DOCTOR OF PHILOSOPHY

Polychlorinated biphenyls and their effects on the early life stages of the European plaice, Pleuronectes platessa L.

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Polychlorinated biphenyls and their effects on the early life stages of the European plaice, *Pleuronectes platessa* L.

A thesis submitted in accordance with the requirement of the University of Wales for the degree of Philosophiae Doctor

by

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Summary

Polychlorinated biphenyls (PCBs) are ubiquitous, persistent, organic environmental pollutants. They are extremely stable, lipophillic compounds that do not break down easily in the environment and bioaccumulate readily in biota and fine sediments, increasing in concentration up the food chain. Research has shown the various effects and implications this bioaccumulation can have in numerous animal species around the globe. Pollutants may be accumulated in eggs before spawning occurs, reducing the female body burden but with potential risks for the next generation. This study aimed to elucidate the potential impacts of these compounds on marine fish by investigating the effects of exposure on reproduction and larval growth, survival, behaviour and physiology using the European plaice, *Pleuronectes platessa* (Linnaeus) as a model species.

Adult plaice and egg samples were sampled from the Irish Sea and juvenile fish from nursery grounds in both the Irish and North Seas in order to collect data on environmental PCB concentrations and supplement data already in the literature. Samples were analysed using gas chromatography-mass spectrometry (GC-MS) and nursery grounds contaminated with PCBs were identified.

Two large scale broodstock experiments involving the oral dosing of female fish over subsequent spawning seasons aimed to determine the potential effects of maternal pollutant transfer on fertilised eggs and larvae. PCB exposure was found to reduce the size at hatch of larvae and resulted in a delay in development compared to larvae from non-dosed females that lasted until metamorphosis. Routine swimming activity of exposed larvae was significantly less than that of fish from control females though the differences had disappeared by metamorphosis. Response to vibratory stimuli did not differ between fish from the different treatments.

A relatively new biochemical assay for assessing the impacts of pollution on aquatic organisms called the cellular energy allocation (CEA) method was used to determine the impact of PCB exposure on the physiology of both larvae exposed through maternal transfer and juvenile 0+ plaice exposed through the diet. Both experiments showed a significant reduction in the activity of the electron transport system in exposed fish at the times most likely associated with PCB metabolism. The electron transport system is involved in the production of ATP that provides energy for growth and physical activity.

All experiments were complemented by subsequent analysis of tissue samples by GC-MS to determine PCB body burdens and compare them to environmental concentrations. Significant correlations of PCB concentration were found with many of the parameters that showed significant differences between treatments in the experimental work.

Implications of exposure to PCBs for species in the environment are discussed as well as the future and importance of integrated ecotoxicological studies and the continued release of new and discontinued chemicals to the environment.

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"Good luck seems to be the most appropriate thing to write, I think you are being rather ambitious."

Initial comment by a CEFAS scientist in response to questions from myself regarding plaice aquaculture, *January 2003*.

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List of Abbreviations

ANCOVA	Analysis of covariance
ANOSIM	Analysis of similarity
ANOVA	Analysis of variance
ATP	Adenosine 5'-triphosphate
BBSRC	Biotechnology and Biological Sciences Research Council
BDE	Brominated diphenyl ether
BFR	Brominated fire retardants
CB	Chlorinated biphenyl
CEA	Cellular energy allocation
CEFAS	Centre for Environment, Fisheries and Aquaculture Science
CV	Coefficient of variation
DDE	Dichlorodiphenyl dichloroethene
DDT	Dichlorodiphenyl-trichloroethane
DE	Diphenyl ether
DeBDE	Deca-brominated diphenyl ether
DEFRA	Department of Environment Food and Rural Affairs
dpf	Days post fertilisation
dph	Days post hatch
Ea	Available energy reserves
Ec	Energy consumption
EDC	Endocrine disrupting chemical
EROD	7-ethoxyresorufin-O-deethylase
ETS	Electron transport system
GC	Gas chromatogram
GC/MS	Gas-chromatography/mass-spectrometry
GST	Glutathione *S*-transferase
HPLC	High pressure liquid chromatography
HSD	(Tukey's) honestly significant difference
HxBDE	Hexa-brominated diphenyl ether
Hz	Hertz
ICES	International Council for Exploration of the Sea

INT	p-iodonitrotetrazolium violet
IUPAC	International Union of Pure and Applied Chemists
JMP	Joint Monitoring Program
K_{ow}	Octanol-water partition coefficient
KW	Kruskal-Wallis
LD	Limit of detection
LED	Light emitting diode
LOEC	Lowest observed effect concentration
MAFF	Ministry of Agriculture Fisheries and Food
MDS	Multidimensional scaling
MeHg	Methylmercury
mRNA	Messenger ribonucleic acid
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NIOZ	Netherlands Institute for Sea Research
NMMP	National Marine Monitoring Program
OCN	Octachloronaphthalene
OECD	Organisation for Economic Co-operation and Development
OSPAR	Oslo Paris Commission
PAH	Polyaromatic hydrocarbons
PBB	Polybrominated biphenyl
PBDE	Polybrominated diphenyl ether
PCA	Principle components analysis
PCB	Polychlorinated biphenyl
PCDD	Polychlorinated dibenzo-*p*-dioxins
PeBDE	Penta-brominated diphenyl ether
pH	Power of hydrogen
POP	Persistent organic pollutant
PPL	Personal procedural licence
PQL	Practical quantification limit
REACH	Registration, Evaluation and Authorisation of CHemicals
RIVO	Netherlands Institute for Fisheries Research
RRT	Relative retention time
RT	Retention time

RV	Research vessel
SD	Standard deviation
SE	Standard error
SfG	Scope for growth
SIM	Selected ion monitoring
SIMPER	Similarity percentages
TBT	Tributyltin
TCA	Trichloroacetic acid
TeBDE	Tetra-brominated diphenyl ether
UK	United Kingdom
USA	United States of America
UV	Ultra-violet
VTG	Vitellogenin
WEEE	Waste Electrical and Electronic Equipment
WHO	World Health Organisation

Chapter 1

An introduction to the polychlorinated biphenyls, their environmental distribution and problems caused by endocrine disrupting chemicals

1.1 Introduction

The following chapter provides an introduction to the chemistry, production and the distribution of polychlorinated biphenyls (PCBs) in the marine environment. Their potential impacts on fish are discussed, as are the general topics of endocrine disruption and contaminant exposure pathways before outlining the intentions of the particular areas of research addressed in this thesis. More detailed discussions surrounding each individual topic are included at the end of each subsequent chapter.

1.2 Physical and chemical properties of PCBs and their uses

PCBs consist of two biphenyl (benzene) rings linked by a single carbon-carbon double bond and with one or more chlorine (Cl) atoms that substitute hydrogen on the remaining 10 carbon atoms labelled 2-6 and 2'-6' (Fig.1.1). The empirical formula for PCBs is $C_{12}H_{10-n}Cl_n$ (n = 1-10) which gives rise to a total of 209 different potential structures called congeners. The congeners are identified by the quantity and position of the Cl atoms on the biphenyl molecule e.g. 2,2',3,3',4-pentachlorobiphenyl. To simplify the identification of PCBs, each congener is given an individual number from 1 to 209 according to rules of the International Union of Pure and Applied Chemists (IUPAC) chemical nomenclature and developed by Ballschmiter & Zell

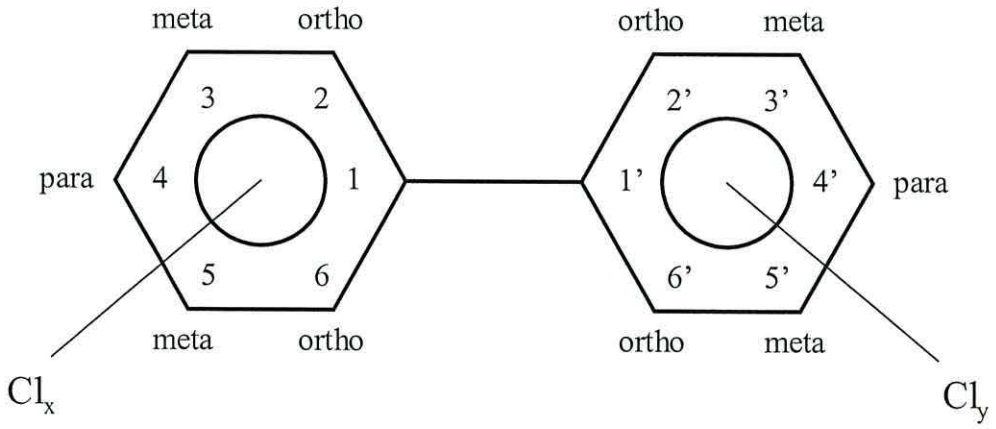


Figure 1.1 Structure of the polychlorinated biphenyl molecule.

(1980) e.g. the previous example would be referred to as CB-82. A list of all 209 PCB congeners and their corresponding IUPAC numbers is given in Table 1.1.

The physicochemical properties of PCBs are related to both the extent of chlorination and the pattern of chlorine substitution within the isomeric groups. Properties such as water solubility, volatility and melting points of some congeners were summarised by Lang (1992) (Table 1.2). A full list of melting points is reported by Bolgar et al. (1995). The more highly chlorinated PCBs are heavier, less soluble, less volatile, less prone to microbial degradation and more lipophilic. Evaporation rates are low, especially for the more chlorinated molecules as viscosity increases with the percentage of chlorine (OSPAR, 2001).

The pattern of chlorine substitution has a strong influence on the toxicity of the PCB congeners. Only a small number are extremely toxic (e.g. 77, 126 and 169) and this is related to the planar structure of these congeners. Fortunately these toxic congeners rarely occur in technical PCB formulations and as such are rarely detected in environmental samples. The structure of most PCB congeners is a non-planar one with preferential chlorine substitution occurring at the *ortho* and *para* positions. The lack of any *ortho* substituted chlorine atoms significantly reduces the minimum steric hindrance of rotation about the 1,1' C-C bond, making a planar configuration easier to obtain (Camacho-Ibar, 1991). Toxicity decreases as planarity decreases due to an increase in *ortho* chlorine substitutions but it does not necessarily disappear. Wilson-Yang et al. (1991) listed 19 congeners (PCBs 28, 52, 60, 77, 81, 105, I14, 118, 123, 126, 138, 156, 157, 158, 166, 167, 169, 170 and 189) with a partially planar configuration that were thought to be responsible for biological activity.

The variation in the three-dimensional structure of PCBs in terms of both the degree of chlorination and chlorination pattern, can influence properties such as the octanol-water partition coefficient (K_{ow}) (Shiu & Mackay, 1986; Hawker & Connell, 1988). In turn, this might go some way to explaining their behaviour following release into the environment and the differences in the way in which congeners partition themselves in different environmental compartments such as aquatic sediments, the water column and the air (Camacho-Ibar, 1991).

Table 1.1 IUPAC numbering of the 209 PCB congeners (No. of isomers of each type is shown in brackets). (Adapted from Camacho-Ibar (1991) and Bolgar *et al.* (1995)).

No.	Structure	No.	Structure	No.	Structure	No.	Structure
Monochlorobiphenyls (3)		53	2,2',5,6'	110	2,3,3',4',6	167	2,3',4,4',5,5'
1	2	54	2,2',6,6'	111	2,3,3',5,5'	168	2,3',4,4',5',6
2	3	55	2,3,3',4	112	2,3,3',5,6	169	3,3',4,4',5,5'
3	4	56	2,3,3',4'	113	2,3,3',5',6		
		57	2,3,3',5	114	2,3,4,4',5	Heptachlorobiphenyls (24)	
Dichlorobiphenyls (12)		58	2,3,3',5'	115	2,3,4,4',6	170	2,2',3,3',4,4',5
4	2,2'	59	2,3,3',6	116	2,3,4,5,6	171	2,2',3,3',4,4',6
5	2,3	60	2,3,4,4'	117	2,3,4',5,6	172	2,2',3,3',4,5,5'
6	2,3'	61	2,3,4,5	118	2,3',4,4',5	173	2,2',3,3',4,5,6
7	2,4	62	2,3,4,6	119	2,3',4,4',6	174	2,2',3,3',4,5,6'
8	2,4'	63	2,3,4',5	120	2,3',4,5,5'	175	2,2',3,3',4,5',6
9	2,5	64	2,3,4',6	121	2,3',4,5',6	176	2,2',3,3',4,6,6'
10	2,6	65	2,3,5,6	122	2',3,3',4,5,	177	2,2',3,3',4',5,6
11	3,3'	66	2,3',4,4'	123	2',3,4,4',5	178	2,2',3,3',5,5',6
12	3,4	67	2,3',4,5	124	2',3,4,5,5'	179	2,2',3,3',5,6,6'
13	3,4'	68	2,3',4,5'	125	2',3,4,5,6'	180	2,2',3,4,4',5,5'
14	3,5	69	2,3',4,6	126	3,3',4,4',5	181	2,2',3,4,4',5,6
15	4,4'	70	2,3',4',5	127	3,3',4,5,5'	182	2,2',3,4,4',5,6'
		71	2,3',4',6			183	2,2',3,4,4',5',6
Trichlorobiphenyls (24)		72	2,3',5,5'	Hexachlorobiphenyls (42)		184	2,2',3,4,4',6,6'
16	2,2',3	73	2,3',5',6	128	2,2',3,3',4,4'	185	2,2',3,4,5,5',6
17	2,2',4	74	2,4,4',5	129	2,2',3,3',4,5	186	2,2',3,4,5,6,6'
18	2,2',5	75	2,4,4',6	130	2,2',3,3',4,5'	187	2,2',3,4',5,5',6
19	2,2',6	76	2',3,4,5	131	2,2',3,3',4,6	188	2,2',3,4',5,6,6'
20	2,3,3'	77	3,3',4,4'	132	2,2',3,3',4,6'	189	2,3,3',4,4',5,5'
21	2,3,4	78	3,3',4,5'	133	2,2',3,3',5,5'	190	2,3,3',4,4',5,6
22	2,3,4'	79	3,3',4,5'	134	2,2',3,3',5,6	191	2,3,3',4,4',5',6
23	2,3,5	80	3,3',5,5'	135	2,2',3,3',5,6'	192	2,3,3',4,5,5',6
24	2,3,6	81	3,4,4',5	136	2,2',3,3',6,6'	193	2,3,3',4',5,5',6
25	2,3',4			137	2,2',3,4,4',5		
26	2,3',5	Pentachlorobiphenyls (46)		138	2,2',3,4,4',5'	Octachlorobiphenyls (12)	
27	2,3',6	82	2,2',3,3',4	139	2,2',3,4,4',6	194	2,2',3,3',4,4',5,5'
28	2,4,4'	83	2,2',3,3',5	140	2,2',3,4,4',6'	195	2,2',3,3',4,4',5,6
29	2,4,5	84	2,2',3,3',6	141	2,2',3,4,5,5'	196	2,2',3,3',4,4',5',6'
30	2,4,6	85	2,2',3,4,4'	142	2,2',3,4,5,6	197	2,2',3,3',4,4',6,6'
31	2,4',5	86	2,2',3,4,5	143	2,2',3,4,5,6'	198	2,2',3,3',4,5,5',6
32	2,4',6	87	2,2',3,4,5'	144	2,2',3,4,5',6	199	2,2',3,3',4,4',5',6'
33	2',3,4	88	2,2',3,4,6	145	2,2',3,4,6,6'	200	2,2',3,3',4,5,6,6'
34	2',3,5	89	2,2',3,4,6'	146	2,2',3,4',5,5'	201	2,2',3,3',4,5',6,6'
35	3,3',4	90	2,2',3,4',5	147	2,2',3,4',5,6	202	2,2',3,3',5,5',6,6'
36	3,3',5	91	2,2',3,4',6	148	2,2',3,4',5,6'	203	2,2',3,4,4',5,5',6
37	3,4,4'	92	2,2',3,5,5'	149	2,2',3,4',5',6	204	2,2',3,4,4',5,6,6'
38	3,4,5'	93	2,2',3,5,6	150	2,2',3,4',6,6'	205	2,3,3',4,4',5,5',6
39	3,4',5	94	2,2',3,5,6'	151	2,2',3,5,5',6		
		95	2,2',3,5',6	152	2,2',3,5,6,6'	Nonachlorobiphenyls (3)	
Tetrachlorobiphenyls (42)		96	2,2',3,6,6'	153	2,2',4,4',5,5'	206	2,2',3,3',4,4',5,5',6
40	2,2',3,3'	97	2,2',3',4,5	154	2,2',4,4',5,6'	207	2,2',3,3',4,4',5,6,6'
41	2,2',3,4	98	2,2',3',4,6	155	2,2',4,4',6,6'	208	2,2',3,3',4,5,5',6,6'
42	2,2',3,4'	99	2,2',4,4',5	156	2,3,3',4,4',5		
43	2,2',3,5	100	2,2',4,4',6	157	2,3,3',4,4',5'	Decachlorobiphenyls (1)	
44	2,2',3,5'	101	2,2',4,5,5'	158	2,3,3',4,4',6	209	2,2',3,3',4,4',5,5',6,6'
45	2,2',3,6	102	2,2',4,5,6'	159	2,3,3',4,5,5'		
46	2,2',3,6'	103	2,2',4,5',6	160	2,3,3',4,5,6		
47	2,2',4,4'	104	2,2',4,6,6'	161	2,3,3',4,5',6		
48	2,2',4,5	105	2,3,3',4,4'	162	2,3,3',4',5,5'		
49	2,2',4,5'	106	2,3,3',4,5	163	2,3,3',4',5,6		
50	2,2',4,6	107	2,3,3',4,5'	164	2,3,3',4',5',6		
51	2,2',4,6'	108	2,3,3',4,6	165	2,3,3',5,5',6		
52	2,2',5,5'	109	2,3,3',4',5	166	2,3,4,4',5,6		

Table 1.2 Melting points, water solubilities and vapour pressures of some PCB congeners (Lang, 1992). Data not available is indicated by '-'.

Congener no.	Melting point (°C)	Water solubility (μg/L)	Vapour pressure at 25°C (mPa)
1	34	6000	1120
2	25	2400	200
3	78	1200	612
4	61	1100	133
7	24	1100	239
15	149	56	2.53
18	44	410	12.0
29	78	130	43.9
30	63	200	117
40	121	29	9.71
47	83	92	11.4
52	87	29	4.92
66	124	4.6	6.12
77	180	9.2	0.306
86	100	21	0.0771
87	114	4.1	2.13
101	77	10	1.20
105	125 [a]	2.1 [a]	0.904
116	124	5.2	-
118	105	2.1 [a]	1.20
126	125 [a]	1.0 [a]	-
128	150	0.57	0.346
138	79	1.8	0.532
153	103	0.91	0.692
156	141 [a]	0.36 [a]	0.213
171	122	2.0	0.239
180	99 [a]	0.63 [a]	0.129
194	159	0.22	-
208	183	0.018	-
209	306	0.0013	-

[a] Estimates

PCBs were marketed as commercial mixtures of chlorobiphenyls with titles such as Aroclor (USA, UK), Clophen (Germany) and Kanechlor (Japan), Fenchlor (Italy), Delor (Czech Republic) and Sovol (USSR) (Lang, 1992; Rice & O'Keefe, 1995). Commercial mixtures are often complex containing a number of congeners each with its own properties e.g. see Table 1.3 (Rice & O'Keefe, 1995). PCB mixtures are produced by the chlorination of biphenyl using various catalysts and production conditions (heat, pressure and reaction time) (for more information see (Bolgar et al., 1995). Individual mixtures were identified by their percentage of chlorine e.g. Aroclors 1254 and 1221 contained 54% and 21% Cl respectively. PCBs were first synthesized in 1881 by Smith and Shulz and commercial production commenced in 1929 (Hooper et al., 1990). PCB formulations were a commercial success as the compounds are electrically resistant and thermally stable over a large temperature range (WHO, 1992). High *n*-octanol/water partition coefficients (K_{ow}) of the many congeners denote the high lipophilicity and low solubility in water of the commercial mixes (OSPAR, 2001) (Table 1.3). PCBs are resistant to acid-base hydrolysis, oxidation and reduction, resulting in stable and environmentally persistent compounds that can remain over geological time (Hooper et al., 1990).

PCBs were used in electrical insulators, lubricants, hydraulic fluids, plasticisers, inks, varnishes, as flame-retardants in plastics and as preservatives in rubbers and a number of other everyday items (Camacho-Ibar, 1991). Peak production occurred mainly in industrialized countries in the 1960s and 1970s (Tanabe, 1988b). Peak production in the USA was in 1970 when 33,000 tonnes were manufactured. In 1972 sales were restricted by many countries such as Sweden, the U.K. and the U.S.A. and prohibited in Japan when it was discovered that PCBs caused widespread environmental contamination and had impacts on human health (WHO, 1992; Aoki, 2001). PCB manufacture ceased in the UK in 1977 (CEFAS, 2001). However, between 1980-84 over 48,000 tonnes of PCBs were still produced in Europe (WHO, 1992) and in 1987, the Organisation for Economic Co-operation and Development (OECD) nations agreed to (i) ban virtually all new uses of PCBs (ii) accelerate their phasing out from current uses and (iii) control methods of disposal (WHO, 1992). In total, it is estimated that more than one million tonnes of PCBs have been created worldwide (Tanabe, 1988b). An estimated 4% of PCBs produced have been destroyed, 65% are considered to remain in use or land-stocked locations (e.g. refuse tips) and 31% are

Table 1.3 Approximate composition in weight percent of Aroclor preparations and some physical properties of the commercial mixes (from Rice and O'Keefe (1995) and WHO (1992)). *a* – at 24°C

Homologue	Aroclor type						
No. of Cl per biphenyl	1221	1232	1016	1242	1248	1254	1260
0	10						
1	50	26	2	1			
2	35	29	19	13	1		
3	4	24	57	45	21	1	
4	1	15	22	31	49	15	
5				10	27	53	12
6					2	26	42
7						4	38
8							7
9							1
10							
Average no. of Cl per molecule	1.15	2	3	3	4	5	6
Approx. wt. % Cl	21	32	42	42	48	54	60
Water Solubility, mg l^{-1}, 25°C	0.59[a]	0.45	0.42	0.24	0.054	0.021	0.0027
Boiling point, °C	275-320	290-325	325-356	325-366	340-375	365-390	385-420
Appearance at common ambient temperature	Clear, mobile oil	Clear, mobile oil	Clear, mobile oil	Clear, mobile oil	Clear, mobile oil	Light-yellow, viscous oil	Light-yellow, sticky resin

free in the environment (Tanabe, 1988b). Of the latter, 61% are found in the open ocean and it is thought that the land-stocked PCBs could begin to enter the environment at any time (Tanabe, 1988b). PCBs contained in sediments have been observed to re-dissolve slowly into the water column over a period of time especially if sediments are mobilised, thus acting as environmental reservoirs of PCBs (OSPAR, 2001; Rice & O'Keefe, 1995).

1.3 Chemical analyses

Before the 1980s, analytical techniques for PCBs lacked the precision to determine individual congeners and attempts were made to express chemical data in terms of commercial mixture concentrations by matching sample congener profiles with those of commercial formulations. Those methods were often open to error because the PCBs detected in environmental samples may have come from more than one commercial preparation and/or the PCBs themselves may have undergone selective environmental degradation depending on the properties of the particular congeners (Camacho-Ibar, 1991). In addition, the uptake of congeners varies between environmental matrices and can change with time, distorting the original congener configuration of the commercial mixture e.g. Fox et al. (2001) noted the change in congener profiles with depth of sediment cores taken from the Mersey estuary with penta- and hexa-CBs being the most prominent congeners detected except in the most recently deposited sediments where tri-, tetra- and hepta-CBs were also detected. In fact the ICES 7 were so named because of their greater tendency to resist degradation and accumulate in greater proportions in biological tissue and sediment, thus demonstrating the differences between environmental PCB profiles and the original mixtures. Given the large quantity of congeners present in environmental samples, it is common practice to report a minimum of seven congeners specified by the International Council for Exploration of the Seas (ICES) to allow for easy comparison of PCB concentrations across studies. The congeners are referred to as the ICES 7 and are CBs nos. 28, 52, 101, 118, 138, 153, and 180. These congeners are often dominant in both commercial PCB formulations and environmental samples and four

are included in the 19 congeners analysed by Wilson-Yang et al. (1991) that may assume a partially planar configuration.

Modern studies providing more detailed information regarding particular congeners are more useful in assessing PCB contamination and importance in specific situations given current knowledge of individual congener properties e.g. the toxicity of CB-118 due to its partially planar configuration (see section 1.2.1). With improvements in high-resolution mass-spectrometry, individual congener peaks could be determined, which allowed more accurate quantitative analyses of samples.

1.4 Environmental distribution and persistence

PCBs were first detected in environmental samples in the late 1960s (Jensen, 1972; WHO, 1992) and became rapidly regarded as an ubiquitous, global contaminant detected in most marine fish species (Table 1.4) (Rice & O'Keefe, 1995) as well as air, water, soil and sediment samples (WHO, 1992).

Generally, the global, spatial trend is for concentrations of PCBs in the environment to decrease further away from the areas in which they were manufactured and used, as was the case with concentrations of PCB153 in dab (*Limanda limanda*) and flounder (*Platichthys flesus*) livers in the UK (NMP, 1998). Entry into the environment is often via leakage from "closed" electrical or hydraulic systems, leaching from landfill sites following improper disposal, sewage effluents and waste incineration (Mason, 1996; Rice & O'Keefe, 1995). Heating of PCBs also leads to the formation of highly toxic compounds such as polychlorinated dibenzo-*p*-dioxins (PCDDs) (Tanabe, 1988a). The discharge of PCBs and their derivatives into surface waters or atmospheric deposition leads to adsorption onto suspended solids and sediments, especially those rich in organic material and lipids (Rice & O'Keefe, 1995).

Degradation of PCBs can occur through microbial action and photolysis through exposure to UV radiation (Rice & O'Keefe, 1995; WHO, 1992). Biodegradation rates are variable and depend on the degree of chlorination, concentration, microbial

Table 1.4 Examples demonstrating the global distribution of polychlorinated biphenyls (PCBs) detected in marine fish and presented by geographical location. Where mean concentrations are given as a range, they denote the ranges of mean values between sampling sites.

