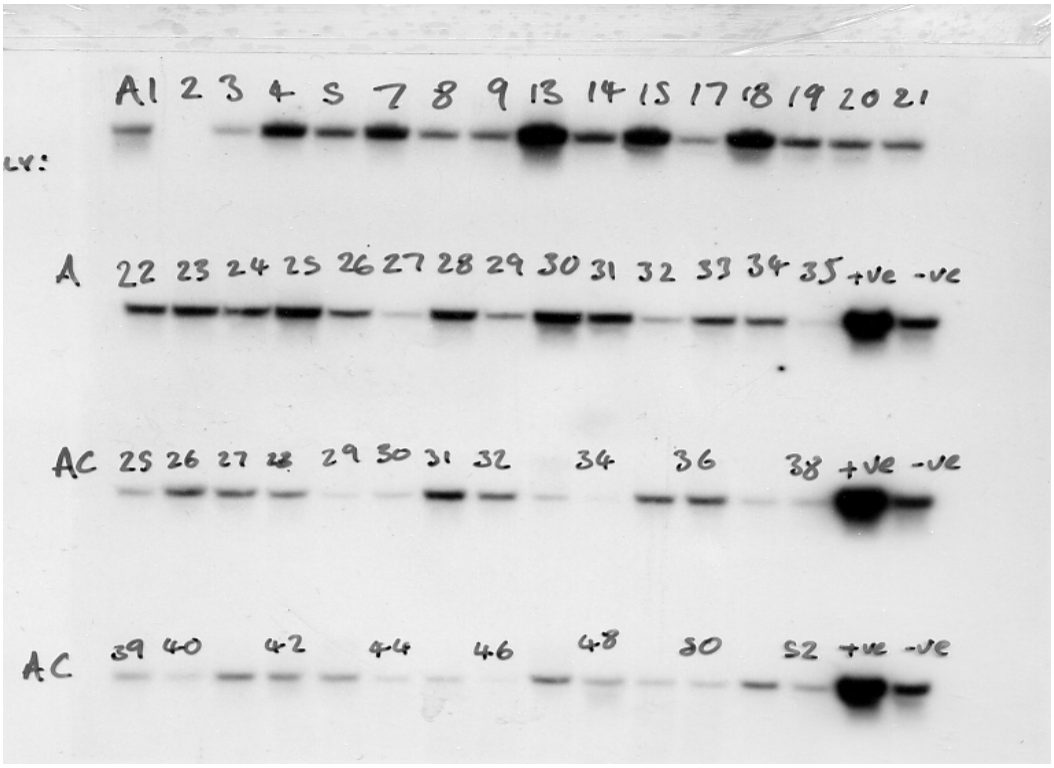

Figure 5.4: An autoradiograph showing Northern blot of 100mg Aroclor 1254 / kg body weight treated (A) and control (AC) flounder hepatic total RNA, probed with ^{32}P labelled CYP1A cDNA probe. Each lane is a separate individual. +ve and –ve samples are adult flounder hepatic total RNA samples following IP treatment with 100mg Aroclor 1254 / kg body weight and vehicle only from a previous trial within our laboratories, respectively, and are multiple aliquots of a single preparation. Each track contains 10µg of total RNA.

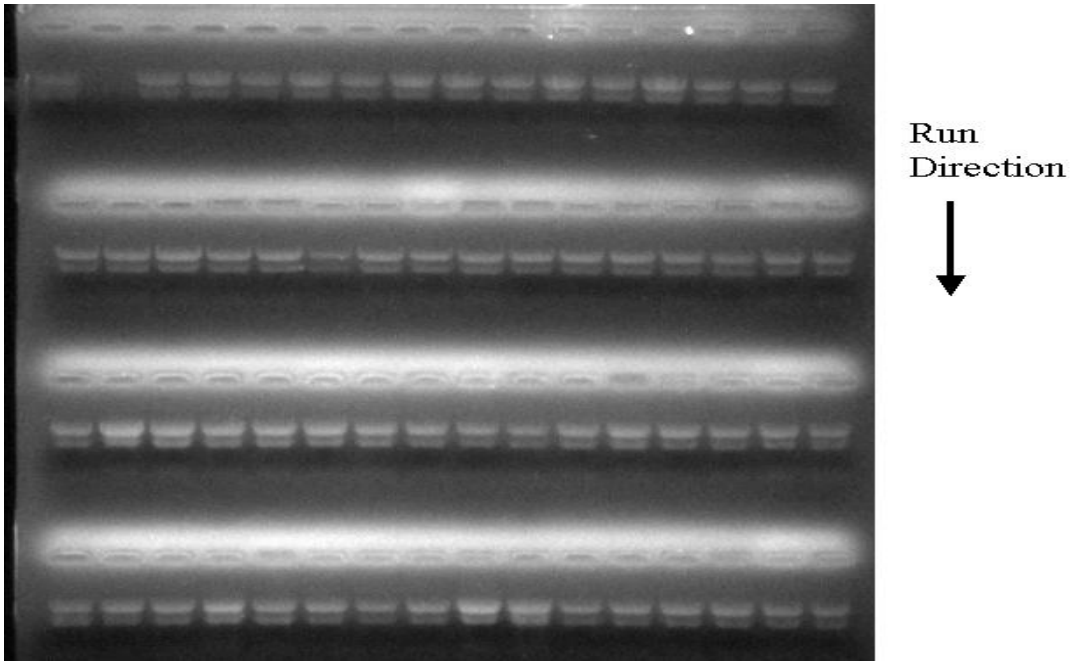

Figure 5.5: A didgitised image of the agarose gel of above blot to allow normalisation of signal to total RNA quantity (10µg total RNA / lane). Order of samples is as above, with positive and negative standards included in the right hand wells for standardisation. Run direction is indicated by an arrow on the right.

Histograms were plotted of Northern blots using Scion Image analysis. An example of a plot (Figure 5.6) is presented below. Data was analysed as described above, and is presented later in this section in Table 5.4. Inter-individual variation in CYP1A levels is presented in Figure 5.10 below.

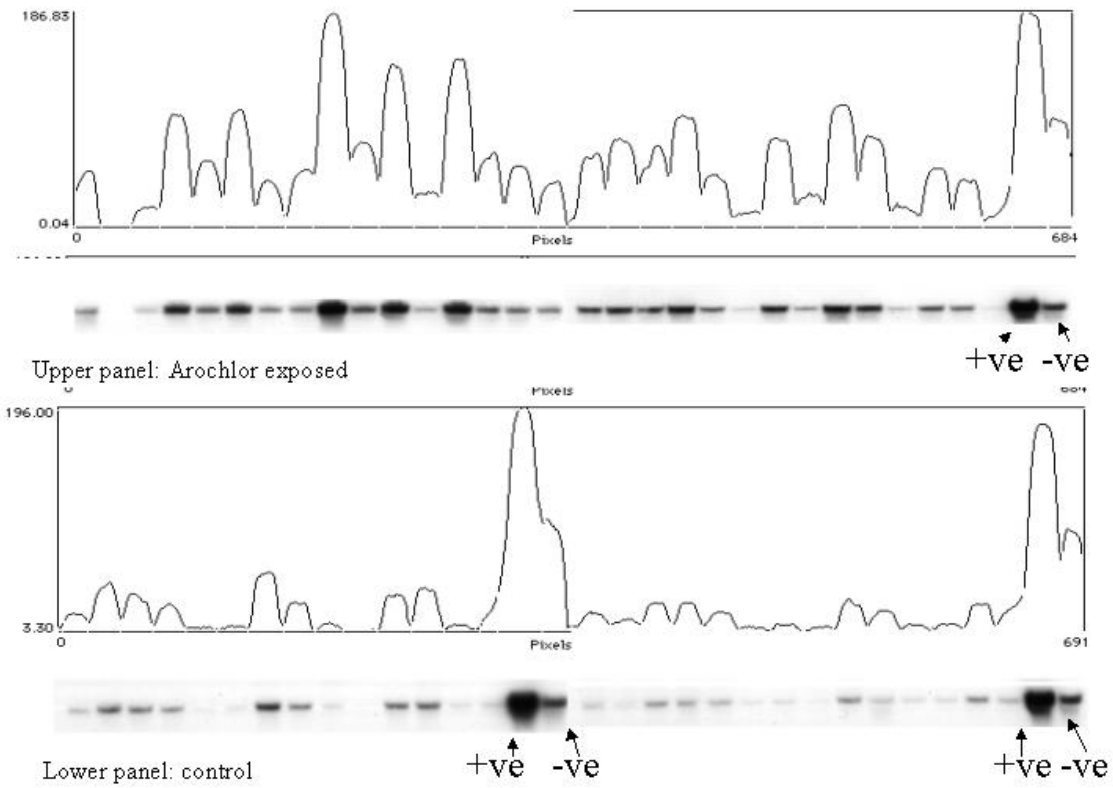

Figure 5.6: An example of a densitometry plot from CYP1A probed Northern blot analysis of Aroclor 1254 treated (upper panel) and control (lower panel) fish. The gel image (from figure 5.4) is included underneath corresponding plot, and each lane / peak represents one individual flounder. Standard positive (+ve) and negative (-ve) samples are highlighted.

5.2.3 GSTA Analysis

GSTA expression was measured in total RNA from the Aroclor 1254 treated fish using Northern blot techniques as carried out for CYP1A above. An example blot is presented below (Figure 5.7).

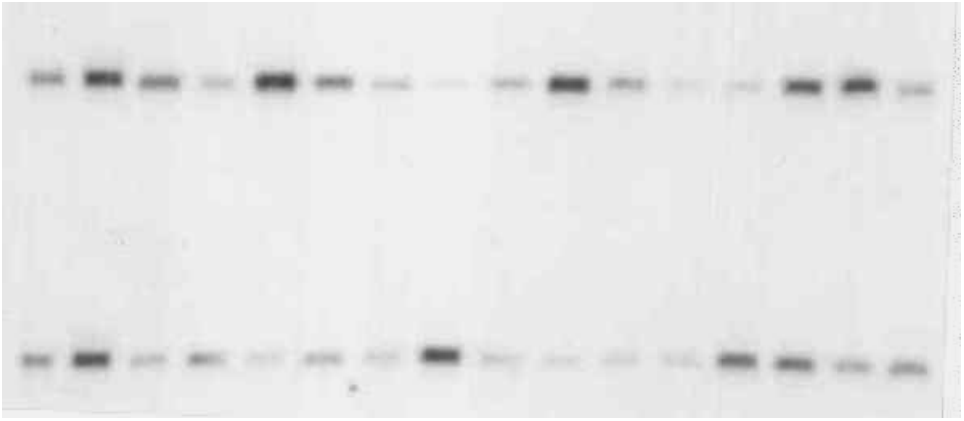

Figure 5.7: An example of an autoradiograph showing Northern blot of 100mg Aroclor 1254 / kg body weight treated flounder hepatic total RNA, probed with ^{32}P labelled GSTA cDNA probe. Each lane is a separate individual. +ve and –ve samples are adult flounder hepatic total RNA samples following IP treatment with 100mg Aroclor 1254 / kg body weight and vehicle only from a previous trial within our laboratories, respectively, and are multiple aliquots of a single preparation.

5.2.4 Transcription factor analysis

AhR and ARNT were analysed using the RT-PCR/Southern blot technique developed in section 4.2 of the current study.

AhR2 RT-PCR and Southern blot analysis

Initial Northern blot analysis carried out to investigate AhR and ARNT expression found no detectable signal when probed with fragments isolated in this study (Chapter 4). Semi-quantitative RT-PCR followed by Southern blot and image analysis provided detectable results. An example of a Southern blot for AhR2 showing exposed fish is included below (Figure 5.8).

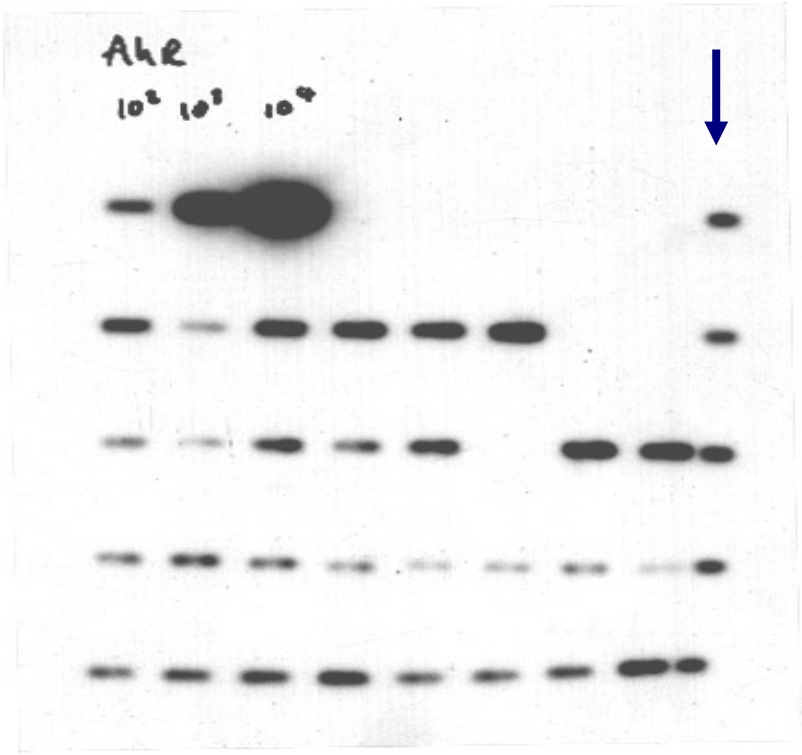

Figure 5.8: Example of an autoradiograph of a Southern blot of AhR2 RT-PCR products from cDNA from flounder exposed to Aroclor 1254. 10^2, 10^3 and 10^4 (top lane) are plasmid standards of known copy number. Each lane is a separate flounder individual. Right hand column is standard sample of known concentration to allow standardisation between gels (indicated with arrow).