Pollutant	Location	Species [a]	Concentration range	Mean concentration (± 1 S.E.)	Author(s)
PCBs	Baltic Sea	Flounder – liver (n = 25) Flounder – ovary (n = 59)	5.0 - 730.0 ng g^{-1} w.w. 5.0 - 317.1 ng g^{-1} w.w.		von Westernhagen *et al.* (1981)
PCBs – 7 congeners	Norway	Plaice – liver (n = 56)		36.3 ng g^{-1} w.w.	Green & Knutzen (2003)
PCBs	Russia	Navaga – whole fish (n = 3) Bullrout (n = 4) White Sea cod (n = 8) White Sea herring (n = 3)	35.5 - 47.1 ng g^{-1} w.w. 14.8 - 32.6 ng g^{-1} w.w. 6.8 - 25.7 ng g^{-1} w.w. 37.8 - 47.6 ng g^{-1} w.w.	40.6 ± 5.92 ng g^{-1} w.w. 23.4 ± 8.16 ng g^{-1} w.w. 15.6 ± 7.57 ng g^{-1} w.w. 41.2 ± 5.57 ng g^{-1} w.w.	Muir *et al.* (2003)
PCBs – Aroclor 1016 & 1254	Hudson River, USA	Atlantic tomcod – liver (n = 12) Atlantic tomcod – ovary (n = 12) Atlantic tomcod – testes (n = 12)	10.94 - 98.22 µg g^{-1} 0.08 - 0.83 µg g^{-1} 0.19 - 7.35 µg g^{-1}		Klauda *et al.* (1981)
PCBs	5 sites along the Hudson River, USA	Mummichog – gonad (n = 200) Mummichog – liver (n = 200) Mummichog – muscle (n = 200)		227 - 3453 ng g^{-1} w.w. 150 - 1333 ng g^{-1} w.w. 37 - 263 ng g^{-1} w.w.	Monosson et al. (2003)
PCBs	4 sites in Puget Sound, USA	English sole – liver (n = 12)		90 - 3470 ng g^{-1} w.w.	Casillas *et al.* (1991)
PCBs	11 sites in North east USA	Winter flounder – ovary (n = 105) Winter flounder – liver (n = 85)		100 - 520 ng g^{-1} w.w. 313 - 2336 ng g^{-1} w.w.	Johnson *et al.* (1992)
13 PCBs extracted from pooled samples	Southern Greenland	Shorthorn sculpin – liver (n = 24) Uvak – liver (n = 10) Spotted wolf fish – liver (n = 1) Starry ray – liver (n = 1)	37.7 - 324.6 ng g^{-1} 119.8 - 178.5 ng g^{-1} 29.6 ng g^{-1} 87.6 ng g^{-1}		Christensen *et al.* (2002)

Contd. overleaf

Table 1.4 *Contd.*

PCBs	Alaska	Pink salmon – whole (n = 7) Arctic charr – whole (n = 5) Arctic cod – whole (n = 12) Fourhorn sculpin – whole (n = 7)		42 ± 21 ng g^{-1} l.w. 52.0 ± 9.4 ng g^{-1} l.w. 67 ± 10 ng g^{-1} l.w. 79 ± 15 ng g^{-1} l.w.	Hoekstra *et al.* (2003)
PCBs	Australia	7 freshwater species – muscle (n = 303)		0.051 - 10.140 µg g^{-1}	Roach & Runcie (1998)
PCBs	Northern Pacific Ocean	Bigeye tuna – liver (n = 10)	7.8 - 19.2 ng g^{-1} liver fat		Hashimoto et al. (2003)
PCBs	5 sites in Cambodia	11 marine fish species – whole body (n = 33)	0.07 - 1.2 ng g^{-1} w.w.		Monirith *et al.* (1999)
PCBs (12 congeners)	Burundi	Ingege – whole body (n = 5)		166.7 ± 37.4 ng g^{-1} l.w.	Manirakiza *et al.* (2002)

(f) = female; (m) = male; w.w. = wet weight; l.w. = lipid weight

[a] SPECIES – Atlantic tomcod, *Microgadus tomcod*; flounder, *Platichthys flesus*; English sole, *Parophrys vetulus*; winter flounder, *Pleuronectes americanus*; ingege, *Oreochromis niloticus*; pink salmon, *Oncorhynchus gorbuscha*; Arctic charr, *Salvelinus alpinus*; Arctic cod, *Boreogadus saida*; fourhorn sculpin, *Myoxocephalus quadricornis*; mummichog, *Fundulus heteroclitus*; bigeye tuna, *Thunnus obesus*; plaice, *Pleuronectes platessa*; shorthorn sculpin or bullrout, *Myoxocephalus scorpius*; uvak, *Gadus ogac*; spotted wolffish, *Anarhichas minor*; starry ray, *Raja radiata*; Navaga, *Eleginus navaga*; White Sea cod, *Gadus morhua marisalbi*; White Sea herring, *Clupea pallasi marisalbi*.

population structure, available nutrients and temperature (Rice & O'Keefe 1995). Generally, the more highly chlorinated PCBs degrade more slowly than the less chlorinated congeners (Rice & O'Keefe, 1995). Half-lives of PCBs also vary greatly depending on the matrix within which they exist, with shorter half-lives occurring in air compared to sediments (WHO, 1992). The biodegradation of PCBs in soils is very slow (Rice & O'Keefe, 1995) making it likely that they will continue to leach into surface waters for many years. The accumulation and breakdown of PCBs within biota can vary with the lipid content of organisms and with differences in metabolism (Rice & O'Keefe, 1995). Niimi & Oliver (1983) calculated the biological half-lives of 31 dichloro- to decachlorobiphenyl congeners in rainbow trout, *Oncorhynchus mykiss*, following administration of single oral doses of between 46-261mg kg^{-1}. Whole-body half-lives increased with the degree of chlorination, from five days to >1000 days (Niimi & Oliver, 1983), which demonstrates their persistence and potential to cause long-term impacts.

Monitoring of the UK coastal waters is co-ordinated by the National Marine Monitoring Program (NMMP) which collates environmental monitoring data from the various agencies e.g. the Centre for Environment, Fisheries and Aquaculture Science (CEFAS) and the Scottish Environmental Protection Agency (SEPA). This monitoring program allows the UK to be used as an example of the environmental distribution and concentration of persistent organic pollutants (POPs). The program has identified areas of the UK with the highest concentrations of PCBs and confirmed that these areas are restricted to historically or presently industrialized estuaries such as the Rivers Tees and Mersey (NMP, 1998). Much of the monitoring of PCB concentrations in UK biota focuses on mussels, dab and flounder, and the NMMP has focused on the single PCB congener 153 due to its suitability as an indicator of general PCB concentrations. Chapter 2 examines the concentrations of PCBs reported in fish species around the UK coast in more detail.

1.5 Exposure pathways

1.5.1 Bioconcentration and biomagnification

Adult fish in the environment can be exposed to contaminants via aqueous transfer across the gill surface (bioconcentration) and dietary exposure through the food chain (biomagnification) (Fu & Wu, 2006). In the case of PCBs that have low solubility, it has been suggested that the less hydrophobic congeners (log K_{ow} <5-6) are more likely to be bioconcentrated, whilst more hydrophobic congeners (log K_{ow} >5-6) are more likely to be biomagnified (Clark et al., 1990; Russell et al., 1999). Fu & Wu (2006) postulated that PCBs detected in Taiwanese mullet (*Liza macrolepis*) in a river estuary had likely been accumulated by both these routes.

PCBs biomagnify with increasing trophic level through dietary exposure in marine food webs (Muir et al., 2003). The hydrophobic tendencies of the molecules mean that predators, especially at higher trophic levels, retain a large proportion of the contaminants consumed. This has been demonstrated for PCBs in the food chain from invertebrates to fish to seals in the White Sea ecosystem, Russia (Muir et al., 2003). A study of Arctic waters by Hoekstra et al. (2003) highlighted the increasing biomagnification factors with trophic level when comparing organochlorine concentrations, including PCBs, in calanoid copepods, fish, seals and whales. Contaminant exposure routes, an organism's feeding strategy and depuration capacity and the physical-chemical properties of a compound all influence the biomagnification of a substance within any particular organism (Hoekstra et al., 2003). However, biomagnification and bioconcentration are not the only routes of exposure.

1.5.2 Maternal transfer

Most PCB exposure to wildlife occurs in aquatic environments due to adsorption to sediments and other organic matter. PCBs vary in concentration between body tissues and organs of fish or mammals, accumulating in tissues of high lipid content such as

the liver (Table 1.5). Seasonal variation in body burdens can also occur as body fat stores vary with prey availability (Sellstrom et al., 1993). Johnson et al. (1992) observed that PCB levels in the ovaries of winter flounder (*Pseudopleuronectes americanus*) increased from October to December whilst concentrations in the liver decreased, suggesting mobilization of the PCBs to the developing ovaries in conjunction with lipid transfer. This variation in body burdens due to lipid mobilisation has also been recorded for dab (Fonds et al., 1995) and Atlantic croaker (*Micropogonias undulatus*) (Ungerer & Thomas, 1996).

Vitellogenin (VTG) is a female precursor protein produced in the liver, transported to the ovaries and deposited in the eggs during egg production (Fisk & Johnston, 1998). The VTG protein transports lipids, together with lipophilic organochlorines, bound to or into its backbone and transports them into the eggs (Ungerer & Thomas, 1996; Fisk & Johnston, 1998). A second hypothesis for delivery to the eggs is by transport directly from the blood stream but this becomes less likely with increasing molecular hydrophobicity (Fisk & Johnston, 1998). PCBs derived direct from the diet through the blood stream were likely to be the lower chlorinated molecules of which there were fewer present due to lower food intake over the winter when the eggs were maturing (Fisk & Johnston, 1998). Fisk and Johnston (1998) concluded that the most likely source of PCBs in walleye eggs was somatic tissue given the large proportion of highly chlorinated (i.e. highly hydrophobic) congeners present.

Maternal transfer reduces the body burden of a female fish but transfers the pollutants to the eggs, placing the next generation at potential risk of decreased survival (Sumpter et al., 1997; Rose et al., 2003; Weis et al., 2003; Alvarez et al., 2006). The amount of contaminant transferred from a female to the eggs depends on three factors: (i) the contaminant levels in the fish (ii) the fish lipid content (iii) the egg lipid content (Niimi, 1983). In freshwater walleye (*Stizostedion vitreum*), the transfer of organochlorines has been shown to vary between individuals, possibly for the above reasons (Fisk & Johnston, 1998).

1.6 Endocrine disruption and sub-lethal effects

1.6.1 Endocrine disruption

The endocrine (hormone) system in organisms is vital for the control of biochemical processes which, in turn control physiology and development (Colborn et al., 1993). Endocrine disrupting chemicals (EDCs) are defined as "an exogenous substance or a mixture, that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations" (Anonymous, 1996). Certain xenobiotics such as PCBs and dichlorodiphenyl-trichloroethane (DDT) can interact with hormone receptors, thereby disrupting the endocrine system and processes of physiology and development (Lye et al., 1997; Matthiessen et al., 1998). Often, the structure of EDCs may not appear to resemble natural hormones making it hard to predict their effects that often remain unseen until they are in use as was the case with DDT (Sumpter et al., 1997). Disruption of endocrine systems and physiology by xenobiotics such as PCBs and DDT has been the focus of much concern due to the unknown, sub-lethal, chronic effects of chemical compounds released into the environment. The first recorded case in the literature of endocrine disruption was the impacts of DDT on young roosters that prevented them from developing into normal males (Burlington & Lindeman, 1950). Testes of exposed roosters were only 18% of the normal size amongst the observed effects (Burlington & Lindeman, 1950). The suggestion was made that DDT was likely to be acting as a hormone but the study was largely overlooked or ignored as the new evidence did not fit into the ruling scientific concepts at the time regarding biological processes (Colborn et al., 1996). Consequently, DDT was marketed as safe product until its full effects were officially recognised some years later.

One of the major issues faced by ecotoxicologists is the reluctance of endocrine disruptors to "play by the rules" of traditional toxicological studies. The general maxim that "the dose makes the poison" does not often apply and dose-response relationships are often non-linear (Colborn et al., 1996). Response curves may be sigmoidal with an effect only being observed above a certain threshold, which itself can vary between species or with environmental conditions (Colborn et al., 1996).

Calabrese & Baldwin (2003) suggested recently that perhaps the most fundamental shapes of dose-response curves are J-shapes or inverse U-shapes, providing evidence of what is called a "hormetic response" or "hormesis". In these cases, modest effects can be observed at lower concentrations before inhibition occurs at higher doses. Reasons behind these forms of response are not fully understood but may be due to the complex feedback loops that form the endocrine system. A small quantity of an endocrine disrupting chemical might cause a detectable effect but at greater concentrations could overload the hormone system it was affecting, causing it to shut down and show no response until concentrations return to lower levels, allowing the system to recover (Colborn et al., 1996). The difficulty caused by this latter pattern in observed effects is that toxicological studies often only make use of a limited range of doses or endpoints and as such, might overlook the lower concentrations at which sub-lethal effects occur (Calabrese & Baldwin, 2003). The hormetic dose-response should be regarded not as an exception to the rules of toxicology but as "the rule" (Calabrese & Baldwin, 2003). Calabrese & Baldwin (2003) also suggested that future toxicology studies should aim to use more low doses and more subjects to enhance their statistical power to detect significant changes in the parameters measured.

1.6.2 Endocrine disruption in the environment

Despite the problems with assessing toxicities as outlined above, much evidence exists regarding the disruption of endocrine systems at low contaminant concentrations in the environment. Endogenous oestrogens or oestrogen-mimics such as PCBs can block receptors of the major vertebrate oestrogen 17ß-oestradiol (Sumpter et al., 1997; Matthiessen & Sumpter, 1998). Among other functions, 17ß-oestradiol stimulates growth through increased lipid deposition and protein synthesis and deposition, interruption of which may hinder normal development. Mimicking natural oestrogens are not the only endocrine disrupting properties of the 209 PCB congeners as some have also been shown to be anti-oestrogenic (CB-126) (Vaccaro et al., 2005) and anti-androgenic (CB-138) (Bonefeld-Jørgensen et al., 2001).

A further, major effect of endocrine disruptors is that of feminisation which has been observed in flounder in the Tyne, Tees and Mersey estuaries in the U.K. (Matthiessen

et al., 2002). Oestrogens are essential for the development of female secondary characteristics, male brain differentiation and the development of sexual behaviour (Westerlund et al., 2000). In polluted UK estuaries, male flounder have been shown to have significantly elevated levels of VTG in comparison with those sampled from control estuaries (Matthiessen et al., 1998). Exposure of fish to endocrine disrupters has resulted in VTG production in male flounder in the River Tyne and development of hermaphroditic fish following exposure to sewage sludge (species not identified) (Lye et al., 1997). Production of VTG in males of egg-laying species can be used as a sensitive bioindicator of oestrogen exposure (Lye et al., 1997). The male flounder in the Tyne were also recorded as having abnormal sperm and testicular structure (Gill et al., 2002). As the Tyne is recognized as one of the most heavily polluted estuaries in the UK, with respect to oestrogenic compounds along with the Tees and Mersey, these sperm abnormalities are likely to result from EDC exposure (Matthiessen et al., 1998; Gill et al., 2002). Gill et al. (2002) also noted that the presence of oestrogenic chemicals in the Tyne estuary was also the cause of asynchronous development of spermatogenesis, which would be likely to hinder successful reproduction. No suggestions were made as to which substances caused these effects although PCBs are amongst the many endocrine disruptors known to exist in the waters, sediments and biota of the Tyne estuary (Matthiessen et al., 1998).

Cumulative effects of exposure to multiple chemicals are exceedingly important to consider as this situation occurs often in estuarine situations where juvenile fish can be found (Gill et al., 2002). It is often hard to pin-point the exact chemical causes of endocrine and physiological dysfunction due to the synergistic effects of the complex mixtures present (Matthiessen & Sumpter, 1998) and/or the metabolites produced through oxidation, or addition of hydroxyl groups resulting in dioxin-like intermediates (Bergman et al., 1994; Matta et al., 1997). As with the physical and chemical properties of PCBs, toxicity varies with the individual congeners, the coplanar (flat) PCBs being the most toxic, especially those with the Cl ions in the para- and meta- positions (Tanabe et al., 1987). Evidence suggests that less toxic congeners are metabolised, although metabolites may not be less toxic than the parent compounds if solubility is increased (Bergman et al., 1994; Matta et al., 1997). This may mean that the more toxic congeners remain in the food chain, having greater effects on the vulnerable carnivores at the top (Aulerich et al., 1986).

1.6.3 Sub-lethal effects of EDCs

Although environmental concentrations of PCBs are reported to be falling in some areas e.g. in mussels in the Mersey Estuary (Connor et al., 2001), the concentrations at which they may exert subtle, sub-lethal effects on the endocrine systems of different species are not always known. A study by Holm et al. (1993) using 3-spined sticklebacks (*Gasterosteus aculeatus*) dosed over 3.5 months with PCBs (Clophen A50) and polybrominated diphenyl ethers (PBDEs, brominated flame retardants) (Bromkal 70-5DE) (3594 mg kg^{-1} fat and 1630 ± 275mg kg^{-1} fat respectively) recorded reduced spawning success in high-dosed groups to 20% and 25% respectively compared to controls which had 80% success. Clophen A50 also induced EROD activity (enzymes involved in detoxification of compounds, explained further in section 5.2) but Bromkal 70-5DE did not. The concentrations used were several orders of magnitude higher than those found in fish from the environment but were used to see what potential the chemicals had to cause an effect (Holm et al., 1993). A further study by Holm et al. (1994) on effects of the commercial PBDE mixture Bromkal 70-5DE on 3-spined sticklebacks and rainbow trout again indicated that it was not a potent inducer of EROD activity in fish. PBDEs were introduced following the discontinuation of the manufacture of PCBs, have the same nomenclature for the 209 potential congeners and have been detected in the environment on a global scale (de Boer, 1990; Akutsu et al., 2001; Christensen et al., 2002; Law et al., 2002; Hale et al., 2003).

Effects of short-term exposure to commercial Penta-BDE and Octa-BDE mixes on Atlantic salmon EROD activity have shown no significant impacts at concentrations three orders of magnitude higher than fish from the North Sea, indicating low short-term toxicity (Boon et al., 2002). The authors did recognise a need for long-term, multi-life stage studies including egg and larval phases given the increasing environmental concentrations of PBDEs. Studies of PBDEs on non-fish species have shown different results. Larval development and population growth rates of the harpacticoid copepod (*Nitocra spinipes*) were both reduced following six days exposure to the PBDE congeners 47 and 99 (Breitholtz & Wollenberger, 2003). A

study of the penta-BDE product DE-71 in mice resulted in increased EROD activity and in individuals administered the maximum dose, immune response was impaired (Fowles et al., 1994). Thyroid disruption has also been observed in rats following exposure to DE-71 (Zhou et al., 2002) and in humans has been linked to products of PBDE metabolism (Meerts et al., 2000). Given the known impacts on non-fish species, further studies would aid in confirming whether some of these lacks of PBDE impact are the case in all fish species. The studies mentioned above did not investigate the sub-lethal impacts of contaminant exposure on fish behaviour, especially larval behaviour, although Holm et al. (1994) did mention there was no observed effect on adult feeding patterns or behaviour. Contamination has the potential to disrupt the sensory systems of fish, impairing their behaviour and ecological performance.

1.6.4 Behavioural studies

Jones & Reynolds (1997) found that only ca.14% of studies involving fish and pollution between 1978 and 1997 included an analysis of behaviour. Many of these studies focused on freshwater fish (Blaxter & Ten Hallers-Tjabbes, 1992) and most, including those on PCB contamination, concentrate on biochemical, physiological and histological parameters, impacts on reproduction such as fertilization rates, and lethal doses of specific compounds. Such studies may have missed important impacts on surviving larvae that could further affect long-term fitness (Foster & Berlin, 1997; Jones & Reynolds, 1997; Wibe et al., 2001). Behavioural changes may be the integrated result of physiological and biochemical changes (Clotfelter et al., 2004). Zhou et al. (1999) reported impairment of thyroid function in mummichogs (*Fundulus heteroclitus*) from a creek known to be polluted with heavy metals and organic compounds including PCBs, which may have been a factor in the many behavioural differences. Reduced prey-capture and predator avoidance ability and impaired routine swimming activities were observed when compared to fish from a clean reference site.

Fish larvae may use olfactory, auditory, mechanosensory, chemosensory or visual systems to detect predators and prey in their environment (Blaxter & Ten Hallers-

Tjabbes, 1992). Disruption of the development of these systems due to sub-lethal contaminant exposure may result in decreased search efficiencies, strike frequencies, prey capture rates, reaction distances and increased prey handling times (Blaxter & Ten Hallers-Tjabbes, 1992). These in turn may then limit growth rates of larvae or juvenile fish and lower survival rates, as they remain susceptible to factors such as predation for longer periods of time in the wild (Faulk et al., 1999; Zhou et al., 2001). Most behaviour exhibited by fish is due to their sensory system responding to stimuli in the environment. Contaminants may affect the functioning of fish' sensory organs, changing or reducing information reaching the brain, and should be detected by changes in behaviour (Blaxter & Ten Hallers-Tjabbes, 1992).

A review by Blaxter & Ten Hallers-Tjabbes (1992) summarised the effects of pollutants on sensory systems and behaviour of aquatic animals but concentrated mainly on freshwater fish and invertebrates due to the lack of literature on marine fish. Organic compounds such as pesticides, herbicides and PCBs can act on mechanoreceptors, taste buds, olfactory organs or the integuments of fish, affecting the central nervous system and the effectors it controls e.g. muscles or endocrine glands (Blaxter & Ten Hallers-Tjabbes, 1992). Effects such as these can be due to the pollutant(s) increasing the threshold level of stimuli by increasing the signal "noise", over-stimulating the sensory system causing fatigue of sense organs or clogging receptors thereby masking normal stimuli that may otherwise induce a response (Blaxter & Ten Hallers-Tjabbes, 1992). Blaxter & Ten Hallers-Tjabbes (1992) noted the lack of research into the long-term effects on populations of sub-lethal chemical exposure. Such exposure may upset the balance between an organism and its environment with respect to inefficient use of resources such as food and space, predator-prey interactions or impaired protective responses such as hiding or burying. Most fish larvae begin learning to catch prey when they still have a yolk sac and feeding efficiency increases with age (Faulk et al., 1999). Impaired swimming speeds and activity, possibly due to impaired brain development, could hinder this learning process if there is less impulse to feed and the larvae do not try. This delay in learning could leave more larvae susceptible to starvation and predation during the critical period following yolk absorption with potential impacts on subsequent recruitment to adult populations (Faulk et al., 1999; McCarthy et al., 2003; Rose et al., 2003; Alvarez et al., 2006).

Toxic and sub-lethal effects of persistent organic pollutants such as PCBs and DDT accumulating at environmentally relevant concentrations in fish have been well documented (Weis & Weis, 1982; Tanabe et al., 1987; Lye et al., 1997; Faulk et al., 1999; Gill et al., 2002; McCarthy et al., 2003). Embryos and larvae are often the most critical life stage in any fish population since mortality rates influence the level of stock recruitment (Houde, 2002). Increased mortality of these life stages due to additional stresses other than those from the natural environment (e.g. xenobiotic contamination) can be detrimental from a population viewpoint (Weis & Weis, 1989). Contaminant exposure can occur even before fertilization, via maternal transfer of lipoprotein-bound pollutants deposited in the eggs during gonadal recrudescence (oogenesis) (Ungerer & Thomas, 1996). Further understanding of the dynamics of parental transfer of xenobiotics and the resulting impacts on early life stages is important when considering the overall impacts on a species and ultimately population and community ecologies.

1.7 A brief ecology of the plaice

The European plaice (*Pleuronectes platessa*) was selected as the appropriate experimental subject in this study for a number of reasons. Clotfelter et al. (2004) suggested that the use of animals other than the traditional model species, such as zebra fish (*Danio rerio*) or sticklebacks, is a good idea as it increases the knowledge of the ways in which different species respond to contaminants. Although the plaice has been the subject of much research (Wimpenny, 1953; Johnston & Goldspink, 1973; Ehrlich et al., 1976; Gibson & Batty, 1990; Batty & Hoyt, 1995; Burrows & Gibson, 1995; Christensen & Korsgaard, 1999) its use in toxicological studies is less common. However, where the plaice has been used in such studies (Eggens et al., 1996), its use has been advocated over that of flounder (*Platichthys flesus*) given its greater propensity to show a response. Species such as flounder that inhabit estuarine environments for the majority of their lifecycle and/or are constantly exposed to polluted environments can sometimes cease to exhibit certain responses to certain pollutants (Zhou et al., 1998; Yuan et al., 2005). Therefore use of fish from relatively

clean environments can be advantageous when attempting to evaluate chemical toxicity. In addition to this, the plaice is easily maintained in aquarium systems, can be hand-stripped of gametes for in *vitro* fertilisation (Hiddink, 1997) and produces relatively large larvae that can feed on *Artemia salina* immediately following yolk absorption without requiring a rotifer feeding stage (Baynes, CEFAS, pers. comm.).

The biology and ecology of the plaice have been studied in great detail for decades and this section aims to provide just a brief description of plaice ecology with an emphasis on the early life stages. For further in-depth information, Wimpenny (1953) and Gibson (2005) provide much essential information. The taxonomic classification of the European plaice (*Pleuronectes platessa*) is given below:

Class:	Actinopterygii (ray-finned fish)
Order:	Pleuronectiformes (flatfish)
Family:	Pleuronectidae (right-eye flounders)
Sub-family:	Pleuronectinae
Genus:	*Pleuronectes*
Species:	*platessa*
Authority:	Linnaeus (1758)

The adult European plaice is capable of growing up to 100cm standard length and up to 50 years old (Neilsen, 1986). However, as the plaice is one of the UK's most economically important fish species, specimens this size are rarely seen. Plaice have a smooth skin, small scales and a bony ridge behind the eyes. The upper side is often brown coloured with vivid, orange-red spots whilst the underside is white. The lateral line is mostly straight with a slight curve above the pectoral fin.

The plaice inhabits a benthic environment at depths up to 200m in water ranging from 2 to 15°C and has a geographical range from as far north as Greenland and Norway, south to Morocco and east into the Mediterranean (Neilsen, 1986). The plaice will inhabit a variety of mixed sea beds feeding mainly on molluscs and polychaetes and older fish tend to reside in deeper waters (Wimpenny, 1953). Tagging experiments have shown that long migrations occur at various times of year as the adult fish move

between spawning and feeding grounds (Hunter et al., 2003). Even in relatively confined water bodies such as the Irish Sea, it has been demonstrated that plaice are divided into distinct sub-populations (Nash et al., 2000).

Plaice are batch spawners and a single female may release between 10,000 and 700,000 eggs in a spawning season depending on its condition and size (Wimpenny, 1953). Plaice larvae are relatively large (ca.6.5mm) at hatch and internal asymmetry commences as soon as the yolk-sac disappears with the coiling of the intestine (Wimpenny, 1953). External signs of metamorphosis can be seen a few weeks following hatch as the left eye commences its journey over to the right side of the head. As the left eye moves over, so the swimming position of the young larvae changes so that the plane passing through the eyes remains horizontal with the body at an angle, until it has moved through 90° and metamorphosis is complete after approximately six weeks (Wimpenny, 1953). Newly hatched larvae need to be large as they hatch earlier in the year than many other fish species when prey is scarce and large in size (McEvoy & McEvoy, 1991). Feeding may occur before the yolk supply is exhausted but after eight days all the yolk has been absorbed and the larvae feed on plankton. Planktonic food consists of both phytoplankton such as diatoms and flagellates and larval forms of other marine animal species, particularly copepods (Wimpenny, 1953).

During metamorphosis, the larvae begin to migrate towards coastal waters and remain close to the seabed rather than in the planktonic zone. On completion of metamorphosis at 10-17mm in length, the flatfish settle to the seabed in their nursery grounds. A few juvenile fish can often be found in any shallow, sandy inshore area around the UK but particular sites are important nursery grounds, home to tens of thousands of young fish. Such areas are found on the Isle of Man, Morecambe Bay, Red Wharf Bay on Anglesey, and Bran Sands in Tees Bay (Wimpenny, 1953; Macer, 1967; Nash & Geffen, 2000). Some of these sites are in areas affected by organic pollution such as Tees Bay (Allchin et al., 1999). The very nature of being a flatfish means that the gills on one side of the body are always adjacent to the seabed. This might make juvenile fish inhabiting contaminated inshore areas susceptible to both bioconcentration of contaminants in sediments as well as biomagnification as they feed on the organisms within those sediments.

Food for juvenile plaice at first consists of small polychaete, spionid or naiad worms along with copepods though as the fish grow, larger prey are taken and include small bivalves, crustaceans, amphipods and small gastropods (Wimpenny, 1953). Competition for food amongst the juvenile plaice is intense and high population density has noticeable negative impacts on their growth (Wimpenny, 1953). Successful foraging and predator avoidance are key in these environments and disruption of such behaviours by xenobiotic compounds could influence the ecological performance of the juvenile fish (Weis & Weis, 1989; Faulk et al., 1999).

1.8 Project aims

The aims of this project were to (i) collect field data and evaluate the current knowledge of PCB contamination in the European plaice (*Pleuronectes platessa*) around the UK coast, (ii) assess the potential for maternal transfer of PCBs following oral dosing with Aroclor 1254 to the ovaries during oogenesis in plaice, (iii) assess the sub-lethal effects of environmentally realistic PCB concentrations on the offspring of the plaice by measuring multiple interdisciplinary parameters including growth, survival, behaviour and physiology, (iv) compare the sensitivities of the techniques used and identify advantages and disadvantages associated with each and (v) identify areas in which investigations of endocrine disruption caused by xenobiotic compounds can be improved. Integration of the results from this wide spectrum of disciplines would then provide a clearer picture of the potential impacts on the ecology of the plaice and identify areas requiring further, in-depth examination.

Given the evidence from previous studies it was hypothesised that larvae from females dosed with Aroclor 1254 might exhibit reduced survival, inhibited growth patterns and altered behavioural and physiological performance. However, given the tendency for species-specific responses and toxin-specific effects often experienced in such studies (Nebeker et al., 1974; Mayer et al., 1977; Weis & Weis, 1982) it was impossible to predict exactly whether each measured response would be inhibited or enhanced.

Aroclor 1254 was chosen as the PCB mixture to use for the duration of the study. Aroclor 1254 was one of the commercial mixtures produced during the period of peak production (Rice & O'Keefe, 1995) and can closely resemble PCB congener patterns observed in the environment (Fu & Wu, 2006). Aroclor 1254 contains all of the ICES 7 congeners that are frequently detected in most environmental and biological samples (see section 1.3) and was thus considered an appropriate mixture to use in the exposure experiments. Studies investigating the impacts of single congeners are important because they explain the different effects and toxicities of the separate congeners but they are rarely representative of environmental conditions where species are exposed to a wide range of PCB congeners as well as other contaminants.

Chapter 2

Polychlorinated biphenyls in the European plaice, *Pleuronectes platessa* in the United Kingdom

2.1 Abstract

In a field survey, samples of European plaice (*Pleuronectes platessa*) eggs and juvenile and adult plaice were collected from various sites around the UK. Eggs were sampled from two spawning grounds in the Irish Sea and adult plaice were sampled from Conwy Bay, north Wales. Juvenile plaice were sampled from nursery grounds in the Irish Sea from Beckfoot to Red Wharf Bay and in the North Sea between Largo Bay and Spurn Point. Sediment samples were collected at the sampling site at Crammond Island in the Firth of Forth and from the NE Irish Sea. Samples were analysed using gas-chromatography/mass-spectrometry (GC/MS) for the presence of polychlorinated biphenyls (PCBs) and were quantified by summing individual congeners identified by analysis of an Aroclor 1254 (PCB) standard. A total of 65 samples were analysed for PCBs comprising five sediment samples, one pooled egg sample, 49 juvenile samples and livers and ovarian tissue from five adult plaice. No PCBs were detected in the initial analyses. Juvenile and adult samples were then pooled within each site, concentrated to 100μl volumes and re-analysed for PCBs. PCBs were detected in sediments and juvenile plaice from the Firth of Forth (114.45 and 11.80μg kg^{-1} dry wt. respectively), Berwick-on-Tweed (10.16μg kg^{-1} dry wt.), Tees Bay (13.80μg kg^{-1} dry wt.), Spurn Point (46.80μg kg^{-1} dry wt.), Morecambe Bay (12.70μg kg^{-1} dry wt.), Gladstone Dock (22.20μg kg^{-1} dry wt.) and New Brighton (14.40μg kg^{-1} dry wt.) and in no other samples. High limits of detection forced many of these concentrations to be classified as "trace" but the concentrations were in the same order of magnitude as those reported for either adult plaice or other species in the vicinities of the sampling sites and other areas of the UK marine environment. The results demonstrated the exposure of juvenile fish to contamination in some nursery areas.

2.2 Introduction

PCBs are ubiquitous, organic pollutants detected frequently in environmental samples (Haglund et al., 1995; Matthiessen et al., 1998; Muir et al., 2003; Borga & Di Guardo, 2005). Although PCBs have a global distribution, locations with the highest concentrations in environmental samples tend to remain close to major points of product manufacture, use and disposal. As a result, regions with the highest concentrations are often associated with industrialised countries and within these regions, concentrations are often highest in estuarine and coastal areas or near waste disposal sites and sewage effluent outlets (de Boer, 1989). Although no longer in use, PCBs are still found in all types of environmental compartments including sediments (Bruckmeier et al., 1997), biota (Covaci et al., 2006), the water column (Bazzanti et al., 1997) and the air (Rice & O'Keefe, 1995).

The lipophilic nature of PCBs means they often become biomagnified as they accumulate in food webs, especially in the top predators. Hoekstra *et al.* (2003) found PCB concentrations to be 1-2 orders of magnitude higher in top predators in an Arctic marine food web than concentrations in fish and plankton. Although many studies such as those mentioned here provide important information regarding bioaccumulation up the food chain, and/or concentrations in adult fish there have been relatively few studies addressing contaminant concentrations in juvenile and larval fish. Studies that have addressed contamination in larval or juvenile fish include Westin *et al.* (1983) (PCBs), Black *et al.* (1988) (pesticides and PCBs), Fisk and Johnston (1998) (numerous organochlorines), Zhou and Weis (1999) (methyl mercury). The lack of studies is surprising given that the nursery grounds of fish are often located in coastal and estuarine areas where higher levels of contaminants are more likely. The dynamics of the early life-history stages of fish are extremely important in determining future population and community structures and there is a need to further our understanding of larval and juvenile ecology, especially in areas where it may be influenced by chemical pollution.

The embryonic, larval and juvenile stages of fish are critical stages in fish life-history strategies as they provide an effective means of dispersal that can extend a

population's range and aid in mixing the gene pool (Cushing, 1975; Fuiman, 2002). Mortality is often highest in the early life stages and has a strong influence on the strength of subsequent year-class structures of populations (Fuiman, 2002). Any further influences on larval/juvenile mortality from acute or chronic pollution exposure could exert a negative influence on the structure of future adult populations should a reduction in recruitment result from exposure.

In the UK, the plaice is a commercially important species. The main UK spawning grounds are located in the Irish Sea and off Flamborough Head in the North Sea (Wimpenny, 1953). Plaice larvae settle in sandy nursery grounds all around the UK coastline (Macer, 1967; Lockwood, 1974) including some areas recognised to have relatively high concentrations of environmental contaminants e.g. Liverpool Bay (PCBs) (Camacho-Ibar, 1991; Leah et al., 1997) and Tees Bay (PCBs and PBDEs) (Matthiessen et al., 1998; Allchin et al., 1999). Routine monitoring programs carried out by the Centre for Environment, Fisheries and Aquaculture Science (CEFAS) and the National Marine Monitoring Program (NMMP) provide some data on PCB concentrations in adult fish (NMP, 1998; CEFAS, 2003) but to the author's knowledge there are no data available regarding organic pollutant concentrations in juvenile plaice. The aim of this survey was to gather samples from nursery grounds to see if PCBs were more prevalent in areas close to sources of contamination. Adult and egg samples were also collected to supplement existing data and determine whether the concentrations detected were high enough to be a cause for concern.

2.3 Methods

2.3.1 Sample Collection

Fourteen sites were sampled for juvenile plaice in the Irish and North seas (Table 2.1). These sites were located in plaice nursery areas. Plaice eggs were sampled in the Irish Sea from the plaice spawning grounds off the Isle of Man (54° 19.5 N, 03° 47.3 W) and off the Great Orme, North Wales (53° 37.0 N, 03° 38.0 W) between March and April 2003. A Gulf VII plankton sampler was towed at 1.5-2 knots in a V-profile between the surface and seabed at a maximum depth of 25-50m. Eggs were identified and sorted onboard and stored at –20°C in hexane-washed glass jars prior to analysis. A total of 475 eggs were collected. In the Irish Sea, juvenile 0+ fish (juveniles that hatched that year) were sampled with a 1m-wide beam trawl towed behind a quad bike through shallow water at low tide between 22-30 June 2003. In the North Sea, juvenile fish were sampled with push-nets at low tide between 22 and 28 June 2003. The sampling dates over-lapped because the earlier Irish Sea sampling was completed by the CEFAS team before the North Sea sampling was complete. Fish were removed from the nets, wrapped in clean aluminium foil, stored in plastic bags before freezing immediately at –20°C in a portable freezer. A surface sediment sample (ca. 100g) was collected from the top 5cm of sediment at Crammond Island, placed in a hexane-washed glass jar and frozen at –20°C. Coarse, sandy samples from the other North Sea sites were not retained as these would not be expected to contain sufficiently high concentrations of PCBs for analysis (Matthiessen et al., 1998).

Sediment samples were collected in the Irish Sea aboard the RV *Prince Madog* between 2 and 5 April 2003 (53° 30.6 N, -03° 43.8 W; 54° 35.0 N, -03° 39.0 W; 53° 42.0 N, -03° 09.0 W; 53° 28.2 N, -03° 19.2 W). Adult plaice were sampled from the Irish Sea off the North Wales coast in Conwy Bay (53° 18.6 N, 03° 56.9 W) on 31 January 2004 aboard the RV *Prince Madog*. A 4m-beam trawl was towed at four knots. Captured fish were measured, weighed and wrapped in clean aluminium foil before being frozen at –20°C prior to analysis. Fig. 2.1 illustrates the locations of all the samples acquired and details of the juvenile and adult samples collected are presented in Table 2.2.

Table 2.1 Information on the sites chosen for sampling juvenile plaice in the North and Irish seas in June 2003.

Site no.	Site	OS Grid Ref.	Description
1 & 2	Largo Bay (1) & Crammond Island (2) (Firth of Forth)	NO 382 004 NT 193 774	Very industrial area. Crammond Island is in close proximity to an oil terminal (<500m). Largo Bay samples taken next to a power station. Sites recommended for sampling by the Scottish Environmental Protection Agency (SEPA).
3	Berwick-on-Tweed	NU 005 520	River mouth included as a reference site. No known inputs of PCBs.
4	Tynemouth	NZ 378 675	One of the most heavily impacted estuaries in the UK (along with the Mersey and Tees) with endocrine disrupting chemicals (Matthiessen et al., 1998).
5	Bran Sands (Tees Bay)	NZ 554 267	At the mouth of the Tees estuary. PCBs previously detected in flounder liver samples (Matthiessen et al., 1998).
6	Spurn Head	TA 398 111	At the very end of the Humber, a known location for juvenile plaice. Within the Humber catchment is a landfill site receiving car waste (Allchin et al., 1999).
7	Beckfoot	NY 095 495	Situated in the Solway Firth fed by the Rivers Nith and Eden. The River Nith receives effluent from a manufacturer of rubber and tyres making use of pentabrominated diphenyl ethers (Allchin et al., 1999). PCB contamination unknown.
8	Seascale	NY 035 010	Just south of Sellafield nuclear reprocessing plant and near mouths of the rivers Irt, Mite and Esk. Organic contamination history unknown but 0+ plaice known to use this area (Fox, pers. comm.).
9	Morecambe Bay	SD 431 645	Vast tidal sand flats in Morecambe Bay, an ideal site for collecting juvenile plaice (Fox, pers. comm.). PCB contamination previously detected in whiting (*Merlangius merlangus*) captured in the bay (CEFAS, 1997b).
10	Mersey (Gladstone Dock - Seaforth Sands)	SJ 310 970	A sandy location good for juvenile fish and just north of Gladstone Dock at the mouth of the Mersey. The Mersey and Liverpool Bay have a long history of PCB contamination (Camacho-Ibar, 1991; Leah et al., 1997; Fox et al., 2001).
11	Mersey (New Brighton)	SJ 312 944	At the mouth of the River Mersey. PCBs known to be present in Liverpool Bay (see above).
12	Rhyl	SJ 001 822	Contamination history unknown but 0+ plaice known to use this area (Fox, pers. comm.).
13	Penmaenmawr	SH 717 768	Contamination history unknown but 0+ plaice known to use this area (Fox, pers. comm.).
14	Red Wharf Bay	SH 547 814	Major nursery ground for 0+ plaice (Macer, 1967). Known PBDE contamination in dab offshore (CEFAS, 2003), but no known local inputs of PCBs.

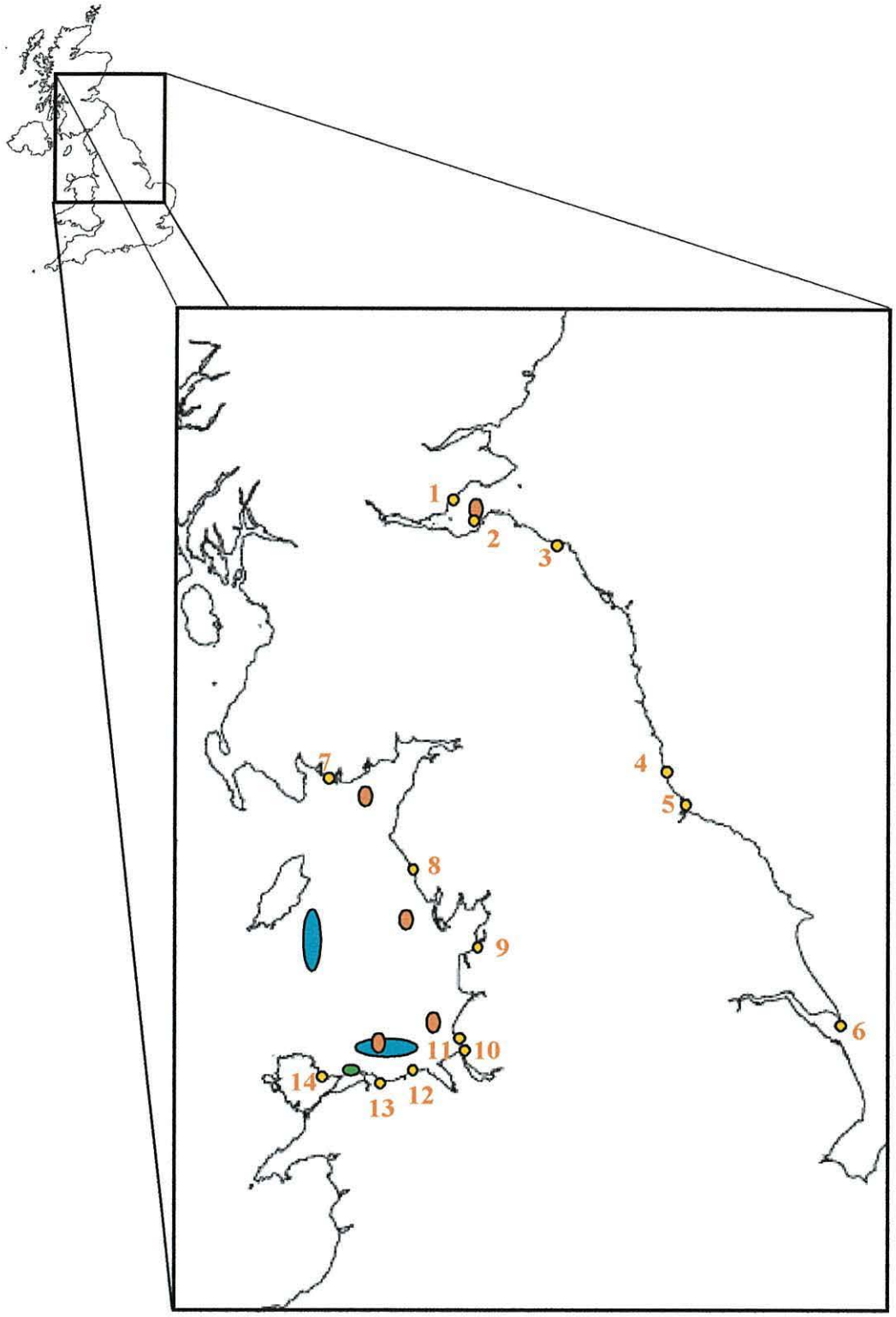


Figure 2.1 Sampling locations for plaice eggs (blue ovals), juvenile plaice (yellow circles), adult plaice (green oval) and sediments (orange oval) in the Irish and North Seas in 2003 and 2004. Ordnance Survey grid references and site details of juvenile sampling locations numbered 1-14 are given in Table 2.1.

Table 2.2 The number of juvenile 0+ plaice and adult plaice sampled in each size class (total length, mm) from each field site for analysis by gas-chromatography/mass-spectrometry of polychlorinated biphenyls. Individual lengths are given where appropriate.

Irish Sea Site	Total length (mm)	No. of fish sampled	North Sea Site	Total length (mm)	No. of fish sampled
Beckfoot	30 – 35	10	Largo Bay	<30	14
	35 – 40	4		30 – 35	9
	40 – 45	8		35 – 40	4
	45+	6		40 – 45	6
Seascale	30 – 35	15	Crammond Island	<30	5
	35 – 40	4		30 – 35	14
	40 – 45	3		35 – 40	12
	45+	7		>40	4
Morecambe Bay	30 – 35	2	Berwick-on-Tweed	<30	4
	35 – 40	5		30 – 35	13
	40 – 45	11		35 – 40	7
	45+	8		>40	2
Mersey (Gladstone Dock)	40 – 45	2	Tyne	<30	5
	45 – 50	8			
	88	1			
Mersey (New Brighton)	45 – 50	5	Tees (Bran Sands)	30 – 35	10
	55 – 60	7		40 – 45	6
	79	1		45 – 50	5
				50 – 55	4
Rhyl	35 – 40	2	Humber (Spurn Point)	<30	16
	45+	8		30 – 35	7
				77	1
				95	1
Penmaenmawr	35 – 40	6			
	40 – 45	4			
	45 – 50	10			
	50 – 55	5			
Red Wharf Bay	30 – 35	7	Adult fish (Conwy Bay, Irish Sea)	248	1
	35 – 40	8		279	1
	40 – 45	3		308	1
	45 – 50	5		313	1
				392	1

2.3.2 Sample extraction and clean-up

The methods used for extraction and analysis of the samples were based on contemporary methods described by de Boer *et al.* (2001). Juvenile plaice were sorted into length (total length) size-classes of <30mm, 30-35mm, 35-40mm, 40-45mm, 45-50mm, 50-55mm and >55mm in order to test for an increase in pollutant body burdens with increasing age. Plaice samples were freeze-dried (Edwards, Super Moduylo) at –40°C for 24-hours and sediment samples were air-dried for 48-hours prior to analysis. All samples were prepared and extracted with glassware that had been rinsed thoroughly in hot tap water before being soaked in hot Decon 90 solution (4-5%) in distilled water overnight before being rinsed again in tap water followed by distilled water and placed in a drying oven at 70-80°C for at least six hours. Before use, all glassware items were rinsed with a 1:1 mixture of hexane and acetone (HPLC grade, Rathburn Chemicals).

Samples were ground with a hexane-washed pestle and mortar and transferred to 18mm x 55mm soxhlet thimbles for extraction in *n*-hexane:acetone 1:1 (v/v). Each sample was spiked with 10μg of octachloronaphthalene (OCN) in 1ml hexane (HPLC grade, Rathburn Chemicals) as an internal standard prior to extraction as a sample matrix spike and allowed to dry. This was designed to test the efficiency of the extraction process. OCN was obtained as a pre-weighed sample of 21.67mg (Supelco) and dissolved in 2ml hexane (HPLC grade, Rathburn Chemicals). Copper turnings (BDH) that had been rinsed with 10% nitric acid (Sigma-Aldrich) followed by distilled water and then dried, were placed in the solvent flask to remove any free sulphur. Sulphur may interfere with the GC/MS determination of PCBs by saturating the detector if it is present at high concentrations (Camacho-Ibar, 1991). Extraction took place over six hours at a rate of 45-50 cycles per hour.

Extracts were reduced by rotary evaporation (Rotavapor RE120, Büchi) and nitrogen gas evaporation to 1ml in volume before alumina (Merck, 70-230 Mesh 90 active neutral, no. 1077) and silica gel (Aldrich Keiselgel, Merck type, 70-230 mesh) clean-up. Alumina and silica gel were prepared by heating them in a muffle furnace (Carbolite CWF1100) at 500°C for 4-hours and 200°C for 24-hours respectively (EA, 2003). Glass chromatography columns (Quickfit, 8mm internal diameter) were

packed with 3.0g of 5% deactivated alumina followed by a 1cm layer of anhydrous sodium sulphate. The sample was placed on the top of the column and eluted with *n*-hexane. Two fractions were collected at 2 and 12ml elution volume. Both fractions were reduced to 1ml by evaporation under a stream of nitrogen gas and the first was eluted over 3% deactivated silica in an equivalent column with *n*-hexane, collecting fractions at 7 and 16ml elution volume. The second silica fraction was combined with the second alumina fraction and reduced to 1ml volume. Both fractions were then stored at –20°C until all samples were ready for analysis.

De Boer *et al.* (2001) state that the first combined fraction should contain the polychlorinated biphenyls and the second fraction should contain tri- to octabrominated diphenyl ethers. Subsequent analyses using biota samples from laboratory experiments found, in our case, that both fractions contained PCBs. Consequently, both final fractions were re-combined to give one sample reduced in volume to 1ml.

2.3.3 Chemical analyses

Quantification of PCBs in the samples was carried out on a gas chromatography-mass spectrometer (GC/MS) in the selected ion-monitoring (SIM) mode. This enables the mass spectrometer to spend more time scanning for specific ion masses every second as opposed to covering the entire range from *m/z* 50 to 550 and improves the signal to noise ratio. This can improve the sensitivity of the analysis by a factor of 100 to 1000 (Hites, 1997). The PCB ions monitored were the tetra-, penta-, hexa- and heptachlorinated biphenyls with atomic parent-ion masses of 292, 326, 360 and 394 respectively. The internal standard OCN with a parent-ion mass of 404 was also detected. A Fisons GC8000 Series GC and Fisons AS800 autosampler were used with a MD800 mass detector. Cool, on-column injection of 1μl volumes was used as this provided the most sensitive method available for detection of potentially low concentrations in the samples (<1μg) though it can be more sensitive to particulate contamination than splitless injection which involves vaporising the sample before transferring a fraction of the vapour to the GC-column (Lang, 1992). Helium was used as the carrier gas with a flow rate of 1ml minute^{-1} through a 30m HT5 (SGE)

column (0.25mm internal diameter) with a methyl-coated pre-column (0.53mm internal diameter) which helps minimise any problems caused by particulate contamination (Lang, 1992). For PCBs, the temperature program was 60°C for 1 min, increasing at 15°C min^{-1} to 170°C, then to 230°C at 4 °C min^{-1} and finally to 350°C at 15°C min^{-1}. The mass spectrometer source temperature was set at 200°C and the interface temperature at 250°C. Chromatograms were examined using MassLab GC/MS Software version 1.4.

To enable accurate PCB congener identification and quantification of samples, a calibration was achieved by injection of an external (i.e. external to the samples being analysed) Aroclor 1254 standard at a concentration of 50μg ml^{-1} together with an OCN standard at 10μg ml^{-1} dissolved in hexane. Aroclor was used as a standard as it contains most of the congeners regularly detected in environmental samples including the ICES 7 congeners and OCN is often used in PCB analyses due to its similar retention time during GC/MS analysis to PCB congeners (Camacho-Ibar, 1991). Also, the use of a compound (OCN) with similar properties to those of the analytes provides a more accurate calibration (Hites, 1997). The use of external standards for quantification (OCN in this case) often allows for detection limits of a few nanograms per sample to be obtained (Hites, 1997). Blank samples of hexane were run to test for contamination of samples by the GC/MS equipment and for use in calculating the detection limits of PCB congeners. Between the processing of each sample, the autosampler needle was rinsed and flushed with the solvent dichloromethane (DCM) 12 times. All results are expressed as μg kg^{-1} dry weight. The commercial mixture of Aroclor 1254 was supplied by CEFAS Burnham Laboratory and originated from Monsanto.

Following the first analyses (originally by size class in the case of the juvenile fish) and a lack of contaminant detection in many of the samples, the samples were combined to form one sample from all the fish at each site. Nitrogen gas evaporation was then used to reduce them in volume to 100μl in glass micro vials (Chromacol) before being re-analysed for PCB content.