ARNT RT-PCR and Southern blot Analysis

Analysis was carried out identically as for AhR, and results are presented below. Figure 5.8 is an example blot.

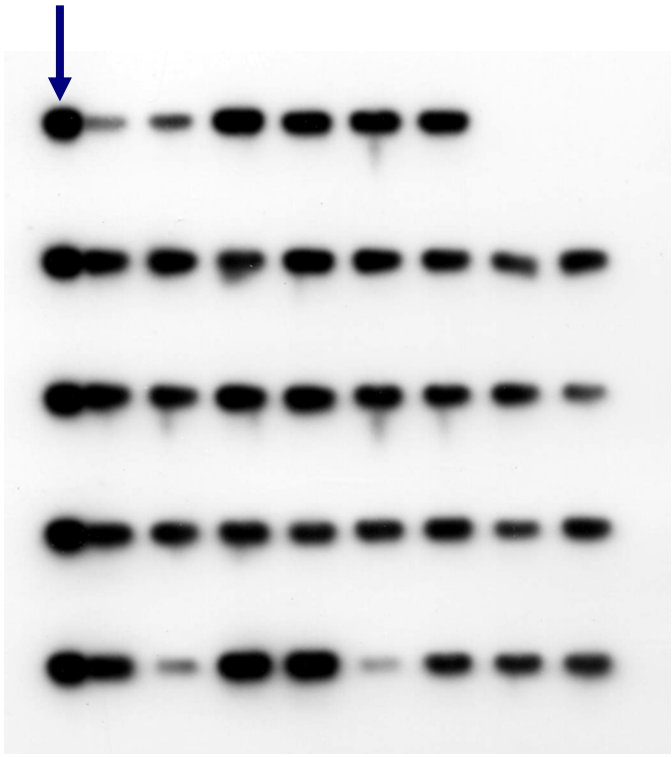

Figure 5.9: Example of an autoradiograph of a Southern blot of ARNT2 RT-PCR products from cDNA from flounder exposed to Aroclor 1254. Left hand column is standard sample of known concentration to allow standardisation between gels (indicated by arrow). Each lane is a separate flounder individual.

5.2.5 Data analysis

5.2.5.1 CYP1A

A large sample variance was found within the Aroclor exposed samples, and this is represented. Large inter-individual variability was observed both in control, and Aroclor 1254 treated fish. Variation in the treated fish was much more exaggerated than in the control fish, as seen in Figure 5.10 below. The sample variance was 6 fold higher in the treated samples compared to the control samples (410 versus 66, respectively), and they were found to be significantly different following F-test analysis ($P < 0.001$, therefore the null hypothesis was rejected). The maximum treated value was three fold higher than the maximum control value.

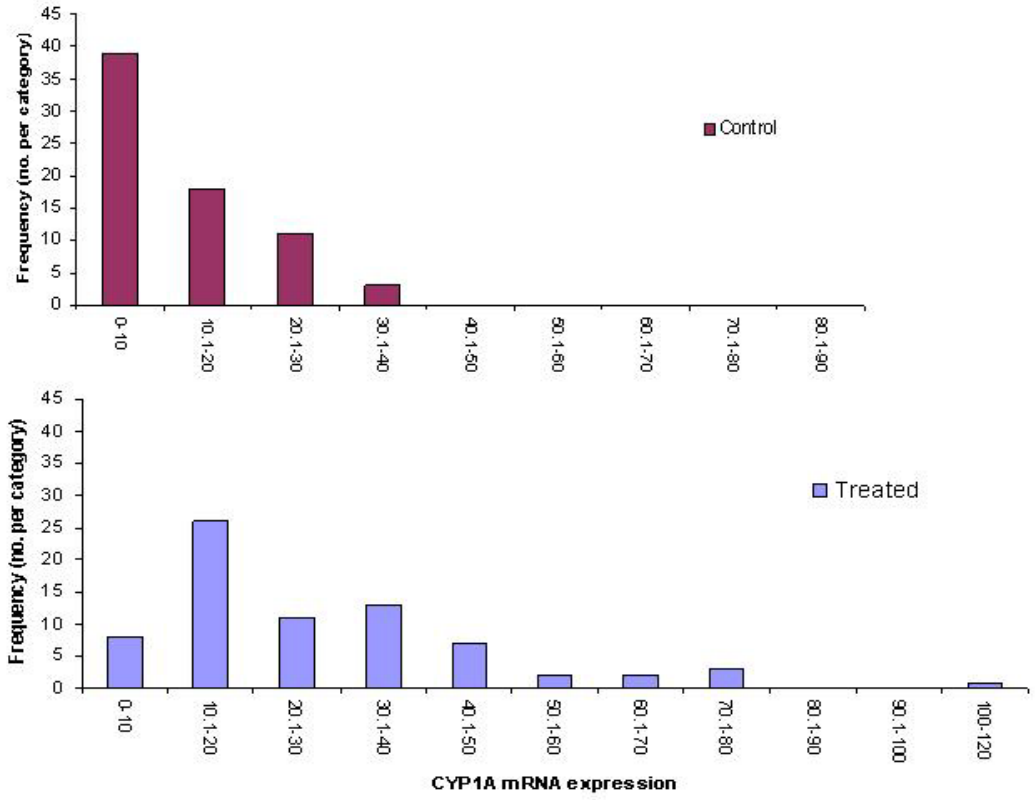

Figure 5.10: CYP1A mRNA expression versus frequency distribution in control (upper panel) and Aroclor 1254 treated (lower panel) flounder. Values are deduced from image analysis of Northern blots presented above.

Treated vs control fish

The values from densitometry analysis were used to calculate mean CYP1A expression values, and results of comparison between the two populations (treated and control) are presented below (Figure 5.11). Normality was tested using GenStat® V6, (an example test is provided below in Figure 5.12), and tests of the data showed that the data was non-normally distributed. Log transformation of the data was carried out using GenStat® prior to any analysis being carried out to allow statistical comparisons to be made.

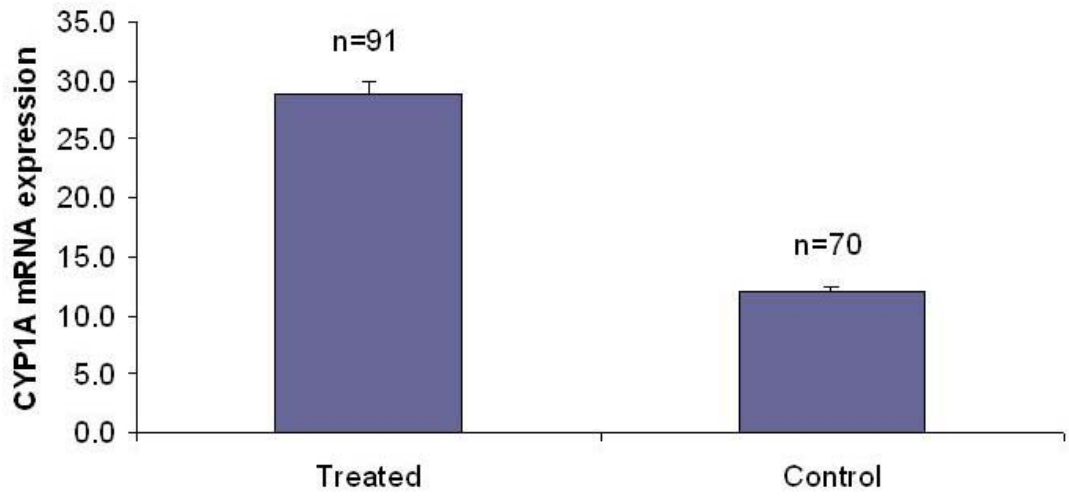

Figure 5.11: Mean CYP1A mRNA expression of control (vehicle injected) fish versus treated (Aroclor 1254 injected) fish (untransformed data). Number of fish per category is indicated above each bar, and error bars are standard errors of the means.

T-test analysis of the log transformed data returned a probability of $P < 0.001$, and the null hypothesis that there was no difference between Aroclor 1254 treated and control samples was rejected. A highly significant induction of CYP1A was observed after exposure to Aroclor 1254.

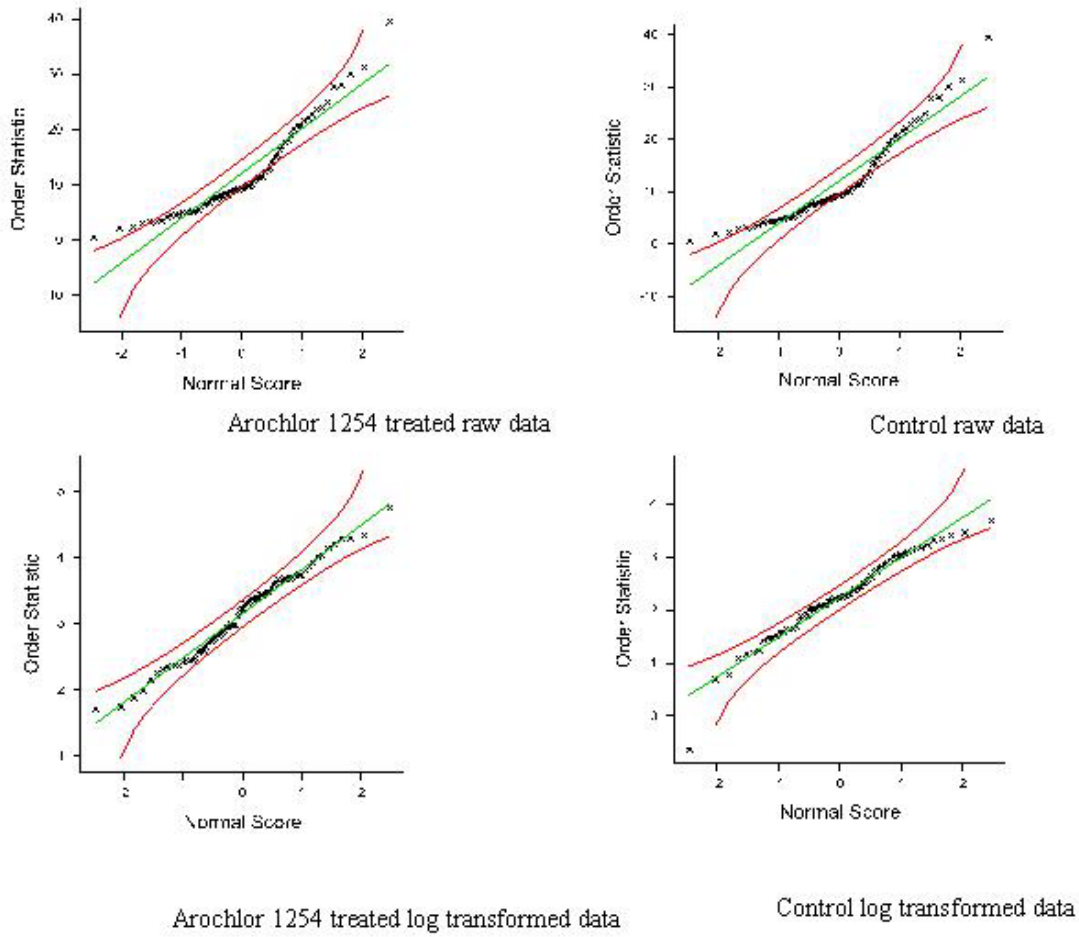

Figure 5.12: Normal Q-Q plot for data (+95% confidence limits). Upper panel is raw data, and lower panel is log transformed data. Note: In upper panels data are outside of the 95% confidence limits, following transformation, data are within 95% confidence limits.

Quantitative (real-time) PCR

Further analysis of CYP1A expression levels was carried out, and quantitative results from real-time PCR for a subset of the samples described above (Aroclor treated) are presented below.