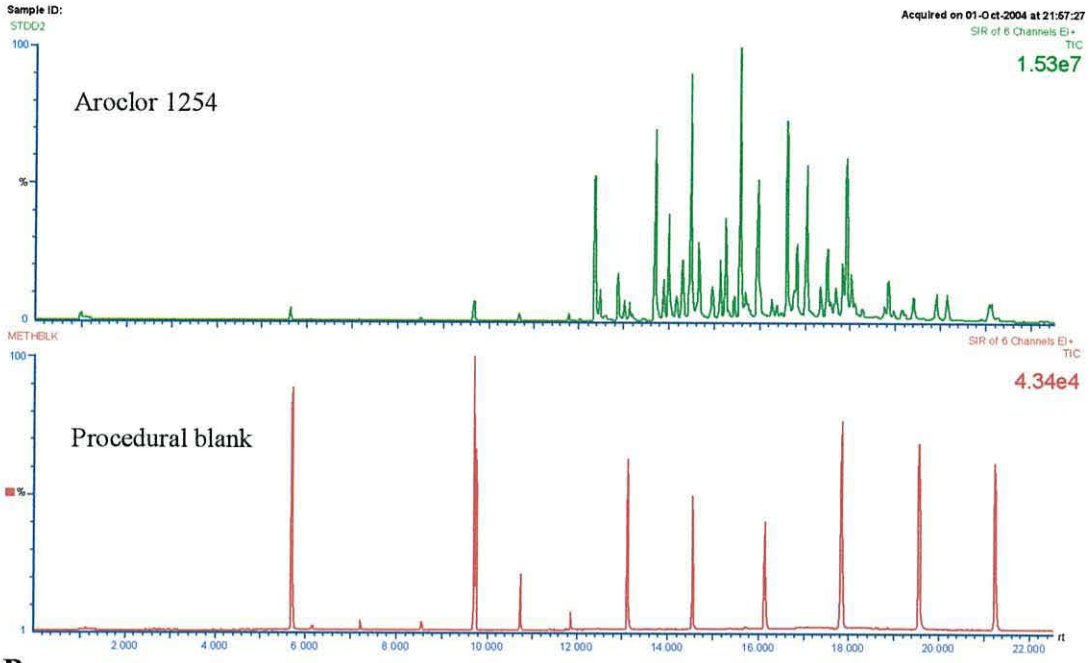
2.3.4 Quality control

A procedural blank was produced by running a 50ml mixture of hexane:acetone (1:1) through an extraction cycle with the glassware used for sample extractions. This was processed in the same way and analysed for contamination caused by possible adhesion of contaminants to the glassware. The procedural blank was run on the GC/MS in parallel with field samples and showed no evidence of PCB contamination from the glassware or solvents being used (Fig. 2.2), indicating that the glass washing and sample preparation procedures prevented cross-contamination between samples. Large, regularly spaced peaks were occasionally but infrequently observed in some samples (blanks and field samples) and were visible in the procedural blank. This was possibly due to infrequent and intermittent loss of material from the solid phase coating the inside of the GC column. These peaks could not be accurately identified due to the use of SIM mode but did not cause problems with the identification of PCB congeners. A 50μg ml^{-1} Aroclor 1254 standard with 10μg ml^{-1} OCN followed by a hexane blank was run at the start middle and end of each batch of 10 samples to ensure quality control. The analysis of one in five samples was also duplicated to ensure validity of concentrations and the internal OCN standard acted as a measure of extraction efficiency.

2.3.5 Congener identification

The Aroclor 1254 standard (Fig. 2.3) was used to aid identification of individual PCB congeners that were detected in the environmental samples and was run alongside the samples. Congeners were identified by comparison of the chromatogram with results of Aroclor 1254 analyses taken from Camacho-Ibar (1991) and the retention times relative to the OCN were compared to those obtained by Mullin et al. (1984) and Camacho-Ibar (1991) (Table 2.3). Six of the ICES 7 congeners were identified in the Aroclor 1254 mixture (CB-52, CB-101, CB-118, CB-138, CB-153, CB-180). The structures of these six congeners are given in Table 2.4. From here on in, the expression "ICES 6" will be used to describe these six congeners. The OCN in the standard mixture, at a concentration of 10μg ml^{-1}, was used to calculate the

A



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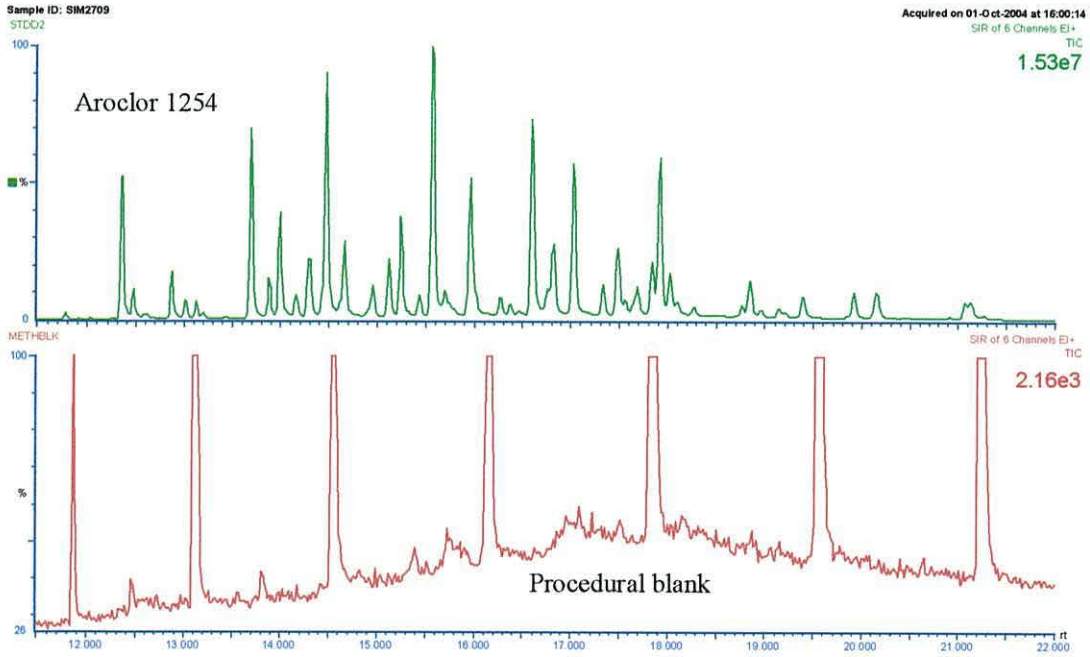


Figure 2.2 Gas chromatograms for Aroclor 1254 (50μg l^{-1}) and the procedural blank (A). A magnified view (B) confirms that the small peaks seen in the procedural blank do not conflict with those in the Aroclor sample. The axes represent time (*x*-axis) and the magnitude of each peak relative to the largest peak detected (*y*-axis). The numbers in the top right corner of each chromatogram represent the magnitude of the largest peak displayed and can be used as an approximate comparison of abundance between chromatograms from the same sample runs. The larger peaks in the procedural blank sample were caused by some contamination in the GC/MS equipment and were rarely seen in subsequent samples or blank runs and caused few problems with chromatogram interpretation.

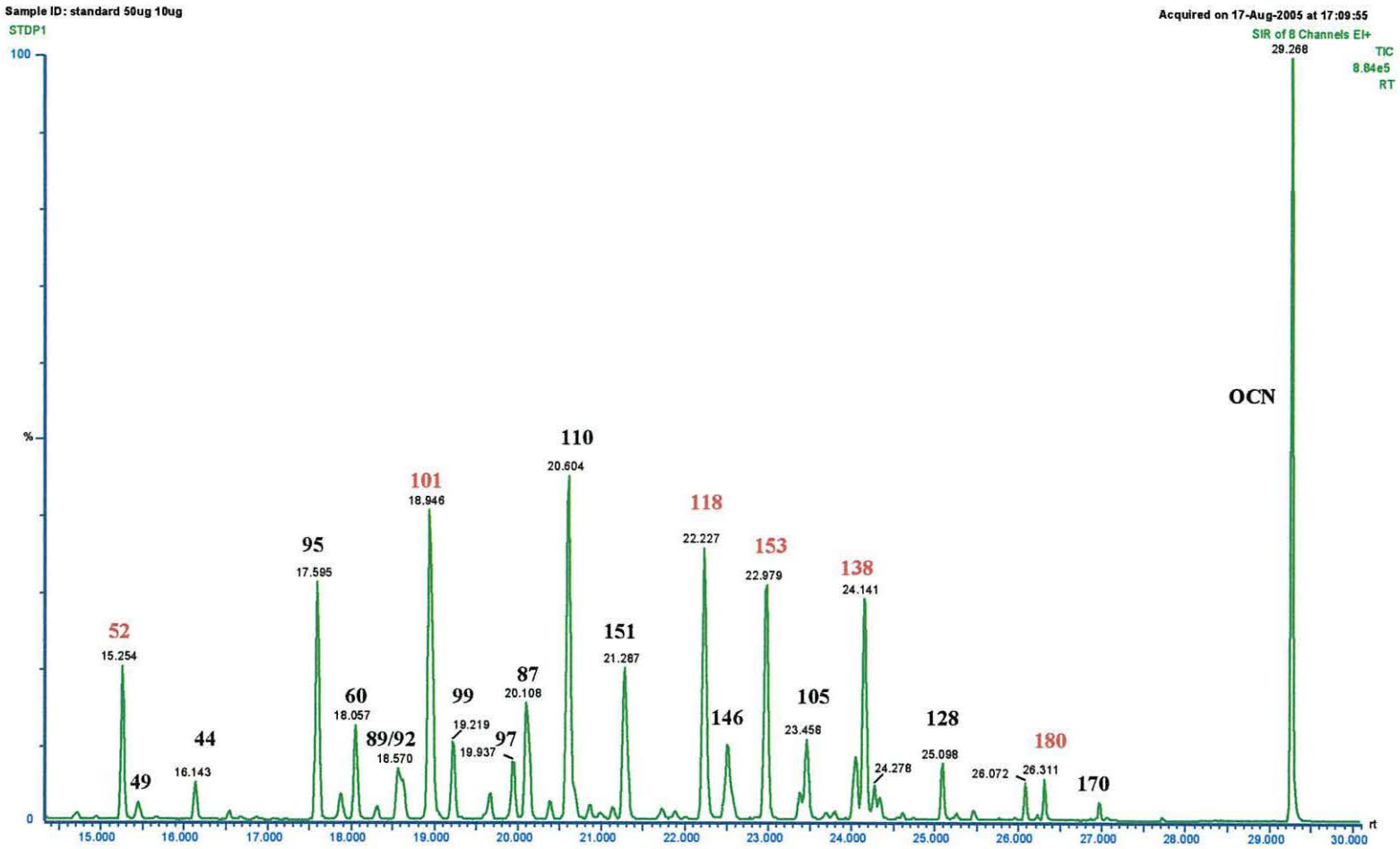


Figure 2.3 GC/MS chromatogram of the commercial PCB mixture Aroclor 1254 with congeners labelled by IUPAC number and identified from Camacho-Ibar, (1991). Six of the ICES 7 congeners that were detected in this study are identified with red text. The internal standard, OCN elutes approximately 2-minutes after CB-170.

Table 2.3 Relative retention times (RRT) to the internal standard (OCN) of the 21 PCB congeners identified in this study (based on three analyses of an Aroclor 1254 standard). Results from Mullin et al. (1984) and Camacho-Ibar (1991) are given to demonstrate the different RRTs obtained using different laboratory equipment and GC programs.

Congener no.	RRT (this study, 25m HT-5 column)	RRT (Mullin et al., 1984)	RRT (Camacho-Ibar, 1991)
52	0.4883-0.4888	0.4557	0.286-0.296
49	0.4933-0.4936	0.4610	0.292-0.303
44	0.5090-0.5094	0.4832	0.318-0.329
95	0.5413-0.5416	0.5464	0.395-0.405
60	0.5531-0.5535	0.5676	0.424-0.435
89	0.5650-0.5657	0.5779	0.433-0.444
92	0.5650-0.5657	0.5742	0.428-0.440
101	0.5722-0.5726	0.5816	0.439-0.449
99	0.5795-0.5798	0.5880	0.449-0.459
97	0.5979-0.5983	0.6100	0.480-0.490
87	0.6025-0.6029	0.6175	0.489-0.498
110	0.6150-0.6158	0.6314	0.509-0.518
151	0.6308-0.6312	0.6499	0.534-0.543
118	0.6558-0.6567	0.6693	0.561-0.570
146	0.6650-0.6654	0.6955	0.597-0.605
153	0.6729-0.6734	0.7036	0.609-0.618
105	0.6913-0.6918	0.7049	0.614-0.622
138	0.7085-0.7089	0.7403	0.664-0.671
128	0.7451-0.7456	0.7761	0.719-0.726
180	0.7967-0.7972	0.8362	0.805-0.808
170	0.8355-0.8360	0.8740	0.854-0.857

Table 2.4 IUPAC congener number and structure of the ICES 7 polychlorinated biphenyl congeners (Bolgar et al., 1995).

Congener number	**Structure**
28*	trichlorobiphenyl [2,4,4']
52	tetrachlorobiphenyl [2,2',5,5']
101	pentachlorobiphenyl [2,2',4,5,5']
118	pentachlorobiphenyl [2,3',4,4',5]
138	hexachlorobiphenyl [2,2',3,4,4',5']
153	hexachlorobiphenyl [2,2',4,4',5,5']
180	heptachlorobiphenyl [2,2',3,4,4',5,5']

* Omitted from the expression 'ICES 6' in this thesis

concentrations of individual congeners detected in environmental samples based on the relative chromatogram peak areas.

2.3.6. Limits of detection

The limit of detection (LD) calculated for each PCB congener was defined as the mean + 3σ of each peak across all three blanks run with each sample batch (Mudge pers. comm.). A practical quantification limit (PQL) was also used to give further certainty in any calculations of congener concentrations. The PQL was defined as 2 x LD (Rong, 2002). The use of these methods of defining the LDs and PQLs are discussed later (see section 3.5.1). Values falling between the LD and PQL were described as "trace" yet still recorded to avoid unnecessary loss of data (Currie, 1999).

2.4 Results

2.4.1 PCB quantification

It was apparent that a small quantity of Aroclor 1254 was carried over between samples as they passed though the GC column during analysis. The hexane blank analysed following each standard often gave readings of PCB and OCN peaks despite the autosampler needle being rinsed and flushed 12 times between each sample. Comparison of the peaks between standards and blanks showed that an estimated 0.1-0.5% of OCN and PCBs were contaminating the blanks (Fig. 2.4) and subsequent field samples which often displayed congener profiles with an exact match to the Aroclor 1254. However, this recurring problem was accounted for when calculating the LD for each congener. Individual PCB congeners were quantified by subtracting the mean peak area for each congener in the blank samples from the measured congener value in each sample. If the result of the blank subtraction was not negative, the remaining area of the congener peak was divided by the mean OCN peak area from the standard runs, which represented $10\mu g\ ml^{-1}$.

The LDs for each of the ICES 6 PCB congeners are given in Table 2.5. Due to the PCB carry-over within the GC/MS equipment, the LDs and PQLs for each congener were relatively high in comparison to limits often stated in similar studies e.g. $<1.0\mu g\ kg^{-1}$ cited by Allchin et al. (1999). This could lead to an increase in Type II errors, i.e. stating that a compound is absent when in fact it is not (Currie, 1999) and this is considered when interpreting the results.

The LDs given for the sediments exceed some of the reported sample concentrations (Table 2.6) for two reasons. Firstly, the LDs for each congener are higher than those seen in similar studies (see above) due to the carry-over of PCBs occurring somewhere within the GC equipment. Had this not occurred, the LDs would likely have been 2-3 orders of magnitude lower, increasing confidence in the detection of very low PCB concentrations. Secondly, the LDs are based on concentrations in the 1ml samples analysed and thus were compared to the concentrations in the field

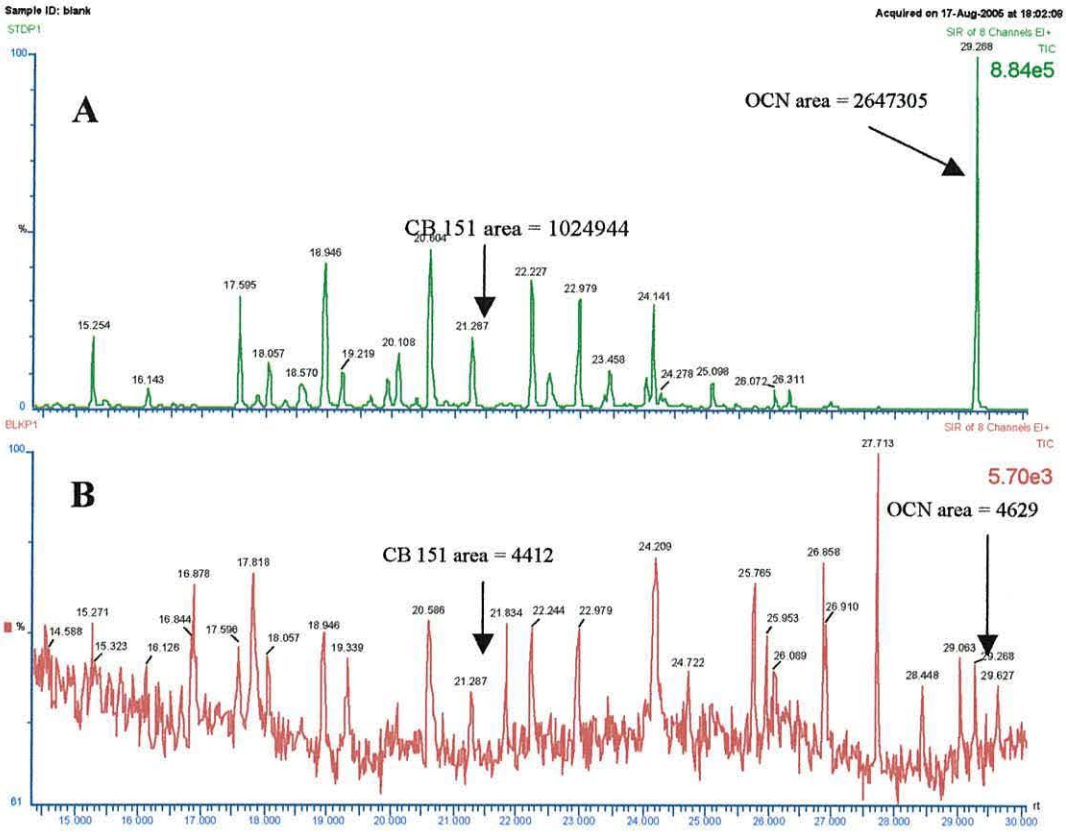


Figure 2.4 Chromatograms demonstrating the carry-over of Aroclor 1254 from the standard (**A**) through to the subsequent blank sample (**B**).

Table 2.5 The limits of detection (LD) of the ICES 6 congeners (ug l^{-1} sample) identified from the Aroclor 1254 standard run alongside field samples of sediments and adult and juvenile European plaice.

Congener	Limit of detection	
	Biota (µg l^{-1} sample)	Sediments (µg l^{-1} sample)
52	35.7	53.5
101	36.2	91.3
118	16.5	95.0
153	60.5	29.6
138	56.2	45.5
180	47.7	3.7

Table 2.6 Results of GC/MS analysis of PCBs ($\mu g\ kg^{-1}$ dry wt.) in plaice egg, juvenile and adult samples from the Irish and North Seas and sediment samples from the Irish Sea and Firth of Forth. Juvenile and adult samples were combined for each site and reduced in volume to 100μl. Entries left blank (-) denote that any PCBs present were below the limit of detection (LD). Trace denotes a value between the LD and practical quantification limit (2xLD). E = eggs, J = juvenile plaice, L = adult livers, Ov = adult ovaries, S = sediment.

Location	Site	Sample	Sample dw (g)	52	101	118	153	138	180	ΣPCB (21 congeners) $\mu g\ kg^{-1}$ dw
North Sea	Largo Bay	J	2.54	-	trace (6.05)	-	-	-	-	trace (11.80)
	Crammond Island	J	2.80	-	-	-	-	-	-	-
	Crammond Island	S	28.82	5.07	12.39	19.89	6.58	-	1.13	114.45
	Berwick-on-Tweed	J	2.64	-	trace (10.16)	-	-	-	-	trace (10.16)
	Tynemouth	J	0.58	-	-	-	-	-	-	-
	Tees Bay	J	3.93	-	trace (3.19)	-	trace (2.62)	trace (1.66)	trace (1.59)	trace (13.8)
	Spurn Point	J	3.59	-	trace (17.89)	-	trace (22.37)	-	-	trace (46.8)
Irish Sea	Beckfoot	J	4.05	-	-	-	-	-	-	-
	Seascale	J	3.35	-	-	-	-	-	-	-
	Morecambe	J	6.56	-	trace (5.36)	-	trace (7.30)	-	-	trace (12.7)
	Gladstone Dock (Mersey)	J	3.90	-	-	trace (2.12)	trace (9.66)	trace (2.43)	trace (4.35)	trace (22.2)
	New Brighton	J	5.01	trace (12.51)	-	-	-	-	-	trace (14.4)
	Rhyl	J	2.88	-	-	-	-	-	-	-
	Penmaenmawr	J	4.72	-	-	-	-	-	-	-
	Red Wharf Bay	J	2.98	-	-	-	-	-	-	-
	Conwy Bay	Adult L	3.18	-	-	-	-	-	-	-
	Conwy Bay	Adult Ov	15.83	-	-	-	-	-	-	-
	NE Irish Sea	E	0.40	-	-	-	-	-	-	-
	NE Irish Sea	S	8.74	-	-	-	-	-	-	-
	NE Irish Sea	S	11.57	-	-	-	-	-	-	-
	NE Irish Sea	S	11.79	-	-	-	-	-	-	-
	NE Irish Sea	S	12.67	-	-	-	-	-	-	-

samples on the same "per ml" or "per sample" basis before adjustments were made for the original amount of material analysed. LDs are expressed here as $\mu g\ l^{-1}$ sample, in order that they are easily comparable to values given in other studies as $\mu g\ kg^{-1}$, whereas in reality the LDs were at the ng ml^{-1} or parts per billion (ppb) level. Samples over 1g would therefore end up with a lower value in terms of concentration $gram^{-1}$. For example, PCBs detected in sediment from the Firth of Forth were over 10x more concentrated than PCBs carried through with the blanks on a per sample basis. However, the concentrations per kg sediment were much lower due to the quantity of sediment used for the extraction (>28g) and the calculation required to account for this. To clarify this, if 40ng was detected in a 0.5g fish sample the final concentration would be $80\mu g\ kg^{-1}$. The same quantity detected in a 10g sample would yield a final concentration of $4\mu g\ kg^{-1}$. Both sample extracts would exceed an LD of 20ng ml^{-1} (or $\mu g\ l^{-1}$ sample) even though the final concentration of the latter is below the LD.

2.4.2 PCB contamination

A total of 70 samples were analysed for PCB contamination. The first analysis of the plaice samples grouped by length provided no evidence of PCB contamination at any of the sites with the exception of sediment collected from near Crammond Island in the Firth of Forth (Table 2.6; Fig. 2.5). Following the combination of the plaice samples within each site and concentration of all samples to $100\mu l$, it was possible to detect small peaks for some congeners, mainly the ICES 6 congeners but concentrations were still very low and not present at all sites. A total of 14 juvenile and adult samples were analysed in this way.

The results of the ICES 6 congeners detected for all sediments and concentrated biota samples as well as ΣPCB(21 congeners) concentrations (given as $\mu g\ kg^{-1}$ dry wt) are given in Table 2.6. Most of these values are given as "trace" as they failed to exceed the practical quantification limit (PQL) that was set to increase the confidence in quantification of results. Entries left blank (-) denote that any PCBs present were not detected and the peak area could not be measured. Given that the concentrations and

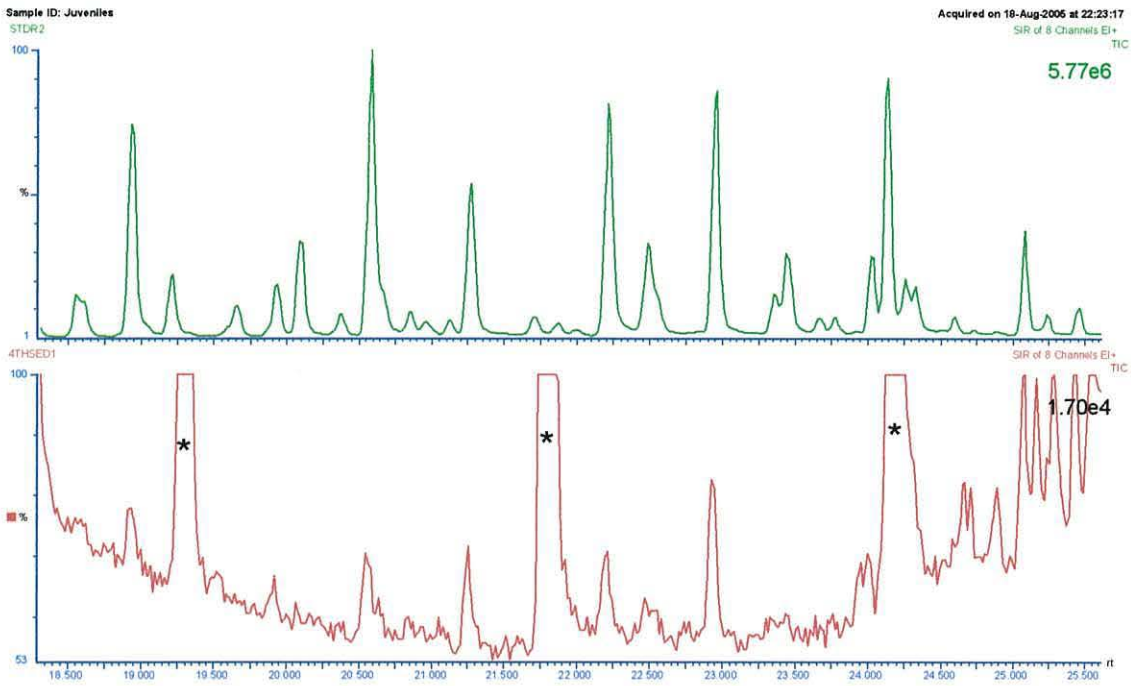


Figure 2.5 Gas chromatograms of the Aroclor 1254 standard (top) and a magnified view of the equivalent section from the sediment sample from the Crammond Island in the Firth of Forth (bottom). Interference from some large peaks prevented identification of some congeners (*) but others can clearly be identified with the same retention times as the Aroclor 1254 congener peaks.

number of congeners detected were so low and the limited data set, it was not feasible to compare congener profiles from the various sites.

2.4.3 Quality control

The Aroclor 1254 standard and blanks run alongside the samples before they were concentrated allowed the accurate determination of 21 PCB congeners based on retention times and the results were used to generate control charts to check method performance. Four CB congeners (52, 110, 153 and 180) were chosen to represent tetra-, penta-, hexa- and heptachlorinated biphenyls. For each standard processed, the ratio of each congener peak area to the OCN peak area was recorded. The mean was then calculated across all the standards and box plots of the data were produced e.g. Fig 2.6. These allowed any changes in equipment sensitivity over the range of PCB ion masses to be identified. Outliers were identified and in these cases the mean OCN values within that sample batch should have been adjusted to correct for inconsistencies in the congener:OCN ratio and bring the values in line with the mean ratio of all standards processed in order to avoid over or under estimation of PCB concentrations. However, the results were very consistent and outliers were only present from two sample batches in which no PCBs were detected, therefore, no corrections were necessary.

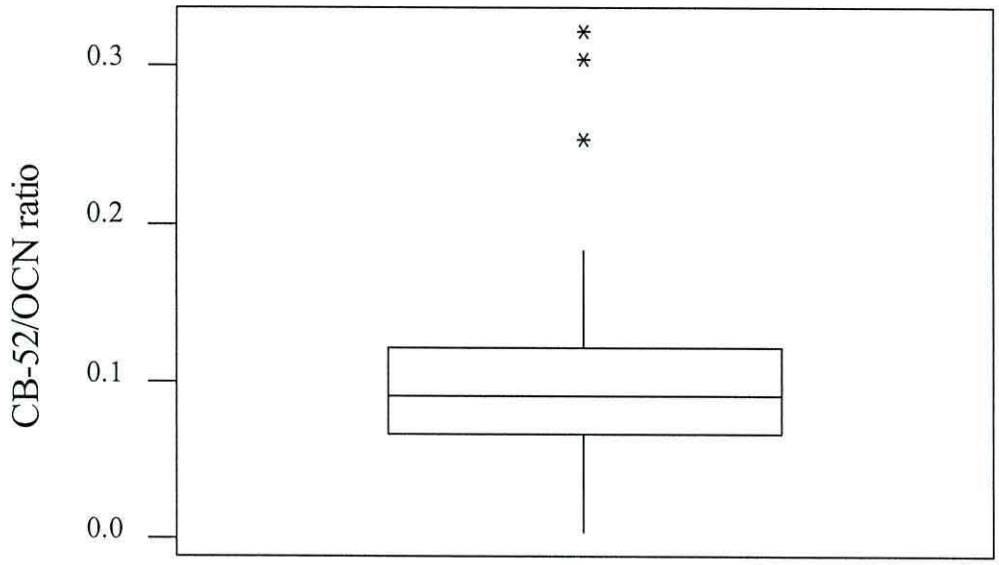


Figure 2.6 Example of the boxplots produced to ensure minimum variation within CB congener:OCN ratios. Outliers (*) were identified and OCN values were adjusted accordingly to bring ratios in line with the main data set.

2.5 Discussion

Of the initial 70 samples analysed in this pilot survey, PCBs were not detected in any samples except in the sediment from Crammond Island in the Firth of Forth (Fig. 2.5). Following the combination of all samples within each site and concentrating them to 100µl, more compounds were determined by the GC/MS methods used. PCBs were detected in juvenile 0+ fish from seven of the fourteen sites sampled (Table 2.6). This does not necessarily indicate that the compounds were not present at the other sites as the detection limits in this study were at least one order of magnitude higher than those often obtained for studies of this type, e.g. 1μg kg^{-1} for PCBs (Hall et al., 1999). This was ultimately due to the carry-over of the Aroclor 1254 standard within the GC/MS equipment, which may have masked the actual presence of contaminants. The result of this issue was that the results presented are likely a conservative estimate rather than an over-estimation of the contaminants present. The calculations of detection limits and analytical methods used in this study are discussed further in section 3.5 along with the results of analyses from laboratory experiments. Even so, given the conservative nature of the results, the sites at which contamination was detected were, in general, the sites where contamination was expected. Typically these sites were all associated with industrial activity (Table 2.1) either upstream or in the near vicinity. Given that the concentrations detected in the biota are described as trace, perhaps more emphasis should be placed on the occurrence of contamination at each site. Nonetheless, it is still reasonable to compare the data between sites and to data in other studies, on a basis of the degree of magnitude of the detected concentrations in order to assess the validity of the results (Allchin et al., 1999).

2.5.1 PCBs in North Sea Samples

PCBs were detected in trace quantities (by these methodological standards) in all North Sea sites except the Tyne. The lack of detection of PCBs in plaice from the mouth of the Tyne is most likely due to the small sample size (n = 5 fish all <1g) due to poor sampling conditions at the time (rough weather). Previous NMMP surveys (1998a) have detected a cocktail of chemicals in sediments and biota in the Tyne

whilst others have found chemicals such as phthalates in water (Matthiessen et al., 1993). Studies of oestrogenic disruption in flounder (*Platichthys flesus*), possibly as a result of this contamination and sewage treatment work discharges, demonstrate that pollution in the Tyne is present in significant quantities (Lye et al., 1999).

PCBs were not detected in fish from Crammond Island and again, this might be due to the small numbers of fish sampled (sampling was difficult at both sites in the Firth of Forth due to the uneven shoreline) resulting in any contaminants extracted being well below the LD. However, the surface sediment sample taken from the same site showed a high concentration of ΣPCBs (21 congeners) indicating heavy contamination at this site. Of the PCBs detected in the sediment from Crammond Island, the ICES 6 congeners comprised 47.6% of the total PCBs detected. CEFAS (1997b) surveys of the UK aquatic environment, use the recommendations by Wells et al. (1989) for classifying sediments based on PCB concentrations therein. They are classified on a dry weight basis as:

$<0.2\ \mu g\ kg^{-1}$	contamination not detected
$0.2 - 20\ \mu g\ kg^{-1}$	slightly contaminated
$21 - 100\ \mu g\ kg^{-1}$	contaminated
$>100\ \mu g\ kg^{-1}$	heavily contaminated

On this basis, the sediment sampled from Crammond Island is heavily contaminated. A further possible reason for lack of detection in the juvenile fish from Crammond Island is that the fish may have had a short residence time at the site at the time of sampling and thus not accumulated enough PCBs to be above the LD set in this analysis. Of the 35 fish sampled from Crammond Island, 31 were <40mm total length compared to only 10 of the 25 sampled from the Tees (Table 2.2) indicating that they may have settled slightly later, although growth rates can be related to food availability (Wimpenny, 1953) which was not surveyed during this study. The much higher concentrations detected in the samples from the Humber (Spurn Point) may be due to the inclusion of two larger 1+ juvenile fish in the combined sample of 77mm and 95mm total length that had been able to accumulate any available contaminants for a longer time period. However, biota samples from the Humber have some of the

highest contaminant concentrations compared to other sites around the UK (Matthiessen et al., 1998; Allchin et al., 1999).

The highest concentrations in the juvenile 0+ fish in the North Sea were detected at Spurn Point at the very end of the Humber estuary and in Tees Bay (Table 2.6). These river estuaries are synonymous with pollution in the UK (Matthiessen et al., 1998) and the detection of PCBs at these sites is not unexpected. The detection of CB-101 in fish from Berwick-on-Tweed was unexpected and warrants further investigation, especially as this is often regarded as a "clean" reference site in other studies of this kind such as Allchin et al. (1999) who investigated concentrations of PBDEs in UK sediments and biota. No other PCB peaks were detected at Berwick, possibly because concentrations were below the LD.

2.5.2 PCBs in Irish Sea Samples

For the Irish Sea, PCBs were detected in trace quantities (again, by these methodological standards) in only the sites associated with the Mersey/Liverpool Bay and Morecambe Bay. Overall, the highest of these trace concentrations were detected near Gladstone Dock at the mouth of the Mersey. PCBs were not detected in any other samples of either juvenile or adult fish, eggs or sediments in the Irish Sea (Table 2.6). With regard to the remaining juvenile samples, this lack of detection might indicate the presence of relatively uncontaminated or "clean" nursery grounds in those areas sampled or that a larger biological sample is required to detect the contaminants at these sites. It is most likely that the latter situation is the case regarding pollutants in the eggs taken from the water column though further sampling would have proved too time consuming and costly. The lack of detection in the adult fish was surprising given that PCBs were detected in smaller juvenile biota samples. Given that the fish were captured in a relatively uncontaminated area they may have simply had contaminant body burdens below the LD. Alternatively, as plaice spend more of their lives offshore as they grow (Wimpenny, 1953) they may be able to depurate PCBs accumulated in the estuarine environments as has been demonstrated with carp (*Cyprinus carpio*) in a clean water depuration study (Hajšloviá et al., 1997). PCBs were not detected in the sediments taken from offshore sites in the Irish Sea. These

were very sandy sediments and would not be expected to contain a detectable quantity of organic contaminants (NMMP, 1998a). Further juvenile sampling and sampling adult fish from other areas associated with pollution such as Liverpool Bay would help elucidate the reasons for the differences observed between juvenile and adult fish. Investigating this was considered too much of a deviation from the main scope of this thesis and was not undertaken especially considering the regular monitoring of PCBs around the UK undertaken by CEFAS.

2.5.3 Comparison to previous studies

Comparison to data acquired from other laboratories should be tentative at least due to varying laboratory methods and detection capabilities but comparisons in terms of order of magnitude are regarded as justifiable (Allchin et al., 1999). CB-153 is a common congener in environmental samples (see section 1.2.1) and is often used as a method of comparison between studies, especially when different or few PCB congeners have been identified (NMMP, 1998a). Concentrations of CB-153 previously reported in flounder livers from the Humber and Tees (8-170μg kg^{-1} and 14μg kg^{-1} wet wt respectively were reported by Matthiessen et al. (1998). Concentrations of CB-153 reported from the Humber and Tees in this study were 22.37 and 2.62μg kg^{-1} dry wt respectively. Using the wet and dry weights of all the sampled fish to convert the dry weights of the field samples to standardised wet weights (Fig. 2.7), it is possible to give approximate concentrations in a wet weight format and make the results between the studies tentatively comparable. Thus, when converted to a wet weight basis, the CB-153 concentrations detected have each dropped by an order of magnitude to approximately 4.87μg kg^{-1} wet wt for the Humber and 0.57μg kg^{-1} wet wt for the Tees samples. Using this method, juvenile fish captured in Morecambe Bay and near Gladstone Dock on the Mersey have CB-153 concentrations of 1.58 and 2.10μg kg^{-1} wet wt respectively. Concentrations of CB-153 in flounder liver from near these sites have been cited as 26 – 57μg kg^{-1} wet wt and 15 – 190μg kg^{-1} wet wt respectively (Matthiessen et al., 1998). Although our study used whole, juvenile fish the concentrations reported are lower than the studies described above. This is to be expected when comparing whole body concentrations

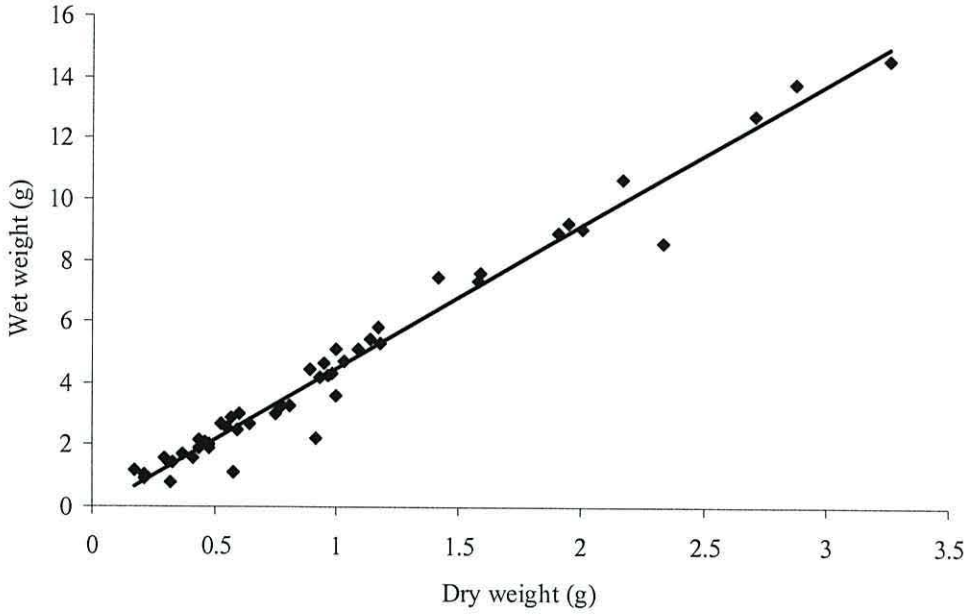


Figure 2.7 Wet weight (g) vs dry weight (g) for samples of multiple juvenile 0+ plaice each weighing approximately 0.8-1.0g weighed, freeze-dried for 48 hours and re-weighed. The equation describing the line is $y = 4.6265x - 0.1225$, $r^2 = 0.9713$, $n = 49$, $p < 0.001$.

to those of livers that have a higher lipid content, putting results from this study in a realistic order of magnitude. Additionally, flounder spend a large proportion of the summer feeding in estuarine and inshore environments (Able et al., 2005) where they will be exposed to a greater quantity of contaminants in comparison to plaice that exist offshore for much of the year (Wimpenny, 1953) where contaminant concentrations are expected to be reduced (de Boer, 1989).

Leah et al. (1997) sampled plaice >25cm from the Mersey estuary and analysed the muscle tissue for ΣPCB(ICES 6) congeners though in that case the six congeners were PCBs 28, 52, 101, 138, 153 and 180. The mean concentrations in the plaice muscle from these sites ranged from 9.6 – 15.1μg kg^{-1} wet wt. If the results from Gladstone Dock on the Mersey were expressed in the same terms, they would equal 3.65μg kg^{-1} wet weight (or 16.44μg kg^{-1} dry wt). Again, results of this study do not appear to be grossly inaccurate in comparison to these other values when considering that the samples were of younger fish and contained all the body organs, of which the liver and brain are likely to have higher contaminant concentrations due to their higher lipid content in comparison to other tissues (Boon & Duinker, 1985). Leah et al. (1997) also reported mean values between 8.2 and 105μg kg^{-1} wet wt for invertebrates living in the Mersey estuary and Liverpool Bay. The fish sampled in this study are likely to have had a shorter residence time in the area resulting in the lower PCB concentrations determined.

Historic surveys of PCBs in the livers of plaice from around the UK yielded values of between 37 and 1200μg kg^{-1} wet weight between 1984 and 1988. The highest concentrations of PCBs were found in Liverpool Bay, and concentrations were more or less stable during the period 1984 – 1988 (CEFAS, 1987; CEFAS, 1990; CEFAS, 1991). The same surveys reported values between 5 and 69 μg kg^{-1} wet weight in plaice muscle tissue from Liverpool Bay and these latter results are comparable with the data collected for juvenile fish in this survey (Table 2.6).

Non-statutory guidelines for ΣPCB (Aroclor 1254) in fish muscle (wet wt; ICES, 1992) set by the OSPAR Commissions Joint Monitoring Programme (JMP) classifies fish in to three categories:

$< 10\mu g\ kg^{-1}$ (low concentration)

$10\text{-}50\mu g\ kg^{-1}$ (medium concentration)

$> 50\mu g\ kg^{-1}$ (upper concentration)

By these guidelines, taking as many of the 21 PCB congeners detectable in this study as possible and based on an Aroclor 1254 standard, the juvenile fish sampled in this study all fell well within the lower bracket of classification with the exception of the fish from the Humber. These concentrations are borderline with ΣPCB(21 congeners) of $10.18\mu g\ kg^{-1}$ wet weight. Depending on the geographical regions to which fish from these areas migrate as they develop, the PCB concentrations and subsequently the classification within these guidelines are likely to change.

The PCB concentrations recorded in the Forth sediment may indicate heavy contamination in the area sampled (see above). In general, the concentrations detected here compare well to those in other studies in terms of order of magnitude. The NMMP surveys of PCBs in UK sediments found concentrations of CB-153 of up to $2.5\mu g\ kg^{-1}$ dry wt in other areas of the Firth of Forth. Although the concentration detected in this study is higher ($6.58\mu g\ kg^{-1}$ dry wt) it is in the same order of magnitude and previously published data has demonstrated that PCB concentrations can show local elevations, especially in areas of high industrial activity (Camacho-Ibar, 1991; CEFAS, 1997b; NMMP, 1998a). Camacho-Ibar (1991) did not take samples in the vicinities of those in this study but reported values ranging between 0.006 and $1.69\mu g\ kg^{-1}$ dry wt for CB-153 in Liverpool Bay and ΣPCB(55 congeners) of $0.08 - 38\mu g\ kg^{-1}$ dry wt. This latter figure is not entirely comparable to the ΣPCB(21 congeners) in this study because of the difference in the number of congeners detected but it does provide an overall idea of the concentrations present. CEFAS (1997b) surveys of sediments found ΣPCB(25 congeners) concentrations of $<0.2 - 11.7\mu g\ kg^{-1}$ dry wt from 15 sites in Cardigan Bay with the exception of $396\mu g\ kg^{-1}$ dry wt detected at Aberystwyth also demonstrating the localised nature of PCB contamination at high concentrations. The PCBs detected in the sediment at Crammond Island as part of this study fall well within the ranges often reported around UK waters (Boon et al., 1985; Camacho-Ibar, 1991; CEFAS, 1997b; NMMP, 1998a; Fox et al., 2001).

2.5.4 Current and predicted trends of environmental PCB concentrations

Fox et al. (2001) investigated the concentrations of the ICES 7 PCBs and other organochlorines in cores of sediment that spanned a period of the past 90 years in the Mersey estuary. PCB concentrations fell progressively over the past 30-50 years from their peak between 1965 and 1975 but still remained in surface sediments despite a cessation in production in 1977 (Fox et al., 2001). Throughout much of the sediment cores, the dominant congeners were penta- and hexachlorinated biphenyls. In recently deposited sediments the proportions of CBs-28, 52 and 180 increased. This signifies that fresh inputs to the environment continue although concentrations in this area have reduced by approximately 5-fold compared to historical high levels. CEFAS monitoring surveys of fish in Liverpool Bay in 1994 concluded that fewer PCB concentrations in cod and whiting muscle remained in the JMP 'upper' categories (see above) though the corresponding whiting liver samples remained in the JMP 'upper' category (>5.0 mg kg^{-1} wet wt) (CEFAS, 1997a). A study conducted by Connor et al. (2001) also demonstrated a decrease in the ICES 7 PCB congeners in mussels sampled from the Mersey and Dee estuaries between 1994 and 1998 (13.9-34.9μg kg^{-1} in 1994 compared to 9.6-31.9 μg kg^{-1} in 1998), thought to be an early indication of improvements in water quality. Studies conducted further away on lake sediments in the Bavarian Forest, southern Germany (Bruckmeier et al., 1997) also exhibit a slight decrease in PCB concentrations since the early-1970s but the more recent samples appeared to show more of a levelling off at around 15x the background levels rather than the sharp decline demonstrated by Fox et al. (2001). These data demonstrate that although concentrations are falling, PCBs continue to persist and may continue to do so for the foreseeable future. Indeed, the detection of PCBs in such young fish in this study, albeit at low concentrations, demonstrates that PCBs are still readily bioaccumulated from the environment and may continue to be a cause for concern (see section 2.5.6). Weber & Goerke (2003) examined the trends of various POPs, including PCBs, in three species of Antarctic fish in 1987 and 1996. The general trend for many compounds, including PCB congeners 138, 153 and 180, was an increase in concentrations in the species studied. Weber & Goerke (2003) indicated that POP concentrations had not yet reached a global equilibrium and the changing levels reflected global redistribution and increasing transfer into Antarctic waters.

2.5.5 Contaminant effects

The results of this study indicate that juvenile plaice are indeed exposed to PCBs in many of their nursery grounds in the UK. It is likely that other contaminants are also present at many of these sites and further investigations are warranted. Studies carried out on adult fish such as dab, plaice and flounder in many of these areas have highlighted some of the possible effects of contaminant exposure. Matthiessen et al. (1998) and Gill et al. (2002) have noted testicular abnormalities in male flounder taken from the Tyne such as thickening of connective tissues, production of female oocytes and increased sperm abnormalities whilst in the same river, Lye et al. (1997) reported the expression of the vitellogenin (VTG) protein in male flounder. VTG production in males is recognised as an indication of endocrine disruption (Sumpter & Jobling, 1995a) and has been reported in flounder from UK estuaries such as the Tyne, Tees and Mersey (Matthiessen et al., 1998). Whilst these end-point effects are relatively easy to detect, determining the cause is not so easy. Fish are exposed to a complex cocktail of xenobiotic compounds in their environment and narrowing the effects observed in the field down to specific compounds can be time-consuming and difficult. PCBs provide a perfect example of this by having 209 possible forms each of which has its own unique set of properties. It is recognised that whilst some PCBs show oestrogenic effects (Arcaro et al., 1999), others are anti-oestrogenic (Arukwe et al., 2001) making the effects of PCB exposure alone difficult to predict .

Studies of contaminant effects on adult fish such as those outlined above are fairly common but relatively few address the potential impacts on larval and juvenile fish. Given the importance of these early life stages (as outlined in section 2.2), it is important to determine if they are also likely to be adversely affected in contaminated environments. As larval and/or juvenile fish grow and develop they are likely to be more susceptible to permanent developmental effects than if they were exposed later in life (Colborn et al., 1993) due to the complex and often rapid changes in development and growth at an early age. High contaminant concentrations in adult fish reported in the literature (see above) indicate that future generations of fish are not just likely to be exposed following settlement in the nursery grounds as reported here but beforehand, even at the embryonic stage due to maternal transfer of

pollutants to the oocytes during oogenesis (see section 1.5.2). The overall aim of the remainder of this thesis is to conduct further research into the effects of exposure to a commercial mixture of PCBs, via maternal transfer and dietary exposure, on larvae of the European plaice with respect to egg and larval survival, behaviour, growth and physiology. Studies of this kind are important as they build an overall picture of the different impacts related to exposure to chemicals. Studies conducted on adult fish might show contaminants to have only short-term or no effects whereas the developing offspring might be at most risk. Fonds et al. (1995) exposed dab (*Limanda limanda*) to the commercial PCB mixture Clophen A40 and found no significant effects with respect to egg production, fertilisation and hatching rates or egg and larval survival but did not test for more subtle effects on parameters such as behavioural or physiological changes. Disruption of these latter factors may have serious implications for larval survival in the field if larvae are unable to feed and/or evade predators efficiently, and warrants further investigation. Research on other flatfish species demonstrated an inability of juvenile fish (50 to 80mm length) to actively avoid sediments containing oil at environmentally relevant concentrations (Moles et al., 1994). This indicates that juvenile fish in contaminated nursery grounds in the UK might not avoid contaminated areas and experience exposure to contaminants via the food chain and/or burying in the sediments. A more detailed investigation of the spatial nature of contamination in nursery grounds and the migration of fish into and out of such areas would help establish the extent of exposure of juvenile populations.

2.5.7 Methods used

A full discussion of the methods used for quantification of the samples is given in section 3.5 but what follows here is a brief discussion of the methods used to identify the individual congeners within the samples and limitations of the results obtained in this survey.

Many different methods for the extraction, clean up and analysis of PCBs in environmental samples have been described in the literature (de Boer, 1989; Ungerer & Thomas, 1996; CEFAS, 2000; EA, 2000; Akutsu et al., 2001; de Boer et al., 2001).

The methods used in this study were reported by de Boer et al. (2001) for the extraction and analysis of both PCBs and PBDEs described as part of an inter-laboratory comparison between three laboratories in the UK and the Netherlands (CEFAS, Netherlands Institute for Sea Research (NIOZ) and Netherlands Institute for Fisheries Research (RIVO)). Each institution employed different methods yet each was shown to provide data comparable to the others. The particular methods chosen for this study were based on those used in the CEFAS Burnham laboratory that were described as being comparable to current state-of-the-art methods, that should provide a framework for good results (de Boer et al., 2001). The CEFAS extraction methods using *n*-hexane:acetone in a 1:1 (v/v) ratio were described as giving the best results with the lowest coefficients of variation (%) between the three laboratories with respect to congener concentrations. In addition, the CEFAS methods were best suited for the laboratory at the School of Ocean Sciences in line with other extraction methods currently employed. Although the samples in this study were only extracted for six hours (half the time reported by de Boer et al. (2001)), the soxhlet apparatus used was smaller in size and produced 45-50 cycles per hour compared to 9-10 cycles per hour reported by de Boer et al. (2001), providing approximately three times as many extraction cycles.

Individual congeners were identified by comparison of the elution order and the order of relative retention times (RRT) to OCN of the congeners in the Aroclor 1254 commercial mixture to those reported by Mullin et al. (1984) and Camacho-Ibar (1991). This was a simple procedure allowing accurate identification of 21 major congeners, which could be used to match up to those detected in the environmental samples based on retention times (RT) and accurately determine the PCB content of those samples. Occasionally problems can occur in terms of identifying individual congeners due to co-elution resulting in one chromatogram peak representing two or more congeners. Camacho-Ibar (1991) noted that problems of co-elution could occur with congeners 28, 52, 101, 118, 138 and 153 amongst others. This was not such a problem in this study as different equipment, capillary columns and GC program cycles were used which allowed good separation of the congeners (Fig. 2.3). Co-elution of congeners 89 and 92 did occur and the concentration for that peak is given as the sum of the two congeners in each case. Only the major peaks in the Aroclor standard were identified as they were the congeners of most interest (e.g. ICES 7) or

present in quantities exceeding 0.5% of the congener profile based on previous reports of the composition of Aroclor 1254 (Schulz et al., 1989; Camacho-Ibar, 1991; Frame et al., 1996). It was necessary to identify the major congeners and to express the concentrations in terms of individual congeners as this allows better comparison to other studies (see section 2.5.3). Different congeners also have different biological effects (Bonefeld-Jørgensen et al., 2001; Jung et al., 2005; Vaccaro et al., 2005) and so reporting data based on individual congeners can help in determining the severity of a PCB contamination problem based on congener effects observed in laboratory studies. Expressing results in terms of a chemical formulation is unrealistic for environmental samples as congener patterns often differ between environmental compartments (e.g. air, water, sediments and biota) and species (Camacho-Ibar, 1991).

Table 2.7 compares the percentages of each congener analysed in this study (out of the 21 congeners analysed) to the percentages detected in other studies following adjustment to include only the same 21 congeners analysed in this study. In general there is a good match in the proportions with the greatest deviation occurring in congeners CB-52, 49, 44, 60 and 95. Although there is some variation in the percentages, consideration must be given to the likelihood of the Aroclor mixtures analysed in each study coming from different production batches (Camacho-Ibar, 1991) and the different analytical techniques and equipment used by each laboratory, for example different GC capillary columns, mass spectrometers and/or GC/MS program settings for the Aroclor 1254 analyses. Although co-elution was not identified as a problem in this study, the partial co-elution of congeners that may be present in very small proportions and which may affect the accuracy of these results to a minor degree, cannot be ruled out completely (Camacho-Ibar, 1991); this study examined 21 congeners, yet a total of 77 congeners were detected in Aroclor 1254 by Schulz et al. (1989) at concentrations exceeding 0.05% (w/w). A total of only 57 separate peaks were apparent in our analyses of Aroclor 1254 (Fig. 2.3), yet given that all the major congeners were identified and the remaining visible peaks form such a major proportion of Aroclor 1254 together with the good matches in proportions detected (Table 2.7), the contribution of any smaller co-eluting peaks is expected to be minimal. Taking the ICES 6 as an example, of those six congeners, Schulz et al.

Table 2.7 Comparison of the PCB congener composition of 21 congeners in Aroclor 1254 as analysed in four different studies. Lot A4 was made from Aroclor 1016 residue and contains elevated percentages of some congeners (non- and mono-ortho congeners) compared to the "normal" Lot G4 (Frame et al., 1996). It is included to further demonstrate the variation in Aroclor mixture compositions. The 'ICES 6' congeners are highlighted in grey.