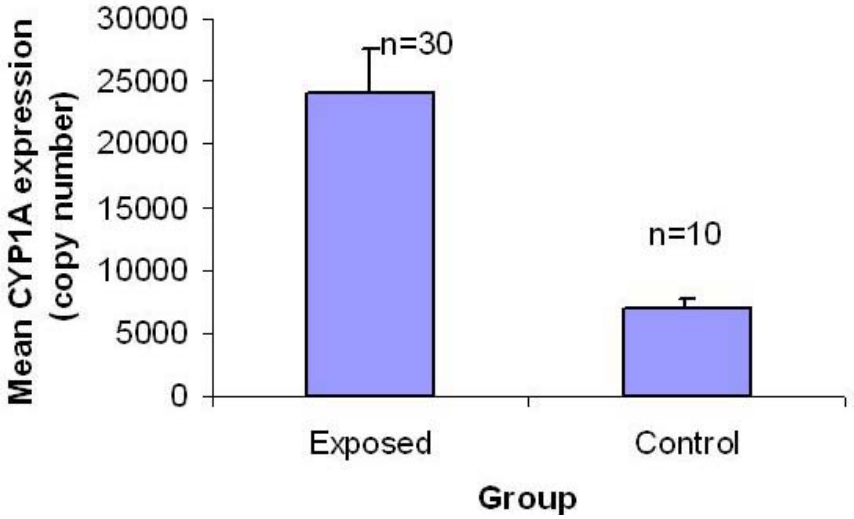

Figure 5.13: Mean CYP1A expression from Aroclor 1254 exposed and control flounder, quantified using real-time PCR (NB: copy number = copy number in cDNA derived from 50ng total RNA). Error bars are standard error of the mean.

Mean values returned from real-time PCR were 7,000 copies from control, and 24,000 copies from the exposed fish indicating a 3.5 fold increase in CYP1A expression in exposed fish. A t-test of the data confirmed that they were highly significantly different ($P < 0.001$). The above samples agreed with the findings from the Northern blot studies, and were correlated to the data obtained from Northern results.

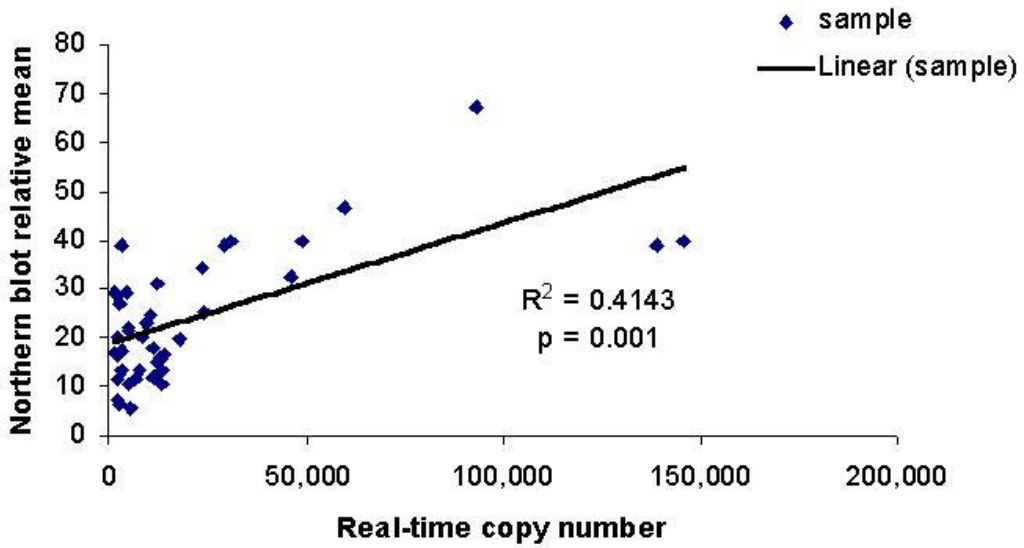

Figure 5.14: Correlation of CYP1A results returned from Northern and real-time PCR analysis – comparison of methodology. R^2 values are signifcant at the P=0.001 level, and values are provided in the legend.

Assays from Northern blot and real-time PCR displayed a highly significant correlation ($p < 0.01$), as illustrated graphically in Figure 5.14 above.

Table 5.3: Expression values of CYP1A transcript levels deduced from image analysis of Northern blots comparing treatment of Aroclor 1254 versus BaP. Values shown include inducer type, mean, minimum, maximum, median, standard deviation of the mean, sample variance and skewness of data.

Inducer	Mean	Min	Max	Median	SD	Variance	Skewness
Benzo(a)pyrene	17.1	5.0	62.2	16	9.9	97.6	2.3
Aroclor 1254	28.49	0	115.11	24.72	20.2	410.51	1.61

5.2.5.2 GSTA

Expression of GSTA was normalised against agarose (18s and 28s RNA) (Figure 5.15) and relative expression was calculated as for CYP1A. Mean relative expression of GSTA mRNA normalised against agarose (total RNA) was calculated as for CYP1A, and is presented in Figure 5.16 below.

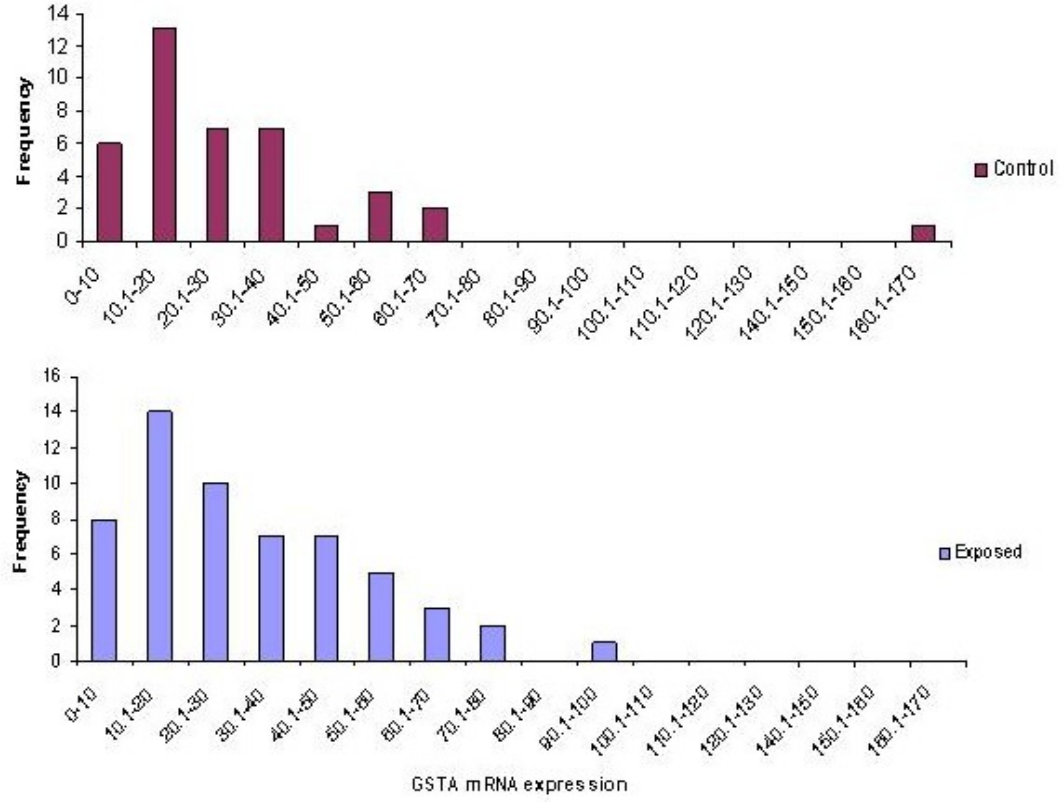

Figure 5.15: GSTA mRNA expression versus frequency distribution in control (upper panel) and Aroclor 1254 IP treated (lower panel) flounder. Values are deduced from image analysis of Northern blots presented above.

Following F-test analysis, variation in GSTA expression was found to be significantly higher (1.8 fold) in control fish than exposed fish ($P = 0.02$). However, this was caused by one outlying individual in the control group which displayed

highly elevated levels, and when this was removed from the analysis, probability was reduced to a non significant level (P = 0.13).

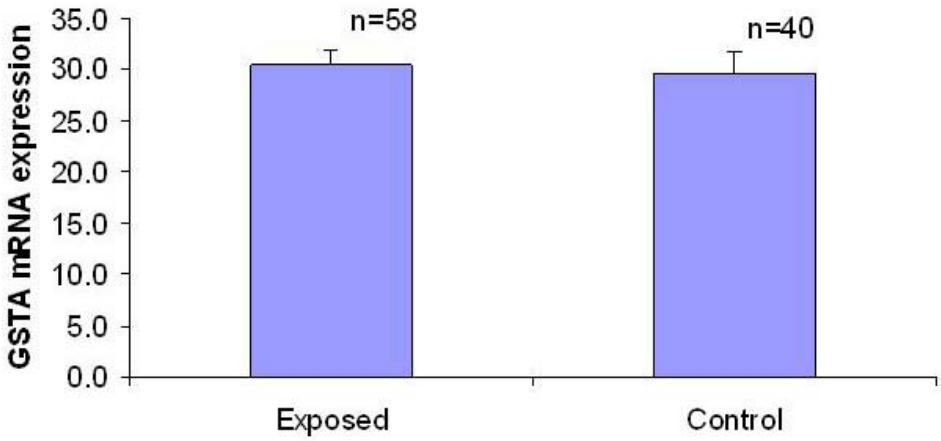

Figure 5.16: Mean expression levels of GSTA mRNA deduced from image analysis of Northern blots for vehicle (control) and Aroclor 1254 IP injected (exposed) flounder. Numbers of animals in each group are shown (above each bar), and error bars are standard errors of the means.

No significant difference was found between GSTA expression levels between Aroclor 1254 treated and control fish, and the null hypothesis was accepted. Further analysis following removal of the outlying control value confirmed the analysis (P = 0.28).

5.2.5.3 Transcription factors

The values from densitometry analysis were used to calculate expression values; data are presented in Tables 5.3 and 5.4.

AhR2 analysis

Densitometry results collected were compared for exposed and control fish (see Figure 5.18). When distribution of expression (plotted as frequency versus

expression) was measured for both control and exposed fish (Figure 5.17), the following plot was obtained.

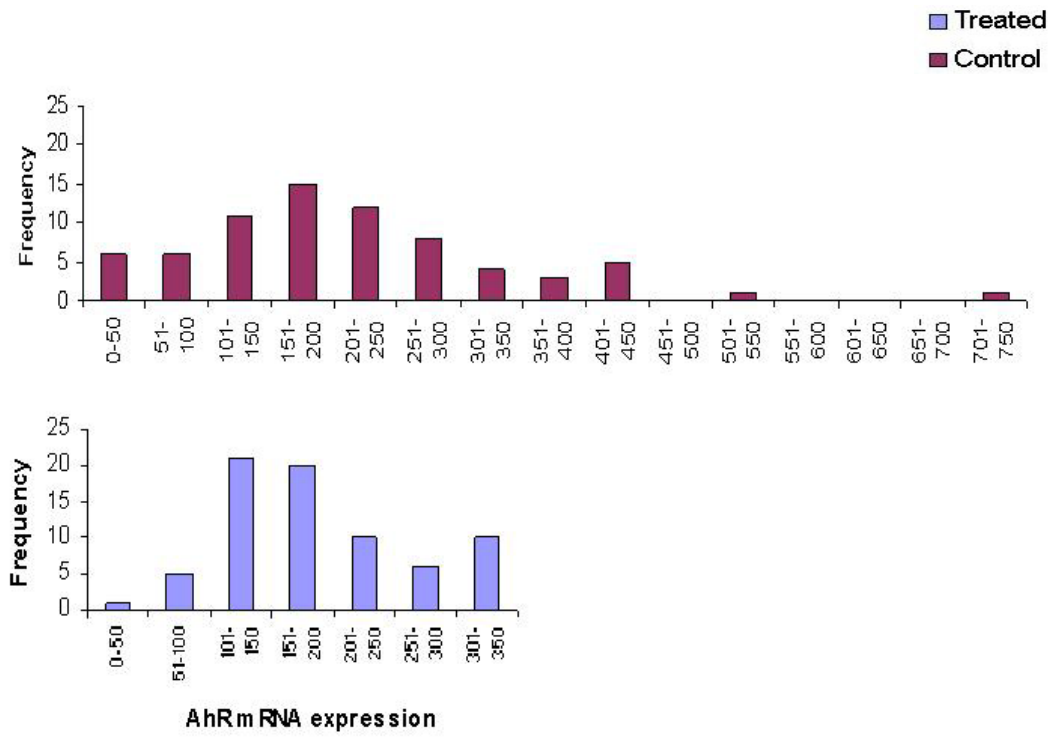

Figure 5.17: AhR mRNA expression versus frequency distribution in control (upper panel) and Aroclor 1254 IP treated (lower panel) flounder. Values are deduced from image analysis of Southern blots of RT-PCR products presented above.

Both exposed and control fish displayed a normal curve for expression levels of AhR. Several control samples were present at higher levels of expression than in the Aroclor 1254 treated group, and the maximum value was two-fold higher in controls than in exposed animals. Sample variance was found to be significantly different following F test analysis ($P < 0.01$), and was found to be ~2.5 fold higher in control than treated fish.