Congener no. (elution order)	Mean ± SD (this study)	Lot A4 (Frame et al., 1996)	Lot G4 (Frame et al., 1996)	Aroclor 1254 (Schulz et al., 1989)	Aroclor 1254 (Camacho-Ibar, 1991)
52	4.62 ± 1.21	1.31	7.98	8.20	8.59
49	0.65 ± 0.28	0.41	1.63	2.60	2.02
44	1.33 ± 0.31	1.06	3.43	3.21	3.66
60	3.51 ± 0.53	1.50	0.27	0.86	0.70
95	7.32 ± 0.90	2.91	9.27	9.53	9.92
89/92	2.99 ± 0.35	0.107	2.04	5.50	8.07
101	10.80 ± 0.99	8.69	11.90	12.57	11.30
99	2.93 ± 0.27	7.17	4.48	5.70	4.55
97	2.33 ± 0.25	4.40	3.89	4.04	3.82
87	4.35 ± 0.29	5.39	5.92	5.99	6.40
110	14.30 ± 0.98	13.32	13.78	9.26	7.69
118	12.24 ± 0.64	21.50	10.90	10.12	8.70
105	4.10 ± 0.41	11.66	4.43	6.06	3.77
151	5.53 ± 0.34	0.35	1.02	1.85	1.22
146	2.59 ± 0.33	0.71	0.99	1.31	1.34
153	7.97 ± 1.49	5.20	5.59	6.75	5.84
138	8.46 ± 1.21	9.41	8.60	5.07	7.75
128	1.74 ± 0.40	2.71	2.11	3.28	2.16
180	1.46 ± 0.58	0.66	0.99	0.60	1.33
170	0.81 ± 0.40	0.55	0.77	0.49	1.14

(1989) stated that in their analyses CB-101 co-eluted with CB-90, CB-153 eluted closely with CBs 132 and 105, CB-118 eluted closely with CB-149 and CB-138 eluted closely with CB-158. By comparing Aroclor 1254 chromatograms such as Fig. 2.3 with those obtained by Camacho-Ibar (1991), it was possible to see that better separation of these particular congeners occurred in this study thereby reducing the problems of major congeners co-eluting with one another. Thus, to take the same approach as Camacho-Ibar (1991), the identification and quantification of the congeners reported from here-on-in can be regarded as accurate.

No contaminants were detected in the field samples until they were combined, concentrated and analysed without running the Aroclor 1254 standard until all the samples were completed to avoid the problems of carry-over. Consequently the quality of the data from the concentrated samples could not be assessed directly due to the lack of concurrent Aroclor 1254 analysis and the samples having 3-4 times the original concentrations of the sample-matrix spiked OCN due to the re-combination of samples. However, given the confidence in the accurate identification of separate PCB congeners and lower LDs (because concurrent Aroclor 1254 standards were not run), the data for PCB congeners detected in the samples can still be regarded as valid. Samples from laboratory experiments were analysed alongside the Aroclor 1254 standard and the quality of those data are discussed in Chapter 3.

The quantity of fish sampled for this survey was limited by restrictions from the Department of Environment Food and Rural Affairs (DEFRA) from whom permission was sought to sample undersized fish from each of the sites. The soxhlet equipment used for these extractions was small in size and allowed for a high frequency of cycles per hour and in theory, a superior extraction efficiency. Collection of larger quantities of biota for extraction would enable better extractions of trace contaminants, especially from sites where none were detected. This would increase confidence in assessments of whether sites were contaminated or not.

2.5.8 Conclusions

The results from this study provide a good indication of the locations around the UK where juvenile plaice are likely to be exposed to PCB contamination. The quantification of the results, although appearing to be in the correct order of magnitude, should be treated tentatively due to the high LDs obtained and the possibility of residual carry-over from the GC/MS equipment used. Further, more extensive sampling would allow more precise determination of the concentrations present. Nevertheless, the results of this and other studies demonstrating the continued presence of these contaminants in the environment suggest that investigation into the effects of PCB contamination on the early life stages of plaice is warranted.

Chapter 3

Bioaccumulation of Aroclor 1254 and maternal transfer of polychlorinated biphenyls in the European plaice, *Pleuronectes platessa*

3.1 Abstract

PCBs are known to accumulate in the marine environment. They are stable, persistent, lipophilic compounds that concentrate in biological tissue, increase in concentration with trophic level and are expected to remain in the environment over geological time. Organochlorines accumulating in female fish can be depurated during vitellogenesis whereby they are transferred to the developing oocytes and expelled from the body during spawning.

Two plaice broodstock experiments were completed during the spawning seasons of 2004 and 2005. These were designed to assess the potential for maternal transfer of PCBs to the ovaries following oral dosing with the commercial PCB mixture Aroclor 1254 at low (0.1-0.175mg kg^{-1} fish $month^{-1}$), medium (1.0-1.75mg kg^{-1} fish $month^{-1}$) and high (2.0-3.5mg kg^{-1} fish $month^{-1}$) doses (n = 15 to 17 females per treatment) and changes in fecundity. Eggs were hand-stripped when the females were judged ripe and at the end of the spawning seasons the broodstock were sacrificed and livers were removed. Egg and liver samples from spawning females (n = 5 to 8 per treatment) were analysed using gas-chromatography/mass-spectrometry (GC/MS) for the presence of PCB congeners. Egg PCB concentrations ranged between 'not detected' and 12.3mg kg^{-1} dry wt. and liver concentrations ranged between 'not detected' and 22.9mg kg^{-1} dry wt. Multivariate analyses of tissue congener patterns revealed significant differences in PCB congener patterns between tissues ($p = 0.001$). Proportions of lower chlorinated congeners increased in the eggs compared to the livers and Aroclor 1254, whilst the proportion of higher chlorinated congeners increased in the livers compared to the eggs and Aroclor 1254. A significant negative correlation existed between individual egg dry weight and egg PCB concentration ($p = 0.003$). Although egg weight and egg production g^{-1} female did not differ in treated fish compared to controls, significant differences did exist between low and high dosed treatments with respect to individual egg weight ($p = 0.0047$). Results suggest that females with low body PCB concentrations produce fewer, heavier eggs and that impacts on reproductive capacity are minimal. However, investigations with the resulting larvae are required to fully evaluate reproductive effects.

3.2 Introduction

The evidence presented in the previous chapter both from the field samples analysed and from the literature, confirms that PCBs are still present in fish around the UK coast. Questions remain as to whether PCBs are the cause of most concern regarding effects of endocrine disruption that have been observed in field-caught fish such as intersex in male roach (*Rutilus rutilus*) (Jobling et al., 2002) and testicular abnormalities in the Tyne flounder (*Platichthys flesus*) population (Lye et al., 1997; Gill et al., 2002). Fish are exposed simultaneously to a range of xenobiotics in the aquatic environment and establishing the effects of particular compounds when synergistic and additive effects occur is challenging. Von Westernhagen et al. (1989) suggested that although concentrations of PCBs can often be an order of magnitude higher than certain pesticides in aquatic organisms, the latter may have a greater impact. In their study of chlorinated hydrocarbons in North Sea whiting (*Merlangius merlangus*), they found DDT to have a greater effect on total hatch rates compared to PCBs even though PCBs were present at higher concentrations. DDE has been reported to have negative effects on the viable hatch of Baltic herring (*Clupea harengus*) at concentrations an order of magnitude lower than PCBs (Hansen et al., 1985). Even so, the high concentrations of PCBs detected in some areas means that they should not be disregarded and warrant further investigation.

It can be difficult when investigating the effects of environmentally realistic concentrations of contaminants to carry out complete investigations into every aspect of an organism's response to exposure, be it chemical, reproductive, developmental, behavioural or physiological in nature. However, if detailed information can be accumulated for as many of these aspects as possible in a particular investigation it can serve to increase our understanding of the full suite of implications for an organism. To give an example, a study by Fonds et al. (1995) investigated the effects of the technical PCB mixture Clophen A40 on the dab (*Limanda limanda*). Detailed information was presented regarding chemical concentrations in parent fish and their eggs together with observed effects on reproduction, fertilisation success and survival of the resulting larvae. The PCB concentrations used in that study were reported to be an order of magnitude greater than those observed in the field. It was predicted that

PCB contamination was unlikely to cause a problem for dab in the North Sea given the concentrations used, lack of observed effects and continued good recruitment. Such conclusions may only be reached with good chemical dosing regimes and sound analytical procedures. Analysis of PCBs on a congener-by-congener basis and across different body tissue types provides a detailed analysis of results and promotes an understanding of the kinetics of contaminants within an organism.

Although Fonds et al. (1995) concluded that PCBs were not a threat to wild dab populations, and von Westernhagen et al. (1989) and Hansen et al. (1985) suggested lower concentrations of other contaminants may cause equally or more severe effects regarding hatch of whiting and herring, other studies have demonstrated that PCB effects can be species-specific. Hogan & Brauhn (1975) reported increased deformities in rainbow trout (*Oncorhynchus mykiss*) fry following hatch from eggs with high residues of Aroclor 1242 and more recently Aroclor 1254 was shown to have a negative influence on the growth and predator evasion skills of Atlantic croaker (*Micropogonias undulatus*) larvae following parental exposure (McCarthy et al., 2003). Given these species-specific effects outlined above, investigations accounting for many species need to be considered when assessing the degree of risk posed by contaminants.

The aims of the procedures reported in this chapter were firstly to address practical issues surrounding dosing female plaice broodstock with Aroclor 1254 in order to achieve PCB body burdens at environmentally realistic concentrations. Secondly, chemical analyses of various tissues would test the hypothesis that a proportion of the PCBs could be depurated during ovulation by transfer into the oocytes. This would then allow more confident conclusions to be drawn regarding whether or not PCB contamination might cause deleterious effects on female reproductive capability in terms of egg production and egg size, or further effects in the offspring of the dosed females (see chapter 4). Investigative multivariate analyses were employed to examine changes in congener profiles between body tissues following dosing and depuration of contaminants into the eggs, to test the hypothesis that smaller, more mobile congeners might be transferred more easily than larger more chlorinated biphenyls.

3.3 Methods

3.3.1 Preliminary dosing experiment

A preliminary experiment was conducted for a number of reasons. Firstly, it was important to know how efficiently the fish would absorb Aroclor 1254 when administered orally and secondly to determine whether these PCBs would enter the aquarium recirculating water system and contaminate the water and filtration systems.

Juvenile 1++ plaice (age estimated by size, n = 16) were collected from the Menai Strait at Port Dinorwic using a 1m beam trawl towed by boat at low speed (1 – 1.5 knots) to avoid damaging the fish. Three fish were killed immediately following capture (weight range 53.4-69.9g), wrapped in clean aluminium foil and frozen at –20°C. Following a 14-day quarantine period, nine healthy fish (weight range 54.6-83.8g) were selected and assigned at random to one of three shallow tanks (100 x 400 x 600mm) within a re-circulating system linked to an aquarium filter and temperature control unit (Teco, RA2000). Water temperature was maintained at 12°C ±1°C and fish were exposed to a 12:12 light:dark photoperiod. The fish were allowed to acclimatise to the tank conditions for a further seven days.

At the end of the acclimation period, fish from tanks A and B were removed one at a time and anaesthetised lightly in an aerated solution of 0.5ml l^{-1} 2-phenoxyethanol using water from the experimental tanks. At all times the fish were handled with rubber gloves and placed on wet towels to reduce skin damage. Fish were exposed to PCBs using the same technique as that described by Fonds et al. (1995). When the fish were anaesthetised, a gelatine capsule containing 1mg of Aroclor 1254 dissolved in 0.15ml vegetable oil was administered orally. Following dosing, fish were transferred to a 60l recovery tank and observed for normal behaviour and regurgitation of the capsule. Recovery was often within five minutes and capsule regurgitation was never observed. Fish were returned to the experimental tanks 15-20 minutes after dosing.

After 14-days the fish in tanks A and B were dosed again and following a further 14-days fish in all three tanks were killed by a Schedule I method. Liver, muscle and gonadal tissue samples were removed from each animal and frozen at –20°C until analysis for PCB content. The Schedule I method of killing fish is the Home Office approved method of dispatching a live fish. It involves a single, sharp blow to the head to stun the fish followed by immediate destruction of the cerebral material by means of a sturdy knife (and seeker as required) and severing of the spinal cord. It is regarded as one of the quickest and most humane methods of killing a large fish such as a plaice. All work was completed under licence from the Home Office (PPL 40/2425).

Throughout the experimental period, nine harbour ragworm (*Nereis diversicolor*) obtained from Red Wharf Bay, Anglesey were added to each tank every other day as food and were always eaten. Every day, faeces were siphoned from each tank and stored separately in hexane-washed glass vials at –20°C for subsequent analysis. At the end of the experiment the filter wool was removed and rinsed with distilled water over filter paper to collect as much organic material as possible for analysis of PCB content.

At the end of the experiment a 2.0 l water sample from the aquarium system underwent liquid-liquid extraction using 1:1 hexane:acetone as a solvent and vigorous shaking in a separating funnel for 10-minutes (S.M.Mudge, pers. comm.). The solvent was then reduced in volume using rotary evaporation and analysed in the same way as the other samples.

The faeces, filter and tissue samples were prepared for analysis by freeze-drying for 24-hours at -40°C before being analysed by GC/MS. The extraction methods used were the same as for the field samples described in section 2.3.2 – 2.3.6, except that the column used was a 25m HT5 (SGE) column with a 0.22mm internal diameter.

3.3.2 Broodstock experiments

Two broodstock experiments were carried out as part of this study in both 2004 and 2005 during the natural spawning season for plaice (Jan – Apr). The methods described below were the same for each experiment unless otherwise stated.

3.3.3 Fish collection and husbandry

The detailed descriptions of the husbandry procedures used comply as far as was possible with guidelines for reporting the results of experiments on fish suggested by Brattelid & Smith (2000). Adult plaice were collected in Conwy Bay, North Wales on board the RV *Prince Madog* in October 2003 and 2004 using a 4m otter trawl net towed at a speed of 3-4 knots. Immediately the catch was emptied on deck, male and female fish were selected that were highly active and showed little or no obvious external damage. These fish were transferred immediately to a $2m^3$ holding-tank at ambient sea temperature. On arrival at the School of Ocean Sciences, Menai Bridge the fish were treated with formalin at 400ppm for 1-hour once only in a 250l holding tank (Grove, D., University of Wales, Bangor, pers. comm.). They were then placed at random into one of three $3.1m^3$ (0.7m x 1.5m x 3.0m) light-grey, rectangular raceways with individual, partially recirculating water systems supplied with seawater at ambient seawater temperature. A large air stone was place in each tank. The fish were then treated with chloramine-T at $5mg\ l^{-1}$ for half an hour for five consecutive days in the raceways (Baynes, pers. comm.). Following each chloramine-T treatment the water flow through the raceways was increased to flush it from the system. These treatments killed any external bacteria or fungi and aided the healing of minor abrasions sustained during capture with the otter trawl.

Seawater was sourced from the Menai Strait and was passed through settlement tanks and sand filters before entering the aquarium system. The replacement rate of the water in the raceways was approximately $180\ l\ hr^{-1}$ and as such there was no build up of ammonia, nitrates or nitrites in the systems and pH and salinity remained constant at 8.0 and 32 psu respectively. Ammonia, nitrate, nitrite and pH were tested every 14-days with testing kits (Palintest Ltd.). Water that did recirculate was filtered through

filtration wool and filter media before re-entering the tanks. Air conditioning cooled the aquarium room to approximately 12°C to minimise the influence of air temperature on the water temperature. Water temperature was maintained as close to ambient as possible to aid gonad maturation by maintaining a high inflow of fresh seawater (see above). Plaice will spawn between 2 and 8°C in the wild (Rijnsdorp & Witthames, 2005). The lowest temperature obtained in 2004 was 8°C and in 2005 was 5.5°C. Photoperiod was maintained at the same light:dark hours to mimic those in the field using 20-watt bulbs on a timer situated 2m above each tank. Fish were fed on a mixture of commercial pellet feed (Dana Feed 1562, supplied by Llyn Aquaculture) and chopped sand eels every other day. Faeces and uneaten food were siphoned from the tanks on the days following feeding.

The fish were given a further seven days to acclimatise to the tanks before being anaesthetised with 2-phenoxyethanol at a concentration of 0.5ml l^{-1}. External parasites were removed and the fish were weighed and tagged with alcian-blue dye (2% w:v) using a hypodermic needle to scratch the epidermal surface. Over the next month, fish were monitored for feeding and general health. Any fish that became infected by pathogens were disposed of using a Schedule I method to avoid infecting others. Less than 10% of the fish in both years were disposed of in this way.

In late November 2003 and 2004, within size classes of <35cm, 35-40cm and >45cm total length, healthy feeding fish were divided randomly amongst the three raceways so that each contained a comparable size range of females. Females were identified by holding individual fish up to the light whilst looking for a dark, finger-like shadow, which denoted the presence of ovarian tissue on the right hand side when the fish were viewed from above. In 2003, the raceways were classified as untreated control fish, medium-dosed fish and high-dosed fish (n = 17 in all tanks). In 2004, only two raceways were used for female broodstock and classified as untreated control fish and low-dosed fish. All male fish were housed in a tank separate from the dosed fish to avoid contaminating them with PCBs through female faeces.

The broodstock tank system was changed before the 2005 experiment. The tanks used were blue, circular, fibreglass tanks, 1.5m in diameter by 1.5m deep, giving a total water volume of approximately 2.7m^3.

3.3.4 PCB exposure

On 19 December 2003, all the fish were again anaesthetised and weighed before being dosed with Aroclor 1254 dissolved in 0.15ml vegetable oil in a gelatine capsule (as described in section 3.3.1). Fish in each size class of <35cm, 35-40cm and >40cm (total length) were weighed and the average weight of each class was calculated. Fish in the 35-40cm size class were approximately 25% heavier than those in the <35cm class, whilst fish >40cm were approximately 75% heavier than the <35cm class. This approximate, percentage increase in weight with increasing size class was applied to the PCB dose administered to size class of fish relative to the smallest (<35cm) class. This ensured that each fish received a dose of PCBs in a similar proportion to the other fish in each treatment tank. Thus, fish in the low dose tank received doses of 1.0, 1.25 or 1.75mg Aroclor 1254 $fish^{-1}$, and those in the high dose tank of 2.0, 2.5 or 3.5mg Aroclor 1254 $fish^{-1}$ depending on the size class to which each fish belonged. Females in the control tank received a 0.15ml placebo dose containing pure vegetable oil. All fish were dosed orally with the appropriate treatment once a month until April when spawning finished. Fish were measured and weighed each time they were dosed throughout this period. Feeding was continued throughout the spawning season every other day with commercial pellet feed though consumption was low in all tanks (visual observation).

In December 2004, the experiment was repeated with even lower doses than those used previously. Due to the change in available facilities at this time, it was only possible to dose fish in one tank (n = 14). Doses administered were 0.1, 0.125 or 0.175mg Aroclor 1254 $fish^{-1}$ depending on the size class of each fish. Again, females in the second tank received placebo capsules containing only vegetable oil (n = 16). Males were housed in a separate holding tank.

At the end of both spawning seasons, female fish in all tanks were killed using a Schedule I method. Liver samples were taken for analysis by GC/MS and frozen in hexane-washed glass vials at -20°C until ready for processing (see Section 3.3.9). From this point onward, dosed female fish from the 2004 experiment will be referred to as medium- and high-dosed fish and dosed fish from the 2005 work, as low-dosed

fish. Data for undosed fish from both years were not combined and kept separate for statistical comparisons between dose classes within each year.

3.3.5 Fish spawning

Plaice were not allowed to spawn naturally in the tanks, as it was necessary to identify the spawns to particular female fish. When females were judged ripe (highly swollen ovaries), they were removed from the holding tanks and anaesthetised lightly in 2-phenoxyethanol as described above and measured and weighed. This occurred between January and April in both years the experiments were run. Eggs were hand-stripped from the fish by applying light pressure by hand from the posterior end of the ovaries moving toward the anterior end. Eggs were collected in a clean glass beaker and weighed.

From each spawn a sub-sample of eggs (ca. 20g) was retained for analysis by GC-MS in a hexane-washed 30ml glass vial and kept at –20°C. A further sample (ca.1g) of eggs was retained and counted within a few minutes of spawning to determine the number of eggs g^{-1} wet wt $spawn^{-1}$. These were then rinsed and dried in an oven (LTE Scientific, OP250-UF) at 60°C for 72-hours to determine the egg dry weights (Fisk & Johnston, 1998). Some multiple spawns were obtained from females that spawned repeatedly but these were only fertilised if fertilisations of previous spawns from the same female had failed. Not all spawns could be fertilised due to aquarium space limitations and the limited number of males available for the experimental work. Although multiple spawns were all sub-sampled for subsequent GC/MS analysis, no investigations were carried out into the changes in PCB concentrations between spawns. This was due to the multiple doses administered throughout the experiment, which would cause fluctuations in total PCB burdens.

Samples for GC/MS analysis were kept in different freezers as a precaution against equipment failure. This proved to be prudent in 2004 when a freezer broke down resulting in the loss of some liver and egg samples before analysis could take place. These samples are indicated where appropriate in section 3.4.

3.3.6 Chemical analysis

Liver and egg samples from the female broodstock were freeze-dried for analysis by GC/MS and processed in the same way as described in sections 2.3.2 – 2.3.6 inclusive. The column used to analyse the egg samples from 2004 was a 25m HT5 column with a 0.25mm internal diameter. All other samples were analysed using a 30m HT5 (SGE) column with a 0.25mm internal diameter, in 2005.

3.3.7 Statistical analyses

Multivariate analyses of chemical data from both the preliminary and broodstock experiments were undertaken using PRIMER software (Clarke & Warwick, 1994). PRIMER is intended mainly for ecological applications but can be used to study data sets such as physico-chemical data where appropriate. The proportion of each PCB congener detected was calculated relative to the 21 congeners identified (see section 2.3.5). Congener proportions in each of the standards, egg, muscle or liver samples analyzed were then arcsine transformed and were the equivalent of "species data" that would be used more commonly in PRIMER analyses. Data were pooled for the broodstock experiments completed in 2004 and 2005. Many of the PCBs detected in the eggs samples from the low-dosed fish and some liver samples (e.g. fish 326 and 358) were at either trace levels or <LD and were therefore not included in the Primer analysis since they may not have provided reliable congener proportions. Data from three additional fish given a high dose during in the 2004/2005 experiment for work presented in Chapter 5 were included in the analysis in order to increase the size of the data set for the livers and eggs. These fish had been treated in exactly the same manner as all other fish described in this chapter and so the addition of their data is a useful aid to help determine patterns of congener distribution.

Multidimensional scaling (MDS) plots were produced from the Bray-Curtis similarity matrices for each of the data sets. A two-way analysis of similarities test (ANOSIM – a non-parametric, multivariate version of ANOVA) was applied to each data set to detect any differences in congener proportions between tissue types and in comparison to the standard mixture with which the fish were originally dosed.

Finally, a SIMPER analysis was applied to the data. The SIMPER analysis involves the computation of the average dissimilarity between each pair of inter-group samples before breaking them down into the separate contribution each congener makes to the dissimilarity between groups. It also determines the contribution to similarity in congener proportions within groups. Multiple *t*-tests were then applied to the data for the ICES 6 congeners to identify specifically where significant differences existed.

In order to show the differences in congener profiles between liver and egg samples and the Aroclor 1254 standard more clearly, the proportional congener data were subjected to principal components analysis (PCA) using SIMCA-P software. This enabled the construction of contribution plots to demonstrate differences in congener proportions between sample types, graphically by subtracting the proportions of each congener in one sample from those in another. Thus, congeners in the first sample having a higher proportion were illustrated as bars above the *x*-axis and those having a lower proportion fall below the *x*-axis.

Following chemical analysis, the egg PCB concentration data were grouped into four categories of ΣPCB(21 congeners) concentration of 0, 0-1, 1-10 and >10 mg kg^{-1} eggs dry wt. Individual egg dry weights were calculated and compared against PCB concentrations based on the groups mentioned above using a nested ANOVA as the females were originally assigned randomly to each treatment group. The data set for egg dry weight from the 2004/2005 experiment was small (n = 11) and so was combined with the data from the 2003/2004 experiment to produce a larger data set and to examine any patterns over the entire concentration range. Minitab Statistical Software version 13.2 was used for all analyses and the significance level was set at $p < 0.05$.

3.4 Results

3.4.1 PCB quantification

As described in section 2.4.1, an estimated 0.1-0.5% of OCN and PCBs were carried over between samples as they passed though the GC column and thus the LD and PQL were calculated as outlined in section 2.3.6. The LD was different for each congener and for each batch of samples because the source of the mass spectrometer required cleaning and re-tuning between each sample batch. Varying quantities of Aroclor 1254 were still carried over each time a standard was run as happened with analysis of the field samples (chapter 2, Fig. 2.4). The re-tuning did not affect quantification, since each set of sample results could be compared with the OCN and Aroclor 1254 standards processed with each sample batch. 21 PCB congeners were identified in each sample (see section 2.3.5, Fig. 2.3). Fig. 3.1 shows the results for CB-153 from one batch of laboratory samples including the LD and PQL for that sample batch. In this particular case all the control liver samples and low dose egg samples fell below the LD meaning that the low concentrations shown cannot be regarded with confidence at present, since they are not greater than the background level as defined by the LD. The three liver samples exceeding both the LD and PQL are from high-dosed females, the progeny of which were used in experimental work described in chapter 5. This figure is discussed further in section 3.5.1.

For every sample batch run through the GC/MS, the coefficient of variation (CV = 100x(SD/mean)) was calculated for each of the ICES 6 congener concentrations in the Aroclor 1254 standard that was run at the start, middle and end of each batch. This was used to compare the amount of variation (%) in congener concentrations in each sample batch with both the other congeners in that batch and other sample batches analysed. The coefficient of variation was used because the GC/MS equipment was re-tuned between each sample batch so making the standard deviations relative to each other makes more sense rather than making comparisons between different sample batch mean values or standard deviations. The values obtained are given in Table 3.1. Most of these values are either approximately 20% or below and are in the

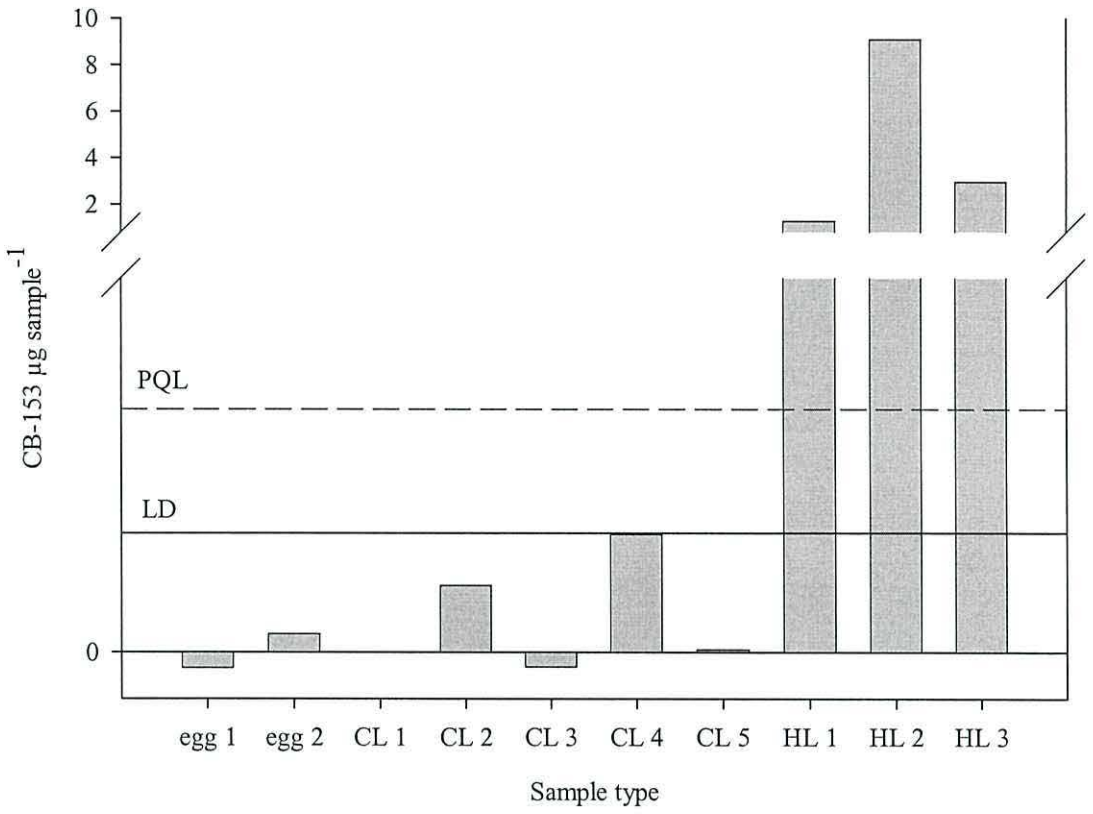


Figure 3.1 Concentrations of CB-153 in samples taken from plaice orally dosed with Aroclor 1254. Samples are either from eggs from fish administered a low dose (0.14-0.19mg kg^{-1} fish $month^{-1}$) or liver samples from control, undosed plaice (CL) or from high dosed fish (HL) dosed with 3.32-4.33mg kg^{-1} fish $month^{-1}$. Values below the LD (solid line, 0.0256µg $sample^{-1}$) are regarded as not detected and values falling below the PQL (dotted line, 0.0512µg $sample^{-1}$) are defined as "trace". Values below 0µg $sample^{-1}$ occur when the blank values exceed those in the samples. Values >PQL can be accepted with confidence.

Table 3.1 Coefficients of variation (%) for the ICES 6 PCB congeners detected in Aroclor 1254 standards by GC/MS from comparison of chromatograms run with each sample batch (n = 3).

Year	Analysis batch code	ICES 6 PCB congeners					
		52	101	118	138	153	180
2004	B	18.8	9.4	12.4	5.2	6.1	4.8
	C	20.6	13.8	11.8	8.9	9.0	4.8
	D	8.4	7.9	9.0	6.0	6.3	9.1
	E	15.7	11.4	13.5	10.0	7.6	5.5
	F	32.7	27.7	29.1	22.9	19.6	9.2
	G	21.5	25.8	12.7	8.6	16.4	6.5
	Overall (n = 17)	34.7	28.7	29.0	24.4	23.0	11.7
2005	A	16.4	7.3	2.3	4.3	4.1	4.5
	B	9.5	7.0	6.0	4.3	8.4	8.5
	C	21.5	21.8	21.7	14.4	14.4	12.2
	D	10.9	9.3	9.8	7.2	8.1	9.9
	E	1.0	2.8	1.6	1.3	4.8	2.1
	F	14.6	14.4	10.3	11.6	7.9	16.8
	G	9.0	13.6	16.1	5.2	13.9	10.1
	H	19.7	15.3	16.4	14.8	15.5	11.4
	Overall (n = 24)	32.4	22.2	19.0	17.9	23.0	23.2
2005	New column used						
	I	7.6	6.7	8.3	8.1	9.7	7.1
	J	30.5	26.8	21.2	17.0	18.6	15.7
	K	2.6	3.1	2.7	1.6	6.0	11.0
	Overall (n = 7)	23.7	22.3	17.6	16.1	17.1	13.7

same range as described as "good" by de Boer et al. (2001) for PBDE congeners. Table 3.1 is discussed further in section 3.5.1.

3.4.2 Preliminary experiment analyses

The LDs were variable (range = 0.5μg l^{-1} sample – 217μg l^{-1} sample; mean LD = 33.7; SD = 42.2) and were often greatest for the more common congeners (e.g. ICES 6). Nonetheless, the preliminary results demonstrated the ready uptake of Aroclor 1254 by plaice and the successful nature of the dosing procedure used without causing physical harm to the fish (Table 3.2). The mean concentration of ΣPCBs (21 congeners) detected in the livers of fish from tanks A and B was 15934.6 ±8332.4μg kg^{-1} dry wt. The high standard deviation was likely to have been caused by the low concentrations detected in one fish from tank A and may also demonstrate the individual variability in PCB uptake and metabolism by plaice. Variation in fish size might be a factor in explaining the concentrations of PCBs detected since the doses were absolute and not relative to body size but given the small sample size (n = 6), it is not possible to see a clear relationship between body size and PCB concentration (Fig. 3.2). Congeners detected in muscle tissues were 1-2 orders of magnitude lower than those in livers and this can be attributed to the lipophillic nature of PCBs whereby they accumulate in organs with a high lipid content such as the liver.

PCBs were detected in liver from one control, undosed fish from whilst another from the control tank contained PCBs that were on average, an order of magnitude lower than those detected in the dosed fish. Aroclor was also detected in the faeces from the undosed fish at an order of magnitude lower than tanks A and B. Since PCBs were not detected in fish from the original field site (PD data in Table 3.2), the PCBs detected in the undosed fish were likely to have come from contamination in the recirculating aquarium system used for the study, most likely particulate matter that passed through the filter wool.

High concentrations of Aroclor were detected in faecal matter collected from tanks A and B and the filter wool demonstrating that only a fraction of the 2mg Aroclor 1254 used to dose the fish actually remained in their systems. Of the total 2mg dose given

Table 3.2 ICES 6 and ΣPCB (21 congeners) concentrations (μg kg^{-1} dry weight) in field-caught juvenile plaice (Port Dinorwic, PD), plaice dosed orally with Aroclor 1254 (2x1mg doses) (TA and TB), undosed fish (TC) and faeces, filter media and water from the aquarium system in which the fish were maintained. L = liver, M = muscle, OV = ovary, F = faeces, f = female, m = male, ns = no sample, concentrations left blank denote no PCBs detected.

Fish	Tissue	Sex	Fish weight (g)	Sample dw (g)	Treatment	ΣPCB dose (mg)	ICES 6 congeners						ΣPCB (21 congeners) μg kg^{-1} dw
							52	101	118	153	138	180	
PD F1	L	f	53.4	0.47	Field	0	-	-	-	-	-	-	-
PD F2	L	f	54.8	0.31	caught	0	-	-	-	-	-	-	-
PD F3	L	f	69.9	0.65		0	-	-	-	-	-	-	-
PD F1	M	f		1.37		0	-	-	-	-	-	-	-
PD F2	M	f		1.34		0	-	-	-	-	-	-	-
PD F3	M	f		1.76		0	-	-	-	-	-	-	-
PD F1-3	OV	f		0.38		0	-	-	-	-	-	-	-
TA F1	L	f	76.2	0.28	Dosed	2	116.4	308.2	236.1	131.1	138.2	14.3	2114.3
TA F2	L	m	68.6	0.34		2	1148.5	2727.1	3098.5	1851.8	1739.4	397.9	23686.4
TA F3	L	f	54.6	0.22		2	657.9	2124.6	2586.8	1334.3	1397.5	231.8	18684.2
TA F1	M	f		1.80		2	40.1	89.2	67.0	30.3	28.6	2.8	630.7
TA F2	M	m		1.65		2	trace (53.8)	trace (123.7)	trace (123.8)	trace (75.6)	trace (75.2)	trace (15.3)	trace (1014.9)
TA F3	M	f		1.40		2	ns	ns	ns	ns	ns	ns	ns
TA F1&3	OV	f		0.23		2	-	-	-	-	-	-	-
TB F1	L	f	64.0	0.31	Dosed	2	775.2	1777.1	1822.3	1090.0	1102.6	194.2	14914.8
TB F2	L	f	63.6	0.34		2	602.6	1389.7	1547.1	976.2	991.2	181.8	11857.1
TB F3	L	f	75.5	0.21		2	942.6	2668.6	3132.3	2031.1	1873.7	488.0	24350.5
TB F1	M	f		1.70		2	-	-	-	-	-	-	-
TB F2	M	f		1.58		2	94.5	161.8	188.4	81.5	159.3	10.0	1452.7
TB F3	M	f		1.42		2	125.0	270.0	trace (279.2)	184.2	181.2	42.9	3352.0
TB F1-3	OV	f		0.26		2	-	-	-	-	-	-	-
TC F1	L	m	83.8	0.14	Undosed	0	-	-	-	-	-	-	-
TC F2	L	f	58.8	0.13		0	trace (88.5)	trace (345.4)	-	-	-	-	trace (433.9)
TC F3	L	f	73.2	0.35		0	276.6	614.6	596.3	277.1	252.6	21.1	4840.2
TC F1	M	m		1.20		0	-	-	-	-	-	-	-
TC F2	M	f		1.24		0	-	-	-	-	-	-	-
TC F3	M	f		1.78		0	-	-	-	-	-	-	-
TC F2&3	OV	f		0.23		0	-	-	-	-	-	-	-
TA F1-3	F			1.90	Dosed	6	5136.8	8276.4	10134.2	4767.5	9264.9	916.7	83247.9
TB F1-3	F			2.26	Dosed	6	2366.5	3740.0	4507.6	2175.8	4082.3	361.3	37752.0
TC F1-3	F			1.72	Undosed	0	124.4	332.7	444.9	204.7	268.0	30.0	2904.2
Filter wool material				1.17		12	3146.2	9164.8	13586.9	9006.6	10473.4	2409.7	99746.7
Water (2.0 l)				-		12	-	-	-	-	-	-	-

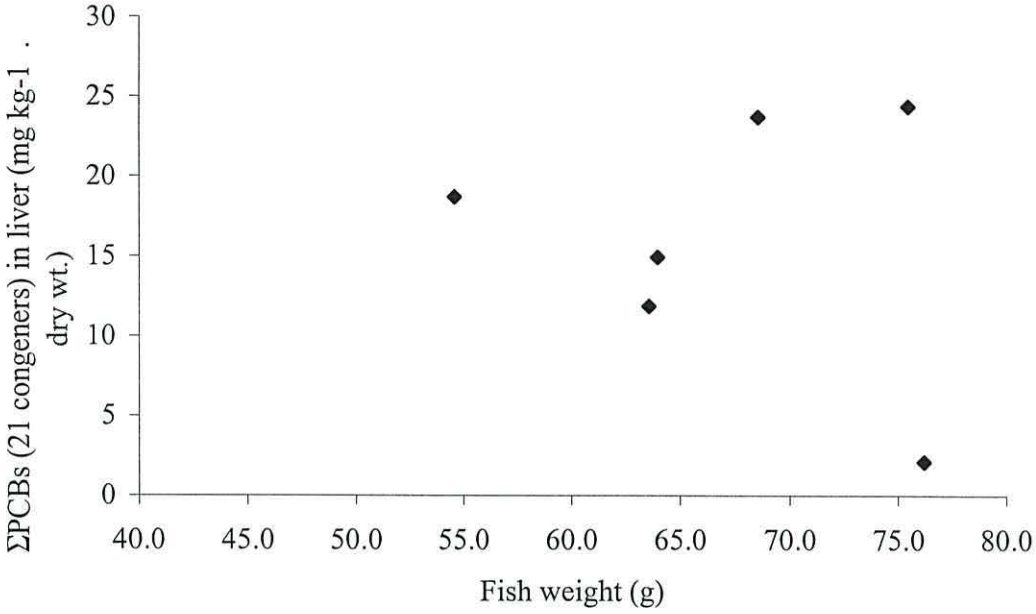


Figure 3.2 ΣPCB (21 congeners) mg kg^{-1} dry weight detected in the livers of plaice dosed with 2x1mg doses of Aroclor 1254.

to each fish, only a total of 0.6 – 8.5μg was detected in the livers two weeks following the final dose, representing only 0.03 – 0.425% of the total original dose. The concentrations detected in the livers were an order of magnitude greater than those detected in dab liver (*Limanda limanda)* taken from around the UK coast (NMMP, 1998b) and the concentrations in muscle, 1-2 orders of magnitude greater than concentrations found in plaice muscle in Liverpool Bay by Leah et al. (1997) (see section 2.5.3 for further environmental concentrations). Overall, the results allowed us to predict that by using similar and lower doses of Aroclor 1254 less frequently on adult fish, that were between one to two orders of magnitude heavier and undergoing gonadal recrudescence, it should be possible to achieve body burdens similar to those reported in the environment.

Analysis of the faeces (Table 3.2) confirmed that a large proportion of the Aroclor dose either passed through the plaice or was excreted readily and would thus require filtration from any water leaving the aquarium system. Concentrations of PCBs in the faeces sampled from tanks containing dosed fish were approximately 10-28x higher than those obtained from undosed fish. PCBs in faeces from tank C are most likely to have been carried in from the other tanks via the recirculating system used. The concentration of PCBs was highest in the filter wool indicating its efficacy in trapping particulate matter that contained PCBs in the water (Table 3.2). No PCBs were detected in the water indicating either extremely low or non-existent quantities. The results from the water, faeces and filter samples provided the information required for the design of larger scale broodstock experiments whereby an isolated water system would be required for each tank and water leaving the tanks would require filtration with multiple layers of filter wool and activated charcoal to filter out material containing PCBs from the water leaving the aquarium.

3.4.3 Broodstock experiment analyses

As with the preliminary experiment, the female broodstock accumulated Aroclor 1254 readily in their body tissues during the dosing period. Table 3.3 gives specific information relating to the ICES 6 concentrations in eggs and ΣPCB (21 congeners) concentrations detected in the livers of the female plaice broodstock for all of

Table 3.3 Polychlorinated biphenyl ICES 6 congener concentrations in the eggs and ΣPCB(21 congeners) in the eggs and livers from female plaice broodstock dosed with Aroclor 1254. Larvae from these particular spawns were subsequently assessed for survival, growth and behavioural performance (see Chapter 4). Abbreviations: n.s. = not sampled; LD = limit of detection (0.6 – 201.0μg l^{-1} sample for ICES 6 congeners); n.d. = not detected.

Year	Fish ID code	Fish length (cm)	Fish weight pre-spawning (g)	Maternal PCB dose (mg kg^{-1} fish $month^{-1}$)	ICES 6 congeners (μg kg^{-1} dw egg)						ΣPCB (21 congeners) μg kg^{-1} dw	
					52	101	118	153	138	180	Eggs	Liver
2004	21_	38.0	596	2.10	326.6	367.5	385.4	163.8	242.8	22.9	3672.7	4048.7
2004	213	37.3	574	2.18	190.1	273.0	276.6	113.1	194.4	16.5	2359.6	1455.1
2004	228	35.8	603	2.07	<LD (37.5)	trace (46.5)	95.10	trace (29.1)	21.9	<LD (0.5)	470.5	323.5
2004	256	39.6	842	1.48	70.9	100.2	93.1	54.8	57.9	5.8	906.9	600.9
2004	278	36.9	636	1.97	407.9	639.8	640.2	277.2	346.7	32.4	6114.5	934.4
2004	225	44.6	1237	1.41	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.s.**
2004	247	45.3	1037	1.69	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	22904.9
2004	257	34.5	393	2.54	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.**	n.s.**
2004	318	35.8	525	4.76	57.3	1461.2	1488.4	829.1	971.4	88.0	12308.6	12205.8
2004	326	36.5	616	4.06	51.1	129.3	141.2	91.2	108.8	22.5	1264.4	n.s. **
2004	336	41.6	930	3.76	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s. *	249.4
2004	347	40.5	756	4.63	115.2	324.2	360.9	193.0	229.4	35.2	2916.2	558.0
2004	358	40.4	906	3.86	81.4	91.3	159.9	<LD (25.8)	<LD (13.8)	<LD (2.9)	892.6	trace (5.9)
2004	368	43.8	1027	3.41	169.0	249.0	262.1	114.4	160.7	15.0	2254.6	364.3
2004	324	34.9	470	4.26	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.**	n.s.**
2004	334	38.0	645	4.65	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.**	n.s.**
2005	423	38.8	646	0.19	n.d.	<LD (7.7)	trace (21.7)	<LD (6.0)	<LD (12.9)	<LD (3.2)	<LD (126.0)	<LD (142.6)
2005	424	44.8	1209	0.14	n.d.	n.d.	trace (4.7)	<LD (4.5)	<LD (4.0)	trace (1.7)	<LD (27.1)	<LD (164.4)
2005	426	35.0	512	0.20	n.d.	n.d.	10.2	<LD (6.7)	trace (8.2)	<LD (2.0)	<LD (68.1)	2652.8
2005	427	38.1	695	0.18	trace (4.06)	trace (15.7)	20.9	14.9	21.7	5.1	<LD (197.5)	659.8
2005	434	35.7	471	0.27	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s. *	4309.4
2004	undosed (n=7)	34.0-44.3	341-977	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2005	undosed (n=5)	31.1-39.2	358-693	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

* No samples were taken due to the small spawn sizes and priority was given to rearing the larvae. ** Sample lost in laboratory incident

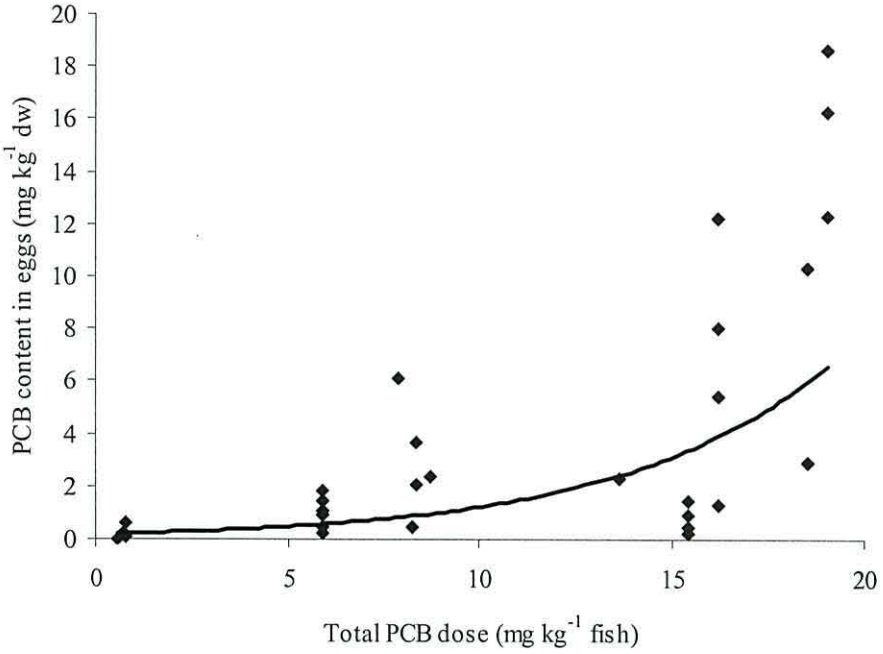
the spawns that were fertilised and used for subsequent analyses of embryo survival and larval survival, growth and behaviour (see Chapter 4). There was high individual variation in the ΣPCB (21 congeners) concentrations within dosing groups by up to two orders of magnitude giving a range of concentrations (Table 3.3, Fig. 3.3 A & B) over which to monitor the possible impacts on survival, growth and behaviour (Chapter 4). No PCBs were detected in the control fish and thus the results for those fish that spawned are summarised at the bottom of Table 3.3. In brief, the range of the LDs for all 21 congeners detected was 0.1 – 262.9μg l^{-1} sample with an overall mean = 25.1μg l^{-1} sample and standard deviation = 36.7μg l^{-1} sample. The LDs for the ICES 6 congeners ranged between 0.6 and 201.0μg l^{-1} sample.

The PCB concentrations detected in eggs and livers are given on a dry weight basis as this is more accurate than presenting the results on a wet weight basis. The female plaice were stripped of eggs when they were judged to be ripe and there may have been (i) some variation in egg water content depending on the timing of hand-stripping and/or (ii) variable amounts of ovarian fluid expelled with the eggs, both making accurate determination of egg wet weights difficult. As total dose across the PCB exposed groups increased, so too did the ΣPCB (21 congeners) concentration detected in the eggs. A significant positive correlation ($r = 0.644$, $p < 0.001$, n=31) existed between total dose administered to individual females (F; mg kg^{-1} fish) and concentration detected in egg samples (E; mg kg^{-1} dry wt.) although an exponential trend line fitted the data better ($r^2 = 0.5147$) than a straight line ($r^2 = 0.4186$) (Fig. 3.3 A):

$$E = 0.1881e^{0.1866F} \qquad (r^2 = 0.5147)$$

There was no significant relationship ($r = 0.357$, $p = 0.211$, n = 15) between total PCB dose and liver PCB concentrations even though the data was probably heavily influenced by the high concentrations in one female liver (Fig 3.3 B). In both cases there was a high degree of scatter among the data points. This might be attributed to individual differences in uptake rates of the Aroclor 1254 or it may be due to the nature of the experiment in which the fish were given multiple doses of Aroclor throughout the spawning season causing fluctuations in total body burdens.

A



B

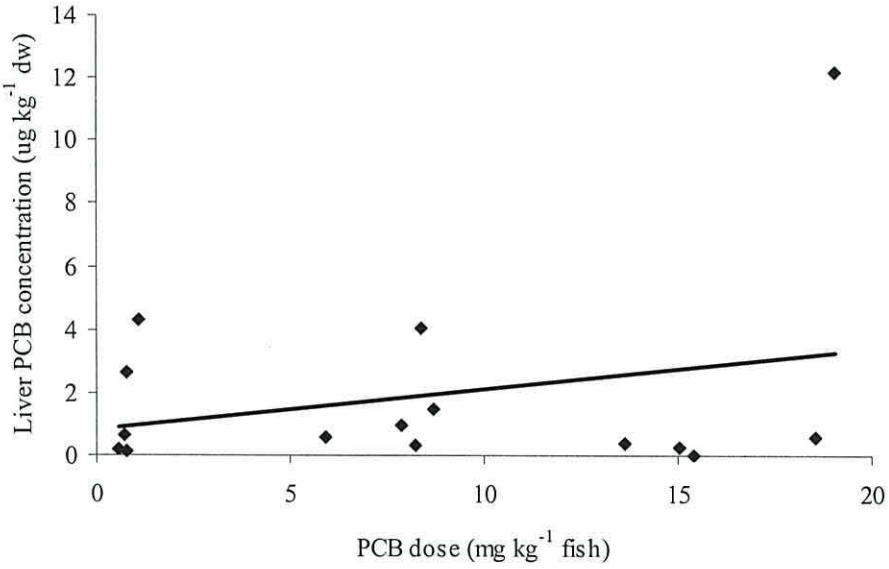


Figure 3.3 Relationships between the total individual PCB doses administered (mg Aroclor 1254 kg^{-1} fish) and PCB concentrations in (**A**) the eggs (mg kg^{-1} dry wt.) $y=0.1881e^{0.1866x}$, $p < 0.001$, $r^2 = 0.515$, n = 31 and (**B**) the livers at the end of the spawning season (mg kg^{-1} dry wt.) $y=0.1276x + 0.8498$, $p = 0.329$, $r^2 = 0.047$, n = 15.

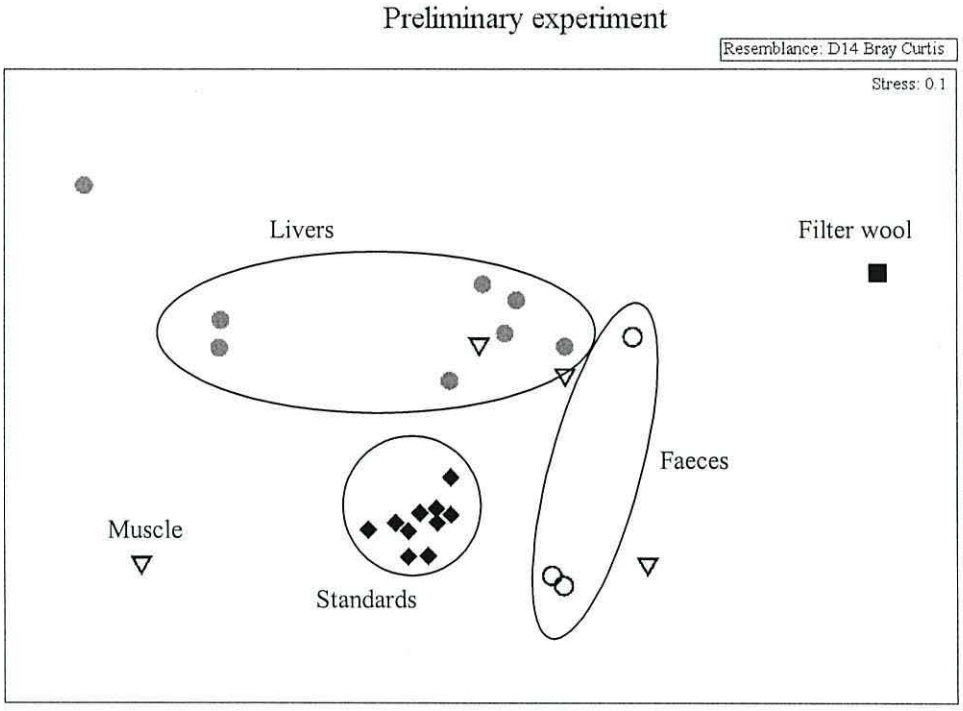
The total PCBs per egg were calculated based on the egg dry weights determined for each spawn (see section 3.3.5). In 2005, the ΣPCBs (21 congeners) per egg in the low-dosed fish ranged from 9-183 pg egg^{-1} (mean = 51, SD = 68, n = 6). In 2004, the concentrations ranged from 63-1524 pg egg^{-1} (mean = 494, SD = 430, n = 11) in the medium-dosed group and 64-4518 pg egg^{-1} (mean = 1738, SD = 1472, n = 14) in the high-dosed group.

3.4.4 Multivariate analyses

Multivariate analysis revealed distinct differences in the congener profiles of the PCBs extracted from different tissue samples (liver, eggs or muscle). Tissue samples also often differed significantly compared to the Aroclor 1254 mixture used to dose the fish in both experiments (Fig 3.4). Table 3.4 also lists the mean proportion and standard deviation of each PCB congener in each sample type analysed from the main broodstock experiment.