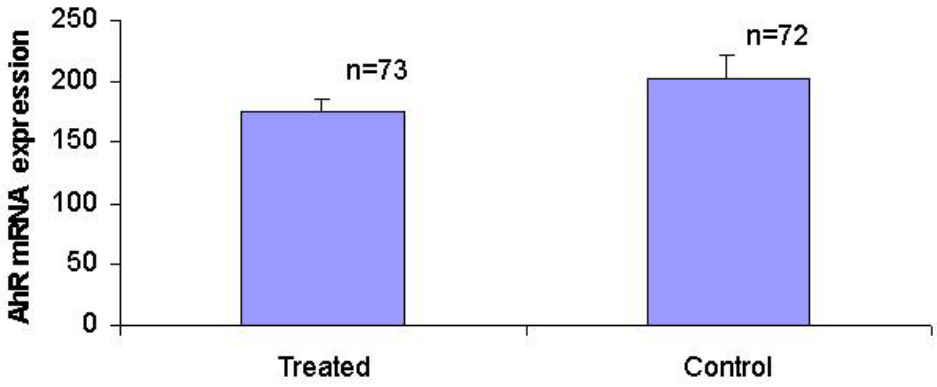

Figure 5.18: Mean expression levels of AhR mRNA deduced from image analysis of Southern blots for vehicle (control) and Aroclor 1254 IP injected (exposed) flounder. Numbers of animals in each group are shown (above each bar), and error bars are standard errors of the means.

No significant difference was found in AhR expression between control and Aroclor treated flounder, and the null hypothesis was accepted.

ARNT2 analysis

Analysis was carried out identically as for AhR, and results are presented below. Following F-test analysis sample variance was found to be significantly higher (1.3 fold) in the control group ($P = 0.02$) than the Aroclor 1254 exposed group.

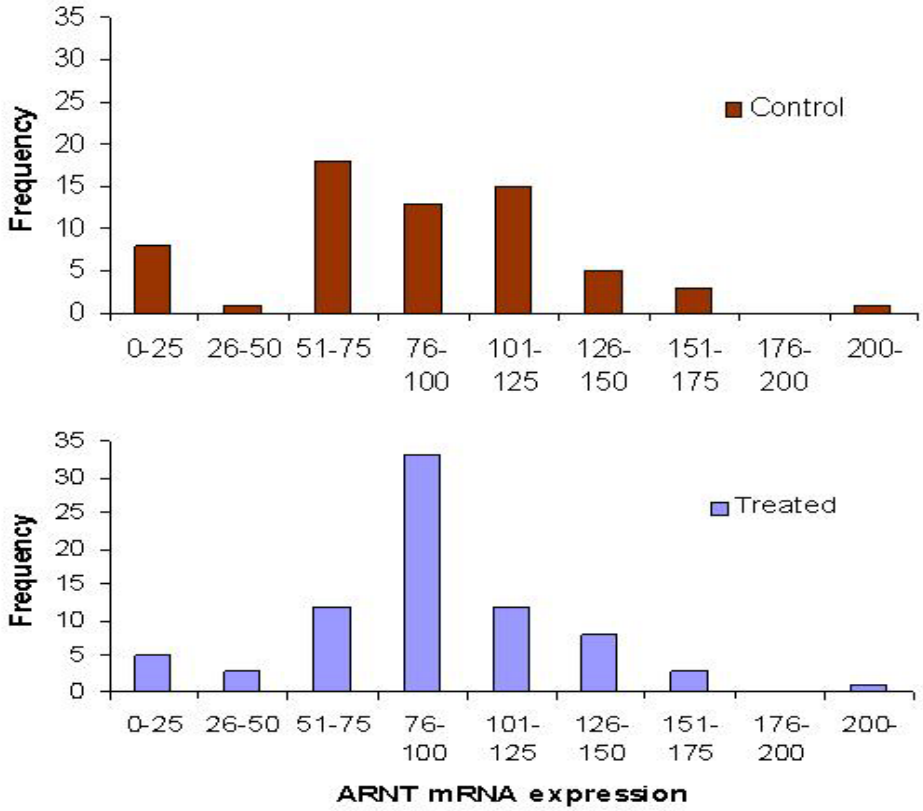

Figure 5.19: ARNT mRNA expression versus frequency distribution in control (upper panel) and Aroclor 1254 IP treated (lower panel) flounder. Values are deduced from image analysis of Southern blots of RT-PCR products presented above.

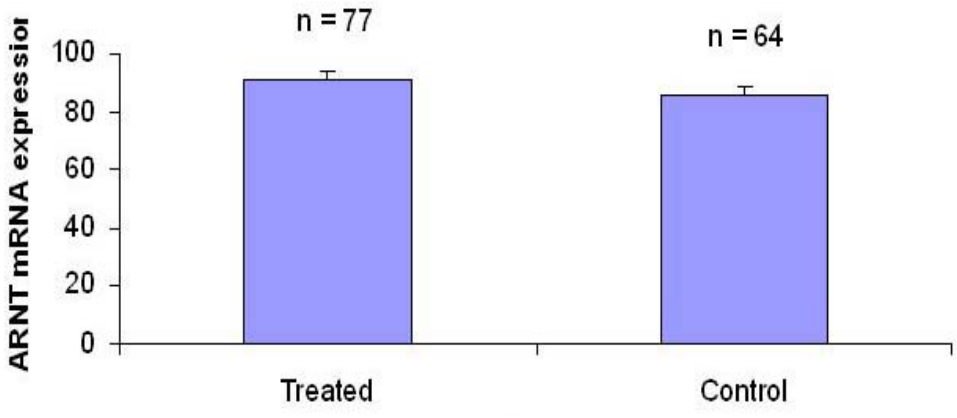

Figure 5.20: Mean expression levels of ARNT mRNA deduced from image analysis of Southern blots for vehicle (control) and Aroclor 1254 IP injected

(exposed) flounder. Numbers of animals in each group are shown (above each bar), and error bars are standard errors of the means.

No significant difference in ARNT expression was found between individuals from exposed or control groups.

5.2.5.4 Summary of data anlaysis

Data from CYP1A, GSTA, AhR2 and ARNT2 analysis are summarised in Table 5.3 and Table 5.4, for control and Aroclor 1254 treated data respectively.

Table 5.3: Basal (control) level expression of *Ah* battery gene transcript expression showing mean, minimum, maximum, median, SD, variation and skewness of data from control fish mRNA expression levels used in various trials.

Gene	Mean	Min	Max	Median	SD	Variance	Skewness
CYP1A	12.01	0.53	39.47	9.39	8.17	66.78	1.14
GSTA	29.58	0	165.27	21.74	27.94	780.69	3.13
AhR	211.32	0	715.5	191.19	129.56	16786.53	1.04
ARNT	91.35	0	233.65	93.21	40.35	1628.47	-0.04

Table 5.4: Expression of *Ah* battery gene transcript expression following treatment with Aroclor 1254 showing mean, minimum, maximum, median, SD, variation and skewness of data

Gene	Mean	Min	Max	Median	SD	Variance	Skewness
CYP1A	28.8	0	115.1	23.1	20.94	438.6	1.53
GSTA	30.58	0	91.86	28.49	20.63	425.76	0.86
AhR	182.10	0	343.76	172.68	81.12	6580.31	0.25
ARNT	91.58	11.10	206.04	89.23	34.70	1204.09	0.30

Table summary

CYP1A gene transcript expression was induced following IP exposure to Aroclor 1254, and accompanied by an increase in variation among samples (i.e. inter-animal variability). Aroclor 1254 appeared to have no inductive effect on GSTA, AhR2 or ARNT2 gene transcript expression levels. Values which were observed as zero (for minimum values) were below the detectable range of analysis possible using the techniques employed in this study.

5.2.6 Interactions of the *Ah* battery gene expression

Values obtained in CYP1A, GSTA, AhR and ARNT analysis were all correlated separately using regression (least squares method, Minitab V.10) analysis to enable any trends or patterns to be identified. No correlations were apparent between transcript levels of any gene / transcription factors which were tested in this study. However, a significant correlation was apparent between AhR2 versus ARNT2 expression.

Correlation of the transcription factors AhR vs ARNT

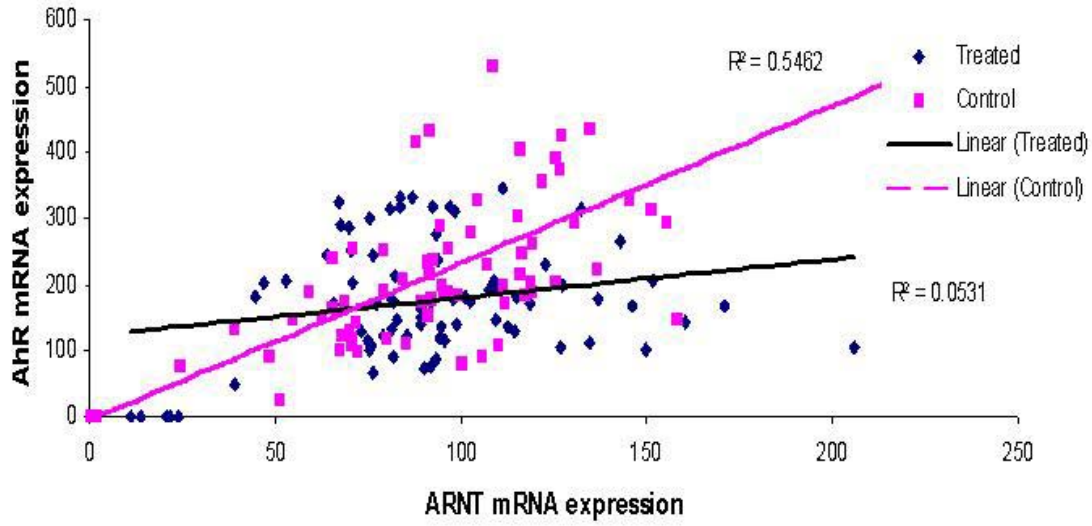

Figure 5.21: Regression analysis of AhR versus ARNT mRNA expression levels deduced from RT-PCR and Southern blot analysis as described above. R^2 values are shown next to each linear regression line. Data are divided into control (pink squares) and exposed (blue diamonds).

No correlation was apparent between AhR and ARNT measured in flounder from the exposed group. However, a strong correlation was found between AhR and ARNT expression in the control group of fish ($R^2 = 0.55$, $P < 0.01$), and an increase in AhR mRNA expression resulted in an increase in ARNT mRNA expression. Multiple regression analyses using the least squares method (in Minitab V.10) were carried out using CYP1A as the dependent data group, and AhR and ARNT as the independent groups. This analysis also returned no significant values.

5.2.7 Regression analysis of detoxification characteristics with physical characteristics of the animal

Regression analysis (least squares method, Minitab V.10) was carried out between physical characteristics of the fish and expression levels of detoxification systems to

ensure that the variation in levels of expression detected in this trial was not due to effects of length or weight of the animal. As immature (juvenile) fish were used in all experiments, it was not possible to correlate any expression levels with sex of the animal (however this should not affect the results has has been found in previous trials using juvenile fish (George *et al.,* 1990). No significant correlations were found between any physical characteristic (length, weight or condition factor) and expression levels of any system (CYP1A, GSTA, AhR or ARNT) (results in Appendix III.3.1).

5.3 Genetic variation in *Ah* battery expression

Fish from exposure trials (from section 5.2) were genotyped, and identification of offspring to their parents was carried out using three of the microsatellite markers isolated in this study (Chapter 3). Two further loci isolated in Plaice (*Pleuronectes platessa*), LIST1007 and LIST1009 (Watts *et al*., 2001) were also employed to increase the power of analysis as it was not possible to match all parents using loci isolated in the current study. Problems were found with interpretation of results returned by LIST1009, and it was later disregarded from the analysis. Using LIST1007, and three loci from this study, parental identification of the trial families was possible.

5.3.1 Parental identification

Microsatellite markers were used as tools in family identification for the cross breeding studies. Offspring were raised and exposed to polluting compounds (see section 5.2) in communal tanks in order to standardise all environmental factors and investigate phenotypic variation in relation to genetic variation (i.e. $V_p - V_e = V_g$). Microsatellite markers were used to produce a genetic profile of the offspring used in these trials which was then compared with those of the parental fish. Primers from the chosen loci were fluorescently labelled (MWG Biotech, UK), and semi-automated genotyping was carried out on an ABI 377 PRSIM™ DNA sequencer (PE Applied Biosystems). Analysis of allele data and comparison of the loci was carried out using 'Family Assignment Package (FAP) version 3.1.1' software (Dr JB Taggart pers. comm., Institute of Aquaculture).

Tissue sampling and collection of samples

Fin clips were taken from the parental fish at the time of setting up the crosses, fixed in absolute ethanol, and transported to the Institute of Aquaculture for storage until required. Tissue samples (as fin clips) were later taken from all offspring used in exposure experiments once sacrificed, and preserved in absolute ethanol until required.

DNA extraction

DNA extractions were carried out according to the optimised method developed in the current study (see Appendix I.1 for protocol). All samples were quantified spectrophotometrically (see Appendix I.2) and diluted to 50ng/µl for simplification of assay.

5.3.2 Analysis

5.3.2.1 Microsatellite PCRs

All reactions were carried out as optimised in Chapter 3, using ten microlitre reactions on a Biometra T-Grad thermal-cycler. The components and the thermal cycle used for each reaction are described below. Four loci were used in parental analysis. Although several trials were conducted to multiplex the PCR reactions using a variety of primer concentrations and conditions, results were found to be much more reliable using traditional single-plex PCR (see also Chapter 3).

Components of standard PCR reaction used for Genescan PCRs

Amount	Component
50ng	Template DNA
1μl	10x *Taq* polymerase buffer (50mM KCl, 10mM Tris-HCl pH 9.0, 0.1% Triton X-100 (Promega, UK)
1.0mM	$MgCl_2$
130μM	each nucleotide
0.75 pmoles	Fluorescently labelled forward primer
0.75 pmoles	Reverse primer
0.5 Units	*Taq* DNA polymerase (Promega)
xμl	dH_2O to total volume of 10μLs

Thermal cycle used for Genescan PCRs:

Temp	Time	Cycles
96°C	3min	
95°C	50s	X6
*Ta°C	50s	
72°C	1min 10s	
94°C	50s	X26
Ta°C	50s	
72°C	1min 10s	
72°C	7min[1]	
60°C	30min[2]	
4°C	pause	

[1]A final extension step is added for Genescan PCRs to ensure all products have extended fully

[2]A soak at 60°C was added to reduce genotyping errors caused by partial adenylation.

*Ta is annealing temperature specific to each primer set (see Table 5.5 below for specific temperatures for each locus)

Gel analysis

Samples were diluted 1 in 10 with dH_2O, and 1.5µl of this dilution product was added to 0.95µl 100% de-ionised formamide (Sigma, UK), 0.29µl Genescan® 350 TAMRA internal lane standard (Perkin Elmer Applied Biosystems (PE)) and 0.2µl of blue dextran loading dye. Samples were then denatured for 5 min at 95ºC, placed immediately on ice, then loaded onto a 4% denaturing polyacrylamide gel (19:1 acrylamide/bis-acrylamide) (Biorad). Electrophoresis was carried out on an ABI PRISM™ 377 DNA sequencer (PE) (36cm plates, 1200 volts, 2hrs 12min using filter set C, with a 40 min pre-run). Gel analysis was carried out using Genescan™ 2.1 (PE) according to the local Southern size calling method, and genotype data generated using Genotyper™ 2.1 (PE).

Table 5.5: Optimised annealing temperatures and visualisation colours of the four microsatellite loci used in genotyping analysis. Visualisation colour is given relative to ABI PRISM 377 filter set C (used in all analyses).

Primer set	Optimised annealing temperature (°C)	Fluor
StPfl001	55	Blue
StPfl002	65	Green
StPfl003	65	Yellow
LIST1007	55	Blue

5.3.2.2 Genescan analysis of microsatellites

All samples were analysed using the three most polymorphic loci isolated within this study, plus LIST1007 (Watts *et al.*, 2001) as described above. Each of the loci for each sample were run 'doubled up' (i.e. Pfl001 and Pfl002 were run in one lane, Pfl003 and LIST1007 were run in another lane) on Genescan gels to multiplex the analysis procedure. Each gel contained parental standards on eight lanes to ensure

consistency and size standard integrity between gels. Using four microsatellite loci it was possible to unambiguously assign the majority of the Aroclor 1254 exposure trial flounder to specific families. For the majority of those individuals unassignable to a single family (i.e. the resulting genotype was shared by several families) it was possible to identify them to a group (i.e. Isle of Man × Isle of Man ('clean' group) or Isle of Man × Mersey ('polluted' group)).

Table 5.6: Assignment of offspring to their parents (as a number, and percentage of the whole group) for the Aroclor 1254 trial. For those which were unassignable to a particular family, a group was allocated where possible. Percentage 'unassigned' are those for which no group or family was possible due to multiple matches or failed genotyping result

	Assigned to family		Assigned to group		Unassigned	
	No.	**%**	**No.**	**%**	**No.**	**%**
Aroclor treated	75	82	90	99	1	1
Control	43	61	53	75	17	24

Once parentage had been assigned, data were further analysed to identify any trends between families, and groups from clean and polluted areas.

5.3.3 CYP1A analysis

As data were non-normally distributed means were compared for all groups using t-tests of transformed data (i.e. both treated and control groups, from polluted and clean parents) to test the two null hypotheses: i) there is no significant difference in CYP1A levels between control and PCB (Aroclor 1254) treated flounder for both clean and polluted groups; and ii) there is no significant difference in CYP1A

expression level between flounder from Mersey × IoM and IoM × IoM (polluted and clean families, respectively).

Results

Results for the control and treated groups were separated into individuals from clean and polluted female parents, and are presented below (Figure 5.22).

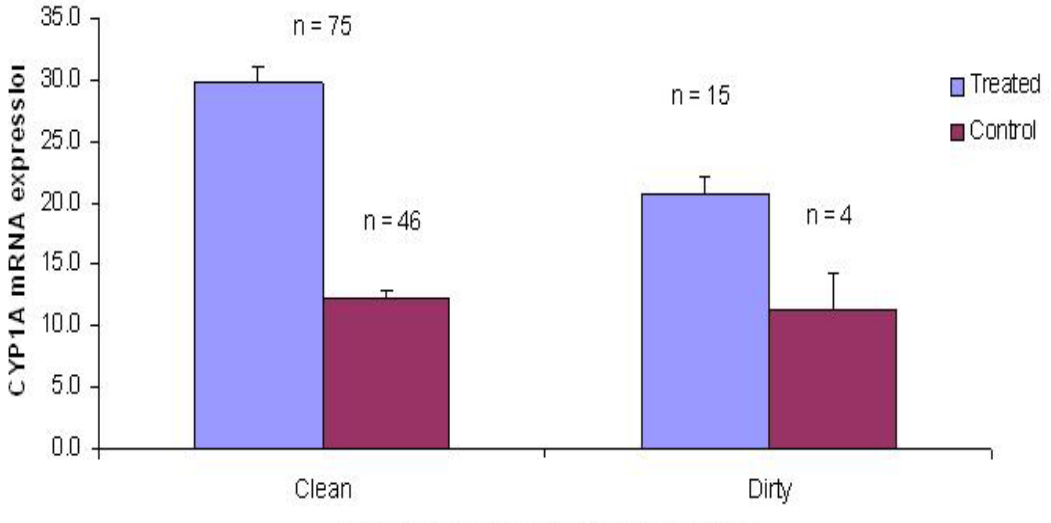

Figure 5.22: Mean CYP1A mRNA expression deduced from Northern blot analysis between 'clean' and 'polluted' groups, divided into results for Aroclor 1254 treated (blue columns) and control fish (red columns). Numbers of individuals per category are indicated, and error bars are standard errors of the means.

All log transformed data were analysed for variance (F-test) to ensure comparisons were possible, and all groups showed equal variances between samples (full F-test results are provided in Appendix III.3). T-tests were then carried out to differentiate means, and data are presented below in Table 5.7 and Table 5.8.

Table 5.7: Hypothesis i – Aroclor 1254 treated versus control animals. Reults of t-test comparisons of mean data. T-statistic and probability values are provided for each comparison made.

Comparison	T statistic	T probability
Treated clean vs. Control clean	6.92	$P < 0.001$
Treated polluted vs. Control clean	2.76	$P < 0.01$

All groups were significantly different from each other at the $P<0.05$ probability level, and null hypotheses were rejected for all groups. Unfortunately numbers of fish were low in the 'control polluted' group, and no statistical analysis could be carried out on the data.

Table 5.8: Hypothesis ii - clean versus polluted. Reults of t-test comparisons of mean data. T-statistic and probability values are provided for each comparison made.

Comparison	T statistic	T probability
Treated polluted vs. Treated clean	1.9	$P < 0.05$

No comparison was possible with individuals from polluted or clean areas in control fish. Post-treatment with Aroclor 1254 there was a significant difference between the CYP1A expression levels. Data were further separated into families, and this is presented in Figure 5.23 below.

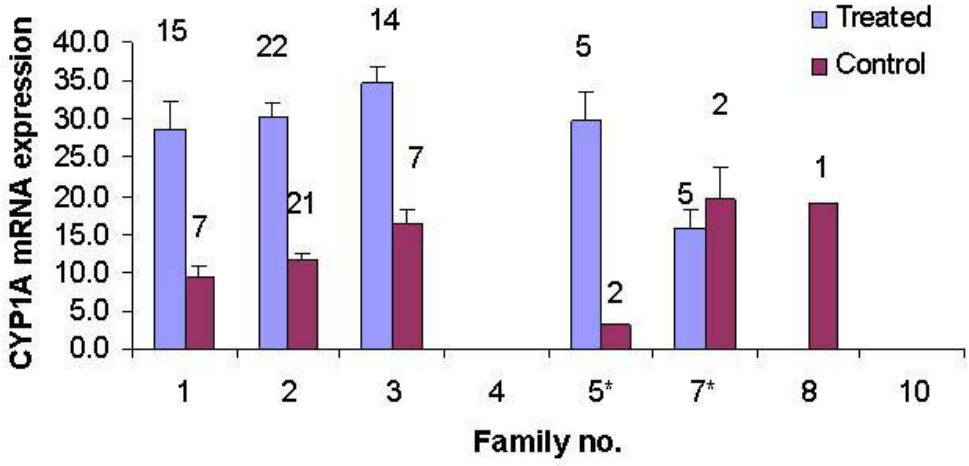

Figure 5.23: CYP1A mRNA expression deduced from Northern blot analysis averaged for each family group. Numbers of individuals per category are indicated above (n=x) (NB: where no number is shown n=0), and error bars are standard errors of the means. Polluted (Mersey × IoM) crosses are marked *.

Expression of CYP1A is consistently up-regulated in the Aroclor treated group across every family, with the exception of family 7 (polluted). Numbers of offspring from family 5 (polluted cross) were too low in the control group to provide any statistical analysis. A significant difference was found between individuals from polluted compared to clean areas post exposure to Aroclor 1254, but no statistical comparisons were possible between families due to low numbers of samples.

5.3.4 GSTA analysis

Data were analysed in groups and plotted as for CYP1A analysis, and are presented below (Figure 5.24).

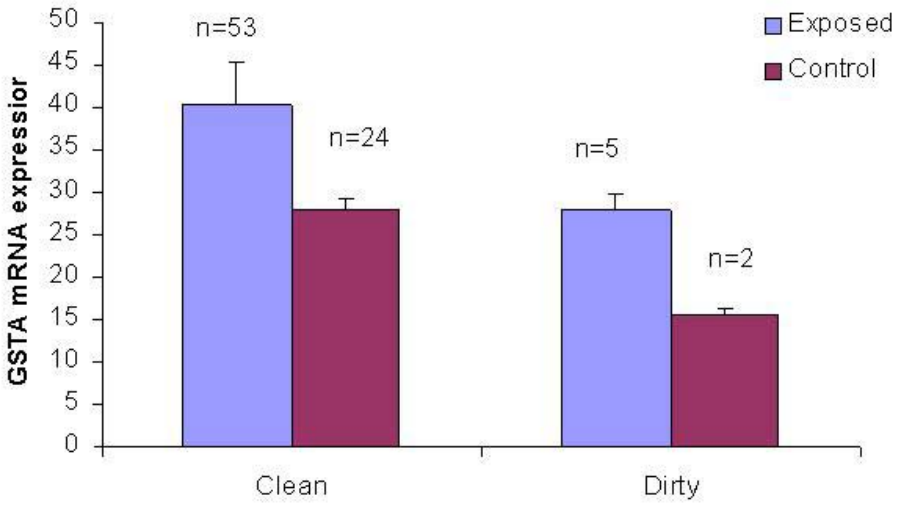

Figure 5.24: Mean GSTA mRNA expression deduced from Northern blot analysis between 'clean' and 'polluted' groups, divided into results for Aroclor 1254 treated (blue columns) and control fish (red columns). Numbers of individuals per category are indicated, and error bars are standard errors of the means.

No significant differences were identified between GSTA expression levels from any of the groups (NB. no analysis was possible using 'polluted control' group due to sample size).

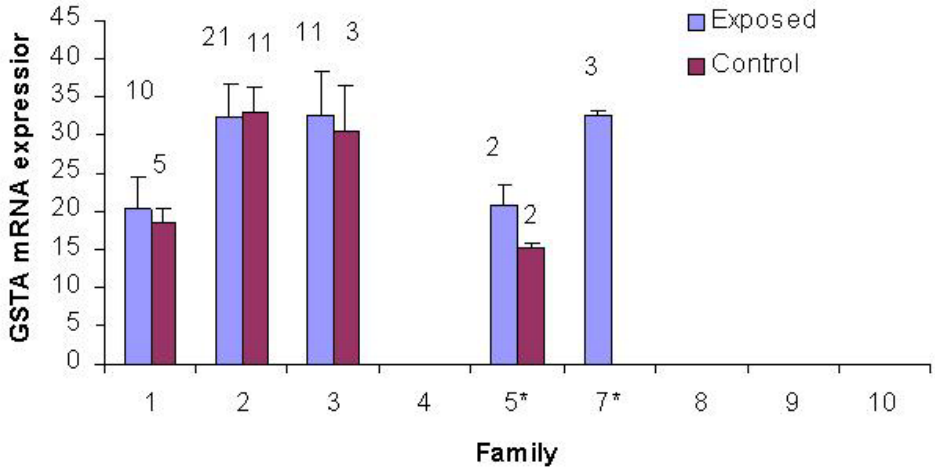

Figure 5.25: GSTA mRNA expression deduced from Northern blot analysis averaged for each family group. Numbers of individuals per category are indicated above (n=x) (NB: where no number is shown n=0), and error bars

are standard errors of the means. Polluted (Mersey × IoM) crosses are marked *.

No significant differences were found between any of the families due to low sample numbers in each group.

5.3.5 AhR expression

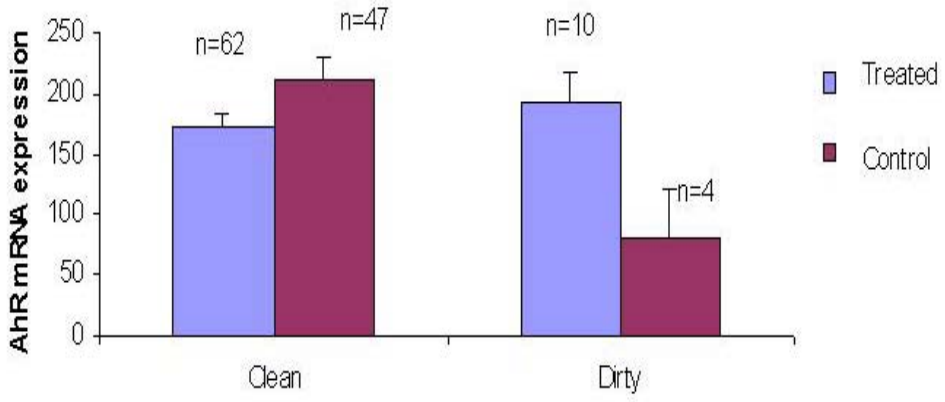

Figure 5.26: Mean AhR mRNA expression deduced from Southern blot analysis between 'clean' and 'polluted' groups, divided into results for Aroclor 1254 treated (blue columns) and control fish (red columns). Numbers of individuals per category are indicated, and error bars are standard errors of the means.

No significant difference was found between any of the groups, and the null hypothesis that area and treatment has no effect on AhR expression was accepted.

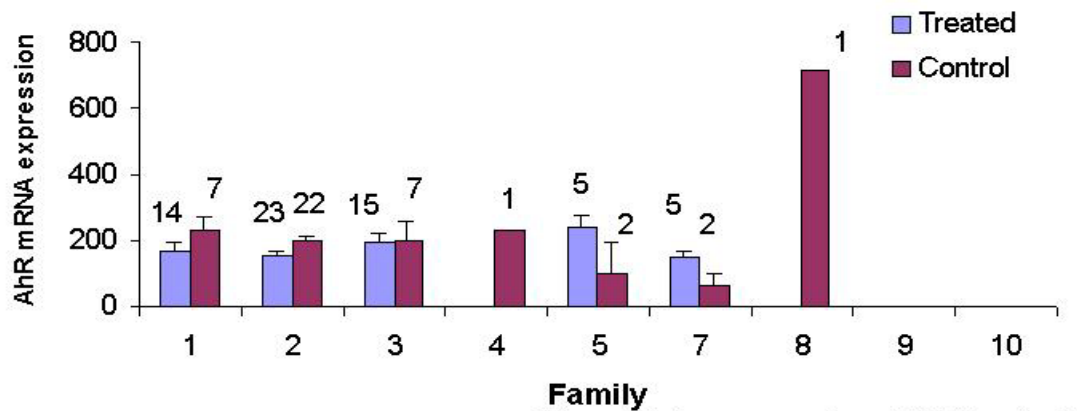

Figure 5.27: Mean AhR mRNA expression deduced from RT-PCR / Southern blot analysis averaged for each family group. Numbers of individuals per category are indicated above (n=x) (NB: where no number is shown n=0), and error bars are standard errors of the means. Polluted (Mersey × IoM) crosses are families 5 and 7.

The highest mean expression demonstrated by any one family was clearly evident in family 8, however as there was only one individual present to represent this family the result was inconclusive. Lowest levels of AhR expression were returned in the 'control polluted' families (5 and 7), but were found to be not significantly different from the others due to low sample numbers.

5.3.6 ARNT expression

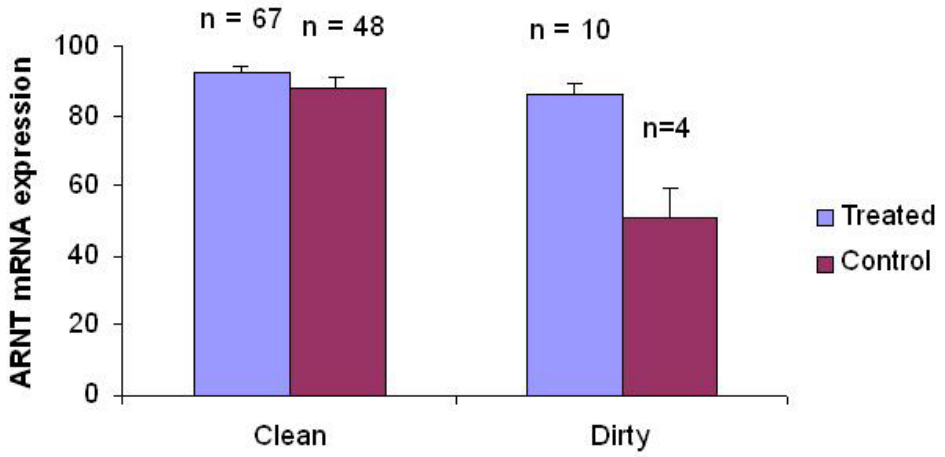

Figure 5.28: Mean ARNT mRNA expression deduced from Southern blot analysis between 'clean' and 'polluted' groups, divided into results for Aroclor 1254 treated (blue columns) and control fish (red columns). Numbers of individuals per category are indicated, and error bars are standard errors of the means.

No significant differences were found between any of the groups. One individual returned a value of zero in the polluted group which reduced the mean, and increased the standard error.

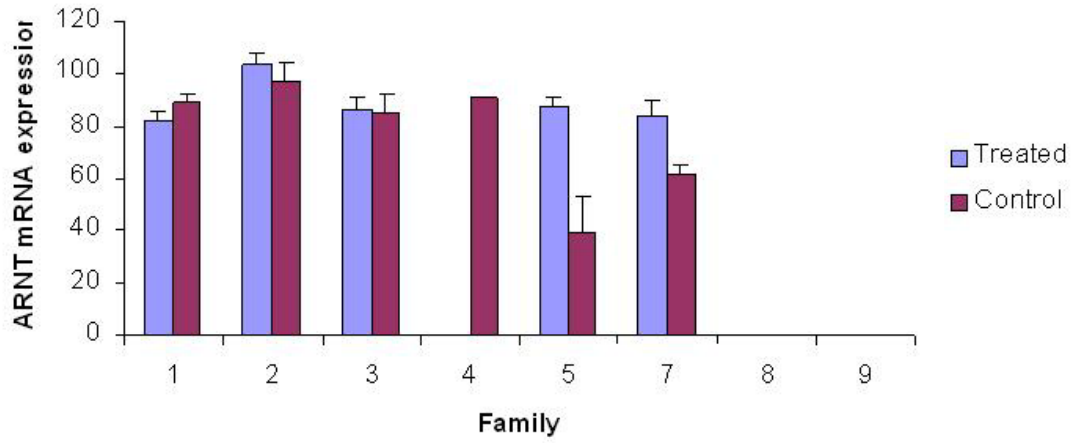

Figure 5.29: Mean ARNT mRNA expression deduced from RT-PCR / Southern blot analysis averaged for each family group. Numbers of individuals per category are indicated above (n=x) (NB: where no number is shown n=0), and error bars are standard errors of the means. Polluted (Mersey × IoM) crosses are families 5 and 7.

No significant patterns could be identified between any of the families used in the trial, and expression level was relatively consistent between groups (one individual in family 5 returned a value of zero which reduced the apparent mean in that group).

5.4 Summary of results

CYP1A analysis

There is a significant induction of CYP1A transcript levels in the European flounder after exposure to the commercial PCB mixture, Aroclor 1254. Induction in fish that originate from polluted x clean area is significantly lower than those from clean x clean crosses. One family (family 7 – polluted x clean cross) demonstrated levels of expression lower than all others in the trial following PCB exposure, however numbers were too low in this group to analyse statistically. Inter-animal variability of CYP1A transcript was high at the basal level, but increased significantly following treatment with Aroclor 1254 (>6 fold higher). Real-time PCR also

illustrated the induction of CYP1A in the fish following exposure to Aroclor 1254. Unfortunately due to technical problems with the Rotorgene machine and consequent lack of numbers of fish it was not possible to assay all individuals from the trial using real-time PCR, or to pick out any trends between families. However, for those fish which were assayed using real-time analysis and Northern blot, the results showed a strong correlation between the two techniques.

Northern blot studies and analysis via densitometry proved that there was an inductive response in European flounder offspring to treatment with Aroclor 1254. Statistical analysis of data indicated that Aroclor 1254 did elevate levels of CYP1A gene transcript (mRNA) significantly in exposed animals when compared with control animals from the same population, in which all other environmental variables had been standardised. No pattern of induction was observed using BaP as an inducing chemical.

GSTA analysis

GSTA was not induced in Aroclor 1254 treated flounder. The conclusion from the data in this trial is that Aroclor 1254 has no effect on expression of GSTA in the European flounder, as found in studies carried out previously in our laboratories (S.G. George, unpublished data).

Transcription factor analysis

There was no detectable significant induction of AhR2 or ARNT2 transcript levels upon exposure to Aroclor 1254. Further analysis of family history identified no trends within / between families for gene expression. A significant correlation was found between AhR2 and ARNT2 gene expression levels in control fish, however this was not evident in the group following treatment with Aroclor 1254. Multiple

regression analysis (least squares method, Minitab V.10) of CYP1A with AhR2 and ARNT2 returned no significant correlation.

5.5 Discussion

PAH exposure

BaP has been demonstrated as causing a dose dependant induction of CYP1A activity in studies of flounder (Husoy, Myers and Goksoyer, 1996) and in numerous mammalian and aquatic species (see introduction). The lack of CYP1A transcript induction observed in the BaP exposure trials in this study could be due to several factors. One possibility is that the particular population used for the study is in some way non-responsive to PAHs. This non-responsiveness is unlikely to be due to prior exposure history as the experimental population originated from both 100% clean populations (1999 breeding trials) and a clean × polluted cross (2000 breeding trials), and no particular pattern was observed. In later trials using two year old fish from the 1999 trial, an induction of CYP1A transcript expression was observed following intraperitoneal injection of 50mg BaP/kg body weight (data not included), and this finding disproves that the population displays a non-responsiveness. Water-borne exposures have proved successful in previous trials within our laboratories and within the literature for various species of fish. An alternative explanation, assuming successful exposure, is that the decreased response was observed due to a tissue specific response. In a water borne exposure CYP1A transcript expression may be more detectable in tissues such as gill which act as a direct interface between the animal and the environment (Van Veld *et al.,* 1997). Reasons for unsuccessful exposure in the current trial could include BaP binding to the plastic tanks, or BaP coming immediately out of solution and settling out of the water column due to the lipophilic nature of the compound. Following dosing of the tanks

during some of the trials, a visible cloudiness was observed which could indicate insolubility of the BaP (see section 5.2.1). As the plastic tanks had been previously used in exposure trials they were assumed suitable. A pre-treatment with acetone has been suggested to minimise any binding that could occur, and for future trials it may be advisable to wash the tanks immediately prior to introducing fish, or searching for a more successful water borne delivery method. If, for either of these reasons, the BaP was not available for uptake from the water by the animals, this would explain the lack of response of CYP1A transcript, and for future trials the suggested method would be via intraperitoneal injection. Due to the lack of CYP1A responsiveness observed following exposure to BaP, no further assays were carried out on other members of the *Ah* gene battery, and the study focussed on PCB (Aroclor 1254) exposures.

PCB exposure

The commercial PCB mix, Aroclor 1254, was used in this trial as a model example to investigate expression of *Ah* battery expression due to successful induction in trials in the current study, and injection was chosen as the preferred method of treatment due to the lack of induction achieved by water-borne exposures for the BaP trials. Determination of CYP1A transcript expression shows a large interanimal variation in both control and Aroclor injected fish, which suggests that in addition to straightforward 'induction' of CYP1A, inherent variability in expression is also increased dramatically. This high level of inter-animal variability would suggest that CYP1A transcript expression in response to PCB exposure is regulated via a complex set of multiple pathways. The significant induction of CYP1A transcript levels following treatment with the commercial PCB mix Aroclor 1254 observed in

these trials was in line with data from previous trials with PCBs. A full discussion of the results of the Aroclor 1254 trial is provided in the general discussion (Chapter 6).

Section 3: Discussion

Chapter 6 General Discussion

6.1 Methods of study

Breeding studies and parental identification

Breeding trials were produced in this study which allowed the effects of PAHs and PCBs to be investigated between families from areas of known exposure history. Communal rearing was carried out, and the fish successfully identified to their known parents using the microsatellites isolated in this study, and one locus which became available during the course of the study. The use of these microsatellite markers allowed successful communal breeding and exposure trials to be carried out, and ensured offspring in the trial remained under standardised environmental conditions throughout.

Development of probes and assays

The cDNA probes available within our laboratories allowed successful quantitation of gene transcript levels for the *Ah* battery genes CYP1A and GSTA, and a novel quantitation method (real-time PCR) was developed for assaying CYP1A gene transcript (mRNA) levels. Although data were limited from the real-time PCR trials, they highlighted the usefulness of such a technique as a potential tool in biomonitoring studies. In addition, (by cloning via RT-PCR based techniques) a novel probe for the flounder ARNT2 gene, and a probe for AhR2 which was not available within our laboratories were devised. A method of quantitation of *Ah* battery transcription factor gene expression was also devised, and detection of differential expression was possible as demonstrated by quantification of standards

from 10^2 to 10^4 copies (chapter 4). This technique allowed further investigation into the possible interactions of AhR2/ARNT2 with differential expression of CYP1A post treatment with Aroclor 1254. By cloning AhR2 and ARNT2 sequences from a number of diverse species it will hopefully allow further studies to draw comparisons between the evolution of the xenobiotic detoxification pathway (Besselink *et al.,* 1998; Hahn, 2002). The flounder ARNT2 partial clone is included in the Genbank database and will allow further comparisons of this type to be made in future. The use of 'relative' values in Northern blot semi-quantitative analysis was sufficient to identify trends between individuals in this study, and is widely accepted as a suitable method of mRNA quantification for such a study, as discussed previously. However, for future studies it would be informative to provide absolutely quantitative results for comparison between laboratories, and thus the technique of real-time PCR deserves further investigation for similar studies.

6.2 Parental identification

Although it was possible to unambiguously identify most of the offspring raised in this trial to their parents using the microsatellites detailed here (*ca.* 99% of treated fish, and 75% of control fish were assigned to 'clean' and 'polluted' parent group), problems were apparent with some individual genotypes. For those individuals unassignable to groups or families, the majority were due to assignment to multiple families. Several individuals were unassignable in the Aroclor trial and control group fish. The fish shared possible genotypes with families 4 and 5, and one individual from the control group shared genotypes with families 3 and 7. Family 4 was not otherwise represented in any of the trial fish. There was a lack of representation of fish from the control polluted group, and further discrimination

between these families would have been desirable (families 3 and 4 were from the clean group, and families 5 and 7 from the polluted group). Families 6 and 9 were not present in the analysis as they were derived from the second Mersey female which produced no offspring. One of the inherent problems in the study regarding genotyping of offspring was a lack of polymorphism found in the markers isolated. As discussed in chapter 3 this may have been an unavoidable consequence of using relatively geographically similar populations, and further polymorphism has since been found at several of the loci in studies outwith the laboratory.

6.3 Aroclor 1254 exposure

CYP1A transcript levels were highly responsive to treatment with Aroclor 1254. Variation in CYP1A transcript levels was increased dramatically following exposure to Aroclor 1254 (>6 fold), which would suggest that although many individuals are responsive to treatment with a PCB mix the degree of responsiveness varies largely within a population. AhR2, ARNT2 and GSTA mRNA appeared non-inducible at the transcript level following treatment with Aroclor 1254. Non-responsiveness of GSTA to PCBs has been observed previously (George, unpublished data). Considerations such as this must therefore be taken into account for any biomonitoring studies which employ CYP1A transcript expression as an indicator. Although the focus of this study was at the molecular (transcript) level, correlations of these data with post-translational (enzyme activity (EROD), or protein) data will further contribute to the understanding of the situation. As juveniles (*ca.* 1-7g) were used in this study it was not possible to quantitate enzyme levels during these trials due to the size limitations of the fish. The lack of sufficient liver tissue from these small flounder prevented a comparison of methodologies,

however these aspects have been covered in numerous trials, and a direct correlation has been demonstrated between CYP1A mRNA levels, protein product and enzyme activity (Leaver, 1996). Post-transcriptional effects have however been observed in previous trials following treatment with certain PCBs which disrupt the active site of the CYP1A enzyme, and cause an apparent reduction in enzyme activity. By studying the expression of the *Ah* gene battery at the transcriptional level these effects can be negated, as it is possible to examine the inherent genetic variability which is present in a population without investigating effects caused by external factors. Variation at the post-transcriptional level does however play a part in the responsiveness of individuals to xenobiotics, and ultimately it is this variation which will determine the toxicity of a substance to an individual. If future trials were carried out with flounder to further examine the possibility of a heritable CYP1A non-responsiveness, it would be informative to broaden them to include phenotypic changes also.

CYP1A transcriptional regulation

In Atlantic tomcod the duration of induction of CYP1A post-exposure to PAHs declined after several days, and disappeared within two weeks, whereas HAHs induced increasing levels of CYP1A mRNA for at least 25-72 days after treatment (Courtenay *et al*., 1999). In rainbow trout CYP1A mRNA expression peaked 5 days after exposure to the PAH 3-methylcholanthrene, and declined to control levels within 5 weeks, but following injection of a commercial PCB mixture (Clophen A50) levels were increased for a 15 week period (Celander and Forlin, 1995). This difference in expressional response to the different groups of compounds suggests that the pathway of activation may be partially responsible, and transcription of

CYP1A may be facilitated by multiple molecular pathways, i.e. there are AhR-dependent, and AhR-independent pathways (Courtenay *et al*., 1999). The existence of an alternative mechanism has also been proposed in studies of the American eel (*Anguilla rostera*) (Schlezinger and Stegeman, 2000). If induction of CYP1A by PCBs was found to act via an AhR independent pathway, it could suggest reasons for the lack of relationship between CYP1A expression, and AhR2/ARNT2 expression observed in this study. A vast number of factors have been described as affecting / regulating transcription of CYP1A via, or independent of, the AhR in humans (see e.g. Ke, Rabson, Germino, Gallo and Tian, 2001; Tian, Ke, Denison, Rabson and Gallo, 1998), including nuclear factor kappa B (NF-κB). Many physiological functions adversely affected by TCDD are also known to be regulated by NF-κB, such as immune activation, maintainance of skin differentiation, control of cell proliferation and survival, as well as induction of xenobiotic metabolising enzymes, and evidence has emerged that NF-κB and the AhR interact and transcriptionally modulate each other. NF-κB is a pleiotropic transcription factor that participates in many of the physiological responses that are affected by TCDD and PAHs suggesting that these two pathways interact (Tian, Rabson and Gallo, 2002). If these factors can be identified in fish these may later prove to be of importance to the fish model, and explain some of the lack of correlation of AhR expression with CYP1A gene expression. The pleiotropic nature of the AhR due to this array of different functions and involvements also further adds to this lack of direct correlation between AhR and CYP1A expression. The huge increase in the availability of molecular data in fishes including the preliminary full sequence releases of the zebra fish (*Danio* rerio) and the pufferfish (*Fugu rubripes*) will allow

easier identification of these transcrition factors involved, and comparison between this and the mammalian model should be possible in the near future.

The AhR Repressor (AhRR)

AhR function may be regulated by structural variations in the AhR itself, in the AhR repressor, the ARNT, or in the AhR target molecules such as CYP1A and GSTA (Mimura, Ema, Sogawa, and Fujii-Kuriyama, 1999; Watanabe, Imoto, Kosungi, Fukudu, Mimura, Fujji, Isaka, Takayama, Sato and Inazawa, 2001), and it may transpire that PCB metabolism is regulated by a more complex interaction of these. Following challenge with the model CYP1A inducer TCDD, AhR has been shown to be upregulated dramatically. The AhR repressor (AhRR) has been described as possibly one the most important elements in the equation, and depressed levels of AhR2 expression in killifish have been described following induction with TCDD and PCBs (Karchner, Franks, Powell and Hahn, 2002). This was due to AhRR to competing with AhR for dimerizing with ARNT and binding to the xenobiotic responsive element (XRE) sequence (Mimura *et al.,* 1999). This pathway provides an extra 'negative feedback loop' and is described in Figure 1.3 (Chapter 1). However, it should be noted that, although directly relevant to CYP1A induction via the AhR, the AhRR functions at a post-transcriptional level, and therefore would not be expected to have caused any decreased induction of AhR gene transcript levels in the current trials. The natural progression of investigation of flounder CYP1A induction would therefore be to investigate the existence of an AhR repressor gene in the flounder, and further correlate expression with of AhR2, ARNT2 and CYP1A.

Spare receptor

Although induction of CYP1A following treatment with dioxin is under the control of the AhR, 'spare' receptor is abundant, and only 1-2% of AhR receptor molecules need be occupied by high intrinsic efficacy agonists for 50% CYP1A induction (Hesterman, Stegeman and Hahn, 2000), presumably related to the pleitropic effects of the AhR. This model may indicate another explanation for the lack of relationship between CYP1A and AhR2 levels observed in the current study. Dioxin has been found to cause a sustained oxidative stress response in the mouse (Shertzer, Nebert, Puga, Ary, Sonntag, Dixon, Robinson, Cianciolo and Dalton, 1998), and AhR is also associated with both constitutive and dioxin-induced mitochondrial reactive oxygen production. These functions are independent of CYP1A (Senft, Dalton, Nebert. Genter, Puga, Hutchinson, Kerzee, Uno and Shertzer, 2002).

The method of quantitation of AhR2 and ARNT2 will benefit from further study due to the very low levels at which they were found (comparison of standard samples indicates levels at <100 copies per reaction in the trials carried out in this study, equivalent to *ca.* 10,000 copies/mg total RNA, although absolute quantitation was not carried out). Following trials with real-time PCR, and contact with co-workers using the system, trials to quantitate AhR2 and ARNT2 levels may benefit from utilisation of molecular beacon or Taqman® technology to improve resolution power due to the limitations of absolute quantitation found using an RT-PCR / Southern blot approach. Numerous studies on *Ah* battery gene expression have demonstrated that the *Ah* transcription pathway is directly responsible for altering levels of CYP1A expression, and therefore this must be investigated further.

CYP1B has been little studied in fish, and may also require investigation, as it has been shown to be induced by PAHs (Leaver and George, 2000), and also regulated by the AhR / ARNT pathway (Hukkanen, 2000), and therefore may influence CYP1A expression indirectly.

Pathways and microarrays

It is apparent that complex gene pathways are responsible for the regulation of transcription of CYP1A and the other *Ah* battery genes, and there still may be many of these unknown, and certainly many unexplored in the piscine model which have been characterised in the mammalian system. One natural future direction of this work is microarray studies which will allow many thousands of genes to be investigated simultaneously following exposure to various xenobiotics. Toxicological testing of rats has suggested that (microarray) assays may prove to be a highly sensitive technique for safety screening of drug candidates and for the classification of environmental toxins (Waring, Jolly, Ciurlionis, Lum, Praestgaard, Morfitt, Buratto, Roberts, Schadt and Ulrich, 2001). This work would allow a more thorough identification of the pathways involved in PCB and pollutant responses in the flounder, and allow profiles of expression to particular toxicants to be created. Ultimately these profiles could allow biomonitoring studies to detect exactly what compounds an animal has been into contact with.

6.4 Genetic basis to pollutant tolerance

Huge species and strain specific differences in sensitivity to TCDD exist, and LD_{50} values vary 5000-fold among different species of animals (1μg/kg for the guinea pig, the most sensitive, to 5000μg/kg for the hamster, the most resistant) (Poland

and Knutson, 1982). Natural populations in polluted areas are possibly subjected to selective pressures for an increased resistance to toxicants, and therefore fish obtained from the Mersey estuary crosses would be expected to demonstrate this to a degree. This can result in the evolution of resistance, which may have important implications for decisions regarding safe ambient toxicant levels (Klerks and Weis, 1987), and which organisms are used to monitor them. Genetic polymorphisms in drug responses have been well documented, and may be caused by polymorphisms in metabolising enzymes or the transcription pathways by which they activated (Gonzalez and Nebert, 1990; Meyer and Gut, 2002). The degree of variability between the drug responses owing to heredity is so large, a branch of science dubbed 'pharmacogenomics' has grown from it (Nebert and Bingham, 2001).

The current study has highlighted a possible genetic component to differential expression of CYP1A levels, as observed in offspring bred from a 'polluted female'. This observed difference may be a result of several factors, including prior maternal exposure which has been passed on in a single generational mechanism, for example through contaminants in egg yolk. The phenomenon observed in the current trials may be independent of the past history of the parental fish. It may be co-incidental that the family which demonstrated decreased induction following challenge with Aroclor 1254 originated from a polluted environment (both of the polluted × clean crosses were half-sib crosses made from a single female parent). Future trials would therefore benefit from larger numbers of families to investigate this further. Unfortunately it was not possible to test *Ah* battery gene transcript expression levels in parental fish due to their location on the IoM. A genetic difference in populations of European flounder has also been suggested in areas of high chemical

contamination (Laroche, Quiniou, Juhel, Auffret and Moraga, 2002), and adaptation and phenotypic expression of the multidrug or xenobiotic resistance type in hepatocarcinomas of European flounder has been described (Kohler, Lauritzen, Bahns, George, Forlin and Van Noorden, 1998).

Several studies have highlighted a decreased response of CYP1A mRNA and EROD induction in flounder (Eggens *et al*., 1996; Besselink *et al.,* 1998), killifish (Van Veld and Westbrook, 1995; Bello, Franks, Stegeman and Hahn, 2000), and Atlantic tomcod (Wirgin *et al*., 1996; Roy *et al.*, 2001) in fish taken from polluted versus reference sites. This phenomenon has been experimentally proven to be heritable in killifish and tomcod (Meyer and Di Giulio, 2002). In addition, a large differentiation has been found between species in induction of CYP1A which may be due to differential pathways of response in the different species (Wirgin *et al*., 1996, Eggens *et al.,* 1996). Pharmacogentic and toxicogenetic (genetic predisposition to toxic response) factors rarely act alone; they produce a phenotype in concert with other variant genes and with environmental factors (Meyer and Gut, 2002), and the complex relationship between receptors and xenobiotic metabolising enzymes requires more study to elucidate effects (Hahn, 2002). In the current trials, by minimising variation caused by environmental effects, it was possible to investigate genetic effects alone, however many questions still remain to be answered. Multi-xenobiotic responses via several pathways have been suggested, and the interactions of P-glycoprotein and CYP1A have been investigated, and although the two are not co-ordinately regulated, these proteins may play complementary roles in cellular detoxification (Bard, Woodin and Stegeman, 2002). Genetic polymorphisms of xenobiotic metabolising enzymes have been the subject

of molecular-epidemiological studies and may contribute to an individuals susceptibility to numerous diseases or toxic compounds (Harth, Bruning, Abel, Koch, Berg, Sachinidis, Bolt, Vetter and Ko, 2001). Demonstrated polymorphisms in CYP1A genes may be a major cause of differential expression (e.g. Wirgin, Kreamer and Garte, 1991; Smart and Daly, 2000), and a future direction of the curent study may include sequence analysis (via traditional sequencing or genotyping studies or real-time PCR) of those samples found to be responsive at a lower level to identify any polymorphisms (e.g. SNPs, as have been found in the UGT gene (Leaver and George, 2002)) which may be present, as has been shown to be the case in humans (Hong and Yang, 1997; Harth *et al*., 2001) and fish (Roy *et al.,* 2001). Polymorphisms in other detoxification enzymes (UGT1B1) have also been proposed as possible mechanisms which at least contribute to a pollutant tolerance phenotype (George and Leaver, 2002). Unfortunately it was not possible to quantitate UGT1B1 expression in the current study due to probe difficulties, but this may be another factor worth investigating further to examine whether any differences are found within expression of this gene. Other phase II enzymes have been studies, and elevations in GSTA levels in a creosote resistant population of killifish from the Elizabeth River (VA, USA) have also been observed, and proposed as another possible mechanism of increased tolerance (Armknecht, Kaattari, and Van Veld, 1998). However no such changes of GSTA transcript expression were evident in this study following treatment with PCBs. Investigation into the possible variation of GSTA genes has been proposed as a further method to understanding the physiology of pollutant response (pers. comm. M. Leaver, University of Stirling).

An important issue which has not been addressed within populations of flounder around the UK which have been used as organisms in biomonitoring studies is the population genetics. This was investigated as a sideline to this project, and a small scale study was carried out on four populations around the UK. No differentiation was found in this trial between individuals from around the east coast, and one population from the south coast (data not presented; Garvey, 2001). This poses important questions to biomonitoring studies, as the most useful organisms in biomonitoring studies are those which do not move far from their native habitat, and inhabit the area which is of interest. With further resolution of the markers identified in this study, or in combination with isolation of additional loci, it should be possible to examine populations of interest in this type of study. A similar population study was conducted on killifish in PAH contaminated and neighbouring sites in the Elizabeth and York rivers in the US. A significant correlation between genetic distance and differences among sites in PAH concentrations was identified, and genetically distinct populations at the heavily PAH contaminated site were found (Mulvey *et al*., 2002). Flounder display a very different life history strategy from the killifish. Frequent mixing and high levels of genetic diversity within populations has been described previously in flounder (Exadactylos and Thorpe, 2000), but killifish form more discrete populations and the level of variability between populations will therefore be much higher (Mulvey *et al*., 2002). The microsatellite loci isolated in the current study are currently being investigated in other areas (as discussed in chapter 3), and will hopefully provide further population data which will be directly relevant to biomonitoring studies regarding mixing of populations. Flounder spawn inshore, then migrate to deeper waters and population mixing occurs, however little is known about movement between spawning

grounds. If any heritability of a change in response to xenobiotics is further observed in flounder, then this issue must be addressed further to identify any possible population link.

6.5 Application of flounder to biomonitoring

The European flounder has been adopted by the national marine monitoring programme (NMMP) as a sentinel species. The NMMP suggests the use of a minimum of 25 individuals per sampling point. As variation in induction of CYP1A transcript levels is so high, data from the current study would suggest that 25 individuals may provide a false representation of the true population and return high standard errors. If low numbers of individuals from a single area of high pollutant burden are selected, this may provide further misleading data due to the lack of induction observed in the current trial following PCB exposure. CYP1A expression data for the NMMP is collected as induction of EROD, which can lead to further variability being detected at the post-transcriptional level (e.g. receptor saturation by high levels of xenobiotic, or induction of repressor genes which may lead to further confusion of results). Gene transcript level via the real-time PCR technique developed may provide a useful insight into pre-translational expression.

6.6 Future directions

Genes involved in the *Ah* pathway

Microarray studies are currently being investigated in the European flounder following challenge with xenobiotics in the EU funded GENIPOL (Genomic tools for bio-monitoring of pollutant coastal impact) project involving a collaboration

with our laboratory in Stirling, and others in Denmark, Israel and Birmingham (UK), and will hopefully offer further insight into the mechanism of action of various xenobiotics upon the transcriptional expression of the *Ah* battery genes, and identify further transcription factors involved in the pathway. This will offer a logical next step in answering some of the questions posed by this study, including the possibility of alternate, AhR independent pathways of PCB metabolism. In order to investigate the direct involvement of the AhR in the PCB metabolic pathway, gel retardation assays could be carried out. Using gel retardation analysis, the binding of the ligand-AhR complex to synthetic radiolabelled oligonucleotides corresponding to the xenobiotic responsive element can be investigated. Analysis of the protein-DNA complexes can then be achieved by standard polyacrylamide gel electrophoresis and autoradiography (see e.g. Besselink *et* al., 1998; Arzuaga and Elskus, 2002).

Application of microsatellites to pharmacogenetics studies

Microsatellites are the tool of choice for raising offspring in communal tanks, and the standardisation of conditions achievable from mixed batch offspring is invaluable to investigations of variability in drug response. Suggestions for future uses of microsatellites could include searching for more informative markers, and further characterisation of the markers isolated in this study. Any genetic component to pollutant responses identified in the flounder would follow the models as illustrated by the development of pesticide resistant insects (e.g. Clarke, 1990), xenobiotic tolerance in numerous species of fish (Meyer and Di Giulio, 2002; Wirgin *et al*., 1996) and indeed drug metabolism in humans (e.g. Nebert and Bingham, 2001). It is crucial to investigate this further if flounder are continued to

be used in biomonitoring studies. Linkage studies could be carried out to identify any microsatellites which could be related to a phenotype which demonstrated a tolerance in pollutants (as has been illustrated by the discovery of markers associated with increased lung cancer occurrence in humans e.g. (Haugen, 1997)). This technique is used readily in pedigree analysis and identification of traits in commercially important stock animals to improve breeding trials, and select for desirable traits. Examples of such markers include increased wool production and disease resistance in sheep and increased meat quality in cattle. Following similar lines, markers may be easily identifiable for a change in pollutant responses in fish. A suite of microsatellites could be generated (as discussed in Chapter 3), and combined with newly emerging technologies of microarraying for genes involved in xenobiotic metabolism. With further study of both areas it may be possible to identify quantitative trait loci (QTLs), and eventually lead to developing a simple PCR based test for these QTLs in relation to testing for an acquired 'resistance' or alteration of response to pollutants. Further breeding studies could be set up to establish 'pedigree' strains of fish with demonstrated differences in responsiveness. Loci could be followed through several generations, as documented in past studies on Ah responsive and non-responsive mice (e.g. Poland and Glover, 1975; Nebert *et al.,* 1972). Combined with currently used tests e.g. quantitative CYP1A analysis which is taken as a measure of exposure to xenobiotics, this strategy could have the potential to correct for misleading results obtained from depressed levels of CYP1A in fish collected from long-term polluted areas: If CYP1A levels were found to be decreased in an area, and the assumption was made that it was a result of inhabiting a non-polluted area, fish could be tested for a marker for a 'non-responsive' phenotype, and the results corrected accordingly. Some of the plaice microsatellites

which were designed in this study were found in intronic areas of the plaice *Ah* battery genes genomic sequence (M. Leaver, unpublished data, and Watts *et al*., 1999), and may be of use in such a study. Further investigation of the loci would be necessary, and correlation with gene expression levels carried out to identify any links.

CYP1A knockout

Another interesting future direction could be to produce CYP1A 'knockout' strains of fish. CYP1A1 deficient mice show no apparent phenotypic differences to wild type, and expression of other *Ah* battery enzymes is unchanged (Dalton, Dieter, Matlib, Childs, Shertzer, Genter and Nebert, 2000). The authors postulated overlapping substrate specificities between CYP1A1 and CYP1A2 as a mechanism which caused this lack of phenotypic variation, and that as CYP1A2 levels remain unchanged in the CYP1A1 deficient strain, basal CYP1A2 in the liver can compensate for the loss of CYP1A1. Conversely, in another study it was demonstrated that sensitivity of CYP1A1 inducibility was unaffected by lack of CYP1A2 expression in CYP1A2 deficient mice (Liang, McKinnon and Nebert, 1997). Due to the absence of a CYP1A2 gene in the fish (or at least not yet discovered, in fish) they could potentially act as an ideal model to study this phenomenon, and by creating a CYP1A knockout line it would be possible to assess whether any other factors may compensate for the lack of CYP1A expression.

Whatever the outcome of such future trials, if and when they are conducted, the issues must be addressed further for continued use of organisms in biomonitoring

studies to gain a more complete understanding of the metabolic pathways involved, and especially in flounder following results of the current trials.

6.7 Conclusion

There is a significant induction of CYP1A transcript levels in the European flounder following exposure to the commercial PCB mix Aroclor 1254. There was no induction of the phase II enzyme GSTA mRNA, or the transcription factors AhR2 and ARNT2, which has been documented following exposure to TCDD. Induction in families originating from a highly contaminated waterway was significantly reduced compared with those originating from families from a pristine environment, indicating a possible heritable response to prior exposure to toxicants. Fish produced from 'polluted females' showed no significant induction of CYP1A mRNA following treatment with Aroclor 1254 which may suggest an adaptation of this population to a heavily polluted area which is heritable to at least the F1 generation. This type of adaptation may have important implications for future biomonitoring studies using this species, and background data must be carefully assessed. As studies on several other species of fish including tomcod and killifish have identified similar findings in the US, and non-responsive populations of flounder have been identified in Europe it is crucial to gather more data in the flounder. Further trials are required on flounder to determine whether the phenomenon of reduced CYP1A transcriptional expression observed in this trial is coincidental to the families which were available in this trial, using larger numbers of fish, and thus a greater representation of each area.

The apparent lack of relationship between CYP1A transcript expression and the transcription factors AhR2 and ARNT2 observed in this trial may indicate that an AhR-independent pathway is responsible for the metabolism of PCBs in the flounder. Further trials are necessary to further elucidate the mechanisms of xenobiotic metabolism, and many gaps in present knowledge remain to be filled. There are certainly more factors involved in the detoxification of PAHs and PCBs than it was possible to investigate in this study, and many more which to be discovered and characterised in the piscine model, but valuable data indicating further areas of investigation have been highlighted, and useful data provided.

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Appendices

(See Appendices PDF)