Pairwise comparisons of the results from the preliminary experiment revealed significant differences in the congener profiles of the original Aroclor 1254 mixture compared to the muscle tissue (ANOSIM, $R = 0.838$, $p = 0.003$), liver tissue ($R = 0.691$, $p = 0.001$) and the faeces ($R = 0.94$, $p = 0.003$). There were no significant differences in any other pairwise comparisons ($p > 0.05$ in all cases) but this is likely to be due to the lack of replicates available as it appears quite clearly from Fig. 3.4 that the congener composition of the filter media was distinctly different from that of the Aroclor 1254 standard. When applying an ANOSIM test with fewer than four replicates it is not possible to obtain a $p<0.05$. Retrospective power analysis (Thomas, 1997) was applied to the non-significant results of the preliminary experiment. In all cases the power value was <0.5 and the calculated sample sizes required to obtain statistical significance ranged between 7 and 27 depending on the differences observed between the congener proportions. In reality, the number of samples obtained from the broodstock experiment depended on the numbers of fish that spawned. However, the sample sizes, n, for each sample type analysed using Primer were: eggs, n = 26, livers, n = 15, standards, n = 27.

A



B

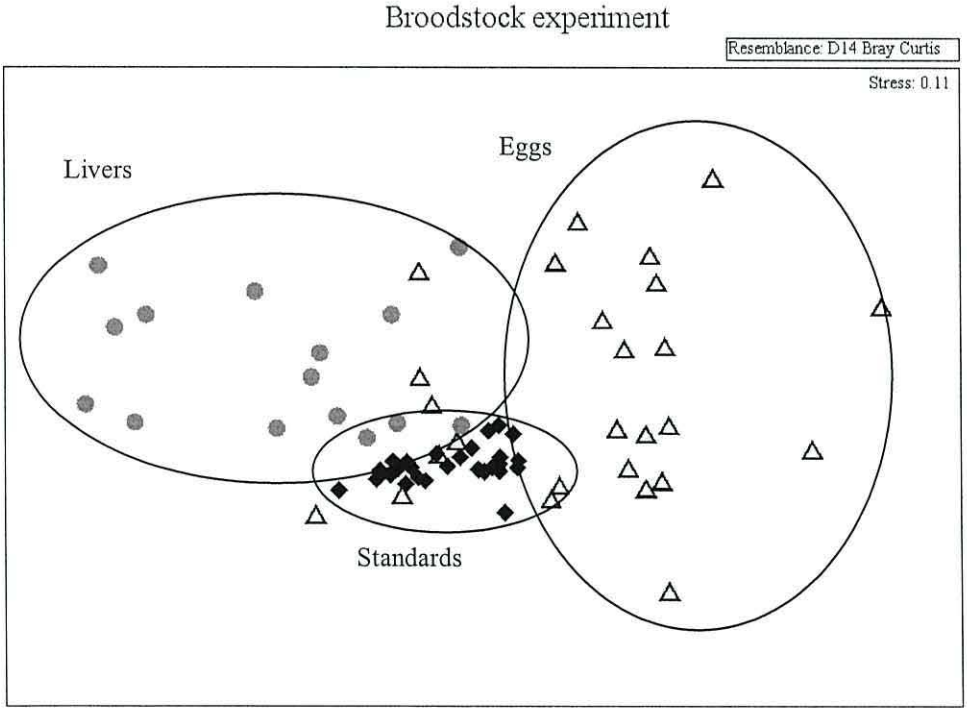


Figure 3.4 2-dimensional MDS plots of the PCB congener proportions in tank filter material (■) and the livers (●), muscle(▽), eggs (△) and faeces (O) of European plaice dosed with the commercial PCB mixture Aroclor 1254 (◆) in the preliminary experiment (A) and the main broodstock experiments (B) in both 2004 and 2005. Each data point shown relates to a sample from an individual fish.

Table 3.4 Mean percentage contributions (± SD) of the 21 PCB congeners identified in Aroclor 1254 and in livers and eggs from female plaice broodstock orally dosed with Aroclor 1254. ICES 6 congeners are highlighted in bold.

Congener no.	Aroclor 1254 (n = 28)	Liver PCBs (n = 15)	Egg PCBs (n = 27)
110	14.30 ± 0.98	15.62 ± 1.24	15.48 ± 1.64
118	12.24 ± 0.64	14.32 ± 1.78	11.62 ± 1.44
101	10.80 ± 0.99	11.17 ± 0.94	10.77 ± 0.80
138	8.46 ± 1.21	9.34 ± 1.22	7.02 ± 1.35
153	7.97 ± 1.49	8.84 ± 1.25	5.50 ± 1.29
95	7.32 ± 0.90	5.59 ± 1.53	7.55 ± 1.07
151	5.53 ± 0.34	4.51 ± 0.81	4.03 ± 1.13
52	4.62 ± 1.21	3.81 ± 1.33	6.44 ± 1.94
87	4.35 ± 0.29	4.04 ± 0.45	4.33 ± 0.75
105	4.10 ± 0.41	4.31 ± 0.70	5.06 ± 1.08
60	3.51 ± 0.53	3.31 ± 1.05	4.79 ± 1.25
89/92	2.99 ± 0.35	2.19 ± 0.54	2.92 ± 0.52
99	2.93 ± 0.27	3.26 ± 0.39	3.34 ± 0.92
146	2.59 ± 0.33	1.94 ± 0.39	2.24 ± 0.57
97	2.33 ± 0.25	1.51 ± 0.48	1.97 ± 0.84
128	1.74 ± 0.40	1.37 ± 0.49	2.32 ± 0.70
180	1.46 ± 0.58	2.25 ± 0.56	0.83 ± 0.49
44	1.33 ± 0.31	0.80 ± 0.47	1.95 ± 0.62
170	0.81 ± 0.40	1.21 ± 0.42	0.40 ± 0.28
49	0.65 ± 0.28	0.61 ± 0.38	1.42 ± 0.92

In the broodstock experiments, significant differences existed between all three sets of samples (Global R = 0.458, $p = 0.001$). Pairwise comparisons confirmed that congener profiles in the livers and eggs were significantly different to original Aroclor mixture (liver, $R = 0.66$, $p = 0.001$; eggs, $R = 0.39$, $p = 0.001$) and that the egg and liver congener profiles also differed significantly from each other ($R = 0.549$, $p = 0.001$).

Results from the SIMPER analyses highlighted the PCB congeners that accounted for the observed differences in the PCB composition between body tissues and in comparison to the original dose of Aroclor 1254 (Tables 3.5-3.11). At least four of the ICES 6 congeners identified (52, 101, 118, 153, 138, 180) regularly made the top 10 that contributed most to the dissimilarity between groups (Tables 3.5-3.11). Often, of these congeners, the proportions in the liver samples were greater than in the original Aroclor 1254 used to dose the fish. Differences in individual PCB uptake rates also resulted in higher variation (greater confidence intervals) in congener proportions in comparison to the Aroclor 1254 (Tables 3.5-3.11). Pairwise comparisons using t-tests were employed to clarify whether the differences in congener proportions were significant between tissue types and the original Aroclor 1254 mixture and were conducted on data from both the preliminary and main experiments (Tables 3.12 and 3.13 respectively). The congeners chosen for comparisons were the ICES 6 and CBs 60 and 110 (mono-ortho substituted PCBs) and CB-146 (tetra-ortho substituted). The Bonferroni correction was applied to the significance level for these comparisons for each congener. Few significant differences were found between congener proportions in the preliminary experiment and this might be attributed to the lower significance level ($p = 0.01$) and the small sample sizes (n = 4 to 10 for each sample type) whereas many differences were detected in the main experiment ($p = 0.016$, n = 15 to 27 for each sample type). The results from the main broodstock experiment show many significant differences in congener proportions between liver, egg and Aroclor 1254 samples, suggesting differences in the ways in which congeners are processed depending on both the degree and pattern of chlorination. Of those examined, most penta- to octa-CBs had increased proportions in the liver compared to the eggs and original Aroclor 1254 mixture. Only CB-52 showed an increased proportion in the eggs compared to Aroclor 1254 and the liver, whilst CB-110 increased in the eggs compared to Aroclor 1254 only. Again, retrospective power analysis was carried out on the non-significant

Tables 3.5-3.8 SIMPER analysis of the top ten PCB congeners contributing most to the dissimilarity between the body tissues sampled and the original Aroclor 1254 used to dose the fish in the preliminary experiment carried out in 2003 on juvenile 1+ plaice. Data are given as arcsin proportions of the ΣPCB (21 congeners) identified ± 95% CI. Congeners in bold indicate ICES 7congeners. Arrows indicate the general increase or decrease in congener proportions in the second column of each table compared to the first column.

3.5 – Aroclor vs muscle tissue

IUPAC Congener no.	Mean proportion in Aroclor 1254		Mean proportion in muscle	Cumulative percentage
138	0.064 ± 0.005	↑	0.077 ± 0.042	11.96
95	0.080 ± 0.002	↓	0.060 ± 0.008	22.14
110	0.141 ± 0.003	↑	0.159 ± 0.004	31.77
92/89	0.035 ± 0.003	↓	0.020 ± 0.019	40.42
153	0.057 ± 0.003	↑	0.064 ± 0.023	47.11
101	0.109 ± 0.004	↑	0.122 ± 0.021	53.51
151	0.048 ± 0.002	↓	0.038 ± 0.014	59.09
105	0.041 ± 0.001	↑	0.050 ± 0.028	64.25
52	0.066 ± 0.004	↓	0.059 ± 0.010	69.29
97	0.025 ± 0.002	↓	0.017 ± 0.008	73.75

3.6 – Aroclor vs liver tissue

IUPAC Congener no.	Mean proportion in Aroclor 1254		Mean proportion in liver	Cumulative percentage
110	0.141 ± 0.003	↑	0.164 ± 0.018	19.19
52	0.066 ± 0.004	↓	0.047 ± 0.008	29.03
101	0.109 ± 0.004	↑	0.120 ± 0.014	37.60
153	0.057 ± 0.003	↑	0.075 ± 0.008	44.89
146	0.028 ± 0.003	↓	0.017 ± 0.004	51.63
95	0.080 ± 0.002	↓	0.064 ± 0.009	58.37
138	0.064 ± 0.005	↑	0.075 ± 0.006	64.62
118	0.118 ± 0.002	↑	0.127 ± 0.009	69.85
44	0.020 ± 0.001	↓	0.012 ± 0.002	74.29
151	0.048 ± 0.002	↓	0.042 ± 0.005	77.66

3.7 – Liver tissue vs muscle tissue

IUPAC Congener no.	Mean proportion in muscle		Mean proportion in liver	Cumulative percentage
110	0.159 ± 0.004	↑	0.164 ± 0.018	11.95
138	0.077 ± 0.042	↓	0.075 ± 0.006	22.58
101	0.122 ± 0.021	↓	0.120 ± 0.014	31.10
153	0.064 ± 0.023	↑	0.075 ± 0.008	38.29
105	0.050 ± 0.028	↓	0.042 ± 0.006	45.16
52	0.059 ± 0.010	↓	0.047 ± 0.008	51.28
118	0.119 ± 0.016	↑	0.127 ± 0.009	56.90
92/89	0.020 ± 0.019	↑	0.031 ± 0.002	62.38
146	0.024 ± 0.012	↓	0.017 ± 0.004	67.36
95	0.060 ± 0.008	↑	0.064 ± 0.009	72.18

3.8 – Aroclor vs faeces

IUPAC Congener no.	Mean proportion in Aroclor 1254		Mean proportion in faeces	Cumulative percentage
138	0.064 ± 0.005	↑	0.104 ± 0.025	26.41
118	0.118 ± 0.002	↑	0.131 ± 0.047	35.65
52	0.066 ± 0.004	↓	0.056 ± 0.028	43.43
110	0.141 ± 0.003	↓	0.138 ± 0.035	50.96
92/89	0.035 ± 0.003	↓	0.025 ± 0.003	58.14
101	0.109 ± 0.004	↓	0.104 ± 0.022	63.92
97	0.025 ± 0.002	↓	0.018 ± 0.003	68.86
153	0.057 ± 0.003	↑	0.062 ± 0.019	72.95
44	0.020 ± 0.001	↓	0.015 ± 0.011	76.82
95	0.080 ± 0.002	↓	0.074 ± 0.012	80.40

Tables 3.9-3.11 SIMPER analysis of the top ten PCB congeners contributing most to the dissimilarity between the body tissues sampled and the original Aroclor 1254 used to dose the broodstock fish in both the 2004 and 2005 experiments. Data are given as arcsin proportions of the ΣPCB (21 congeners) identified ± 95% CI. Arrows indicate the general increase or decrease in congener proportions in the second column of each table compared to the first column.

3.9 – Aroclor vs liver tissue

IUPAC Congener no.	Mean proportion in Aroclor 1254		Mean proportion in liver	Cumulative percentage
118	0.121 ± 0.002	↑	0.144 ± 0.010	11.46
95	0.076 ± 0.003	↓	0.056 ± 0.009	21.30
153	0.073 ± 0.003	↑	0.089 ± 0.007	29.84
52	0.051 ± 0.003	↓	0.038 ± 0.007	37.57
138	0.079 ± 0.003	↑	0.094 ± 0.007	45.30
110	0.142 ± 0.002	↑	0.157 ± 0.007	52.76
180	0.013 ± 0.001	↑	0.023 ± 0.003	57.49
92/89	0.032 ± 0.001	↓	0.022 ± 0.003	62.15
151	0.054 ± 0.001	↓	0.045 ± 0.004	66.78
60	0.038 ± 0.001	↓	0.033 ± 0.006	71.26

3.10 – Aroclor vs egg tissue

IUPAC Congener no.	Mean proportion in Aroclor 1254		Mean proportion in eggs	Cumulative percentage
52	0.051 ± 0.003	↑	0.065 ± 0.008	10.15
153	0.073 ± 0.003	↓	0.055 ± 0.005	19.93
110	0.142 ± 0.002	↑	0.155 ± 0.007	28.88
151	0.054 ± 0.001	↓	0.040 ± 0.005	36.10
138	0.079 ± 0.003	↓	0.070 ± 0.006	42.98
60	0.038 ± 0.001	↑	0.049 ± 0.005	49.41
118	0.121 ± 0.002	↓	0.115 ± 0.004	54.79
105	0.041 ± 0.002	↑	0.050 ± 0.004	59.92
95	0.076 ± 0.003	↔	0.076 ± 0.004	64.55
49	0.008 ± 0.001	↑	0.014 ± 0.004	69.09

3.11 – Liver tissue vs egg tissue

IUPAC Congener no.	Mean proportion in liver		Mean proportion in eggs	Cumulative percentage
153	0.089 ± 0.007	↓	0.055 ± 0.005	11.38
118	0.144 ± 0.010	↓	0.115 ± 0.004	21.53
52	0.038 ± 0.007	↑	0.065 ± 0.008	31.44
138	0.094 ± 0.007	↓	0.070 ± 0.006	39.60
95	0.056 ± 0.009	↑	0.076 ± 0.004	46.97
60	0.033 ± 0.006	↑	0.049 ± 0.005	52.92
110	0.157 ± 0.007	↓	0.155 ± 0.007	58.13
180	0.023 ± 0.003	↓	0.008 ± 0.002	62.98
44	0.008 ± 0.007	↑	0.020 ± 0.003	66.98
151	0.045 ± 0.004	↓	0.040 ± 0.005	70.78

Table 3.12 Results of paired t-tests (assuming unequal variances) on congener proportions between different sample matrices from the preliminary experiment (section 3.3.1). The Bonferroni correction for multiple comparisons was applied to the significance level ($p = 0.01$). n.s.d. = no significant difference.

Congener	Comparison	T-value	d.f.	*p*	Conclusion
52	Muscle vs Liver	2.61	7	0.035	n.s.d.
52	Muscle vs Aroclor	2.14	5	0.085	n.s.d.
52	Liver vs Aroclor	5.75	12	<0.001	Lower proportion in liver
52	Liver vs Faeces	1.16	2	0.367	n.s.d.
52	Faeces vs Aroclor	1.65	2	0.241	n.s.d.
101	Muscle vs Liver	0.46	8	0.655	n.s.d.
101	Muscle vs Aroclor	1.71	3	0.185	n.s.d.
101	Liver vs Aroclor	2.27	7	0.058	n.s.d.
101	Liver vs Faeces	2.59	8	0.032	n.s.d.
101	Faeces vs Aroclor	1.24	2	0.339	n.s.d.
118	Muscle vs Liver	0.88	6	0.411	n.s.d.
118	Muscle vs Aroclor	0.24	3	0.823	n.s.d.
118	Liver vs Aroclor	1.78	8	0.113	n.s.d.
118	Liver vs Faeces	0.61	2	0.606	n.s.d.
118	Faeces vs Aroclor	1.22	2	0.346	n.s.d.
153	Muscle vs Liver	0.83	4	0.452	n.s.d.
153	Muscle vs Aroclor	0.94	3	0.417	n.s.d.
153	Liver vs Aroclor	3.63	8	0.007	Higher proportion in liver
153	Liver vs Faeces	1.65	4	0.174	n.s.d.
153	Faeces vs Aroclor	0.97	2	0.433	n.s.d.
138	Muscle vs Liver	0.56	3	0.612	n.s.d.
138	Muscle vs Aroclor	1.06	3	0.368	n.s.d.
138	Liver vs Aroclor	1.37	8	0.207	n.s.d.
138	Liver vs Faeces	4.84	4	0.008	n.s.d.
138	Faeces vs Aroclor	6.84	2	0.021	Higher proportion in faeces
180	Muscle vs Liver	0.15	5	0.890	n.s.d.
180	Muscle vs Aroclor	0.56	3	0.614	n.s.d.
180	Liver vs Aroclor	1.13	8	0.290	n.s.d.
180	Liver vs Faeces	0.68	7	0.520	n.s.d.
180	Faeces vs Aroclor	1.29	10	0.225	n.s.d.
60	Muscle vs Liver	0.02	5	0.983	n.s.d.
60	Muscle vs Aroclor	1.20	5	0.284	n.s.d.
60	Liver vs Aroclor	1.57	15	0.138	n.s.d.
60	Liver vs Faeces	1.34	2	0.313	n.s.d.
60	Faeces vs Aroclor	0.74	2	0.537	n.s.d.
110	Muscle vs Liver	1.83	7	0.109	n.s.d.
110	Muscle vs Aroclor	8.83	11	<0.001	Higher proportion in muscle
110	Liver vs Aroclor	3.56	7	0.009	Higher proportion in liver
110	Liver vs Faeces	3.04	7	0.019	n.s.d.
110	Faeces vs Aroclor	0.34	2	0.763	n.s.d.
151	Muscle vs Liver	0.97	3	0.382	n.s.d.
151	Muscle vs Aroclor	2.02	3	0.137	n.s.d.
151	Liver vs Aroclor	3.06	11	0.011	n.s.d.
151	Liver vs Faeces	2.51	5	0.054	n.s.d.
151	Faeces vs Aroclor	0.12	3	0.914	n.s.d.

Table 3.13 Results of paired t-tests on congener proportions between different sample matrices from the preliminary experiment (section 3.3.2). The Bonferroni correction for multiple comparisons was applied to the significance level ($p = 0.016$). n.s.d. = no significant difference.

Congener	Comparison	T-value	d.f.	*p*	Conclusion
52	Eggs vs Liver	5.33	37	<0.001	Higher proportion in eggs
52	Eggs vs Aroclor	3.47	34	0.001	Higher proportion in eggs
52	Liver vs Aroclor	3.37	20	0.003	Lower proportion in liver
101	Eggs vs Liver	1.17	23	0.256	n.s.d.
101	Eggs vs Aroclor	0.66	44	0.511	n.s.d.
101	Liver vs Aroclor	0.82	18	0.421	n.s.d.
118	Eggs vs Liver	5.74	19	<0.001	Higher proportion in liver
118	Eggs vs Aroclor	2.90	37	0.006	Lower proportion in eggs
118	Liver vs Aroclor	4.70	15	<0.001	Higher proportion in liver
153	Eggs vs Liver	8.20	30	<0.001	Higher proportion in liver
153	Eggs vs Aroclor	5.89	42	<0.001	Lower proportion in eggs
153	Liver vs Aroclor	4.41	21	<0.001	Higher proportion in liver
138	Eggs vs Liver	5.55	32	<0.001	Higher proportion in liver
138	Eggs vs Aroclor	2.76	36	0.009	Lower proportion in eggs
138	Liver vs Aroclor	4.34	18	<0.001	Higher proportion in liver
180	Eggs vs Liver	8.23	26	<0.001	Higher proportion in liver
180	Eggs vs Aroclor	4.03	43	<0.001	Lower proportion in eggs
180	Liver vs Aroclor	6.11	19	<0.001	Higher proportion in liver
60	Eggs vs Liver	4.24	33	<0.001	Higher proportion in eggs
60	Eggs vs Aroclor	4.24	29	<0.001	Higher proportion in eggs
60	Liver vs Aroclor	1.69	15	0.111	n.s.d.
110	Eggs vs Liver	0.29	36	0.772	n.s.d.
110	Eggs vs Aroclor	4.09	27	<0.001	Higher proportion in eggs
110	Liver vs Aroclor	4.55	15	<0.001	Higher proportion in liver
151	Eggs vs Liver	1.64	37	0.109	n.s.d.
151	Eggs vs Aroclor	6.08	28	<0.001	Lower proportion in eggs
151	Liver vs Aroclor	4.19	16	0.001	Lower proportion in liver

results giving power values <0.4 and indicating that larger number of liver samples were likely required in these cases. Power values for all the significant calculations were >0.9, except three that were >0.74 and demonstrated that sufficient sample sizes had been obtained.

The PCA analysis performed using the SIMCA-P software showed a clear association of the more chlorinated congeners (CBs 118, 138, 153, 170 and 180) with the liver tissue analysed situated to the left of the plot (Figs. 3.5 – 3.6). Contribution plots using fish no. 318 as an example, illustrated the different congener profiles in the different sample types analysed. Fig. 3.7 shows greater proportions of the more highly chlorinated congeners and lower proportions of the less chlorinated congeners in Aroclor 1254 compared to the eggs from a high dosed fish in 2004. Many of the highly chlorinated congeners had a lower proportion in the original Aroclor 1254 mixture when compared to the liver of the same fish (Fig. 3.8). When comparing the egg congener profile to that of the liver from the same female (no. 318) it was clear that the eggs contained higher proportions of the lower chlorinated biphenyls whilst greater proportions of more highly chlorinated congeners were present in the liver (Figs. 3.9). These differences in egg and liver congener profiles were not unique to individual fish and were so consistent that they could be observed even when comparing egg and liver samples from different females (Fig. 3.10).

3.4.5 Reproduction

A significant negative correlation ($r = -0.404$, $p = 0.003$, $n = 54$) existed between egg dry weight and egg PCB concentration (Fig. 3.11) but a positive relationship existed between egg weight and maternal condition factor ($r = 0.548$, $p < 0.001$, $n = 54$) (Fig. 3.12). It should be noted in Fig. 3.11 that a greater number of data points exist at low or zero PCB concentrations compared to the high concentrations and are might be responsible for the high degree of significance obtained. Given the significant correlation of both egg PCB content and maternal condition factor, the data was subjected to ANCOVA with maternal condition factor as the covariate and the treatment groups were based on the egg PCB concentrations (0, 0-1, 1-10, >10 mg kg^{-1} eggs).

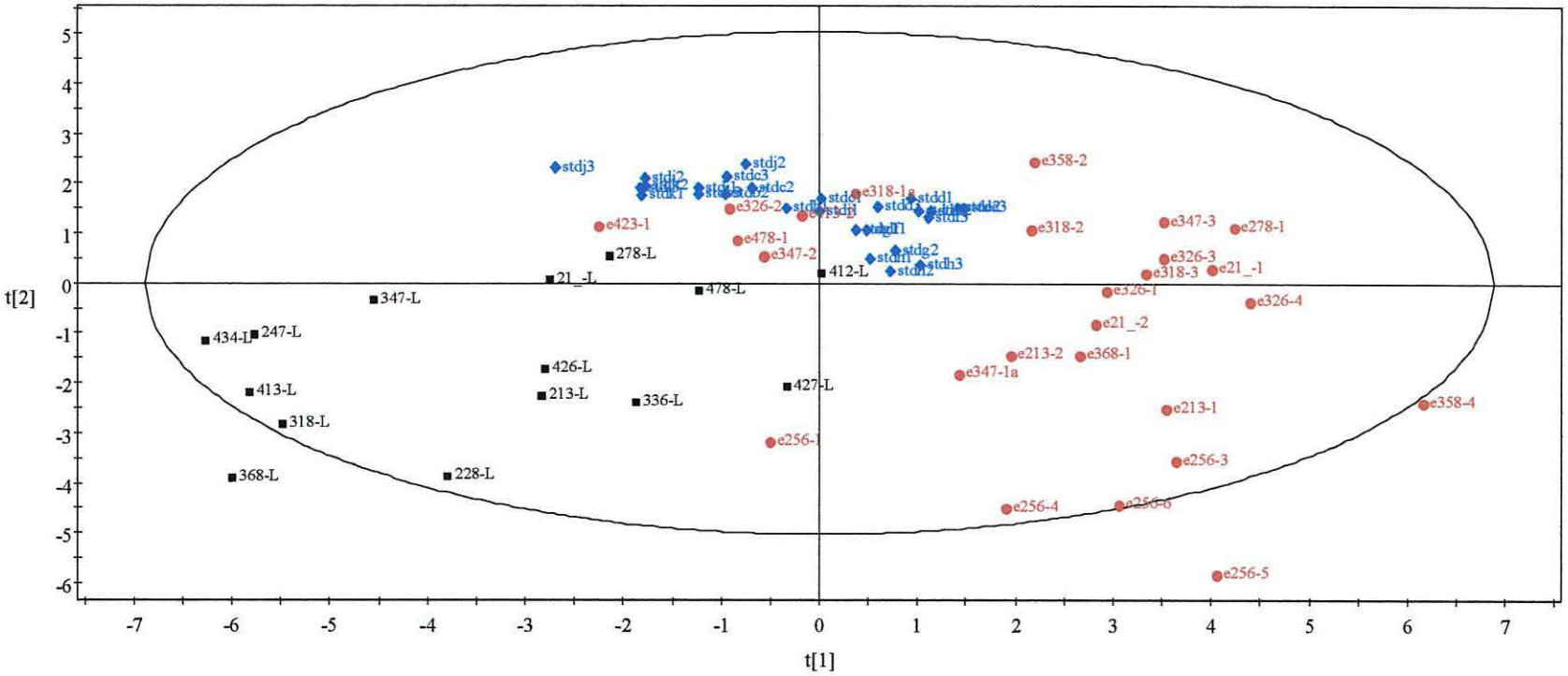


Figure 3.5 Principal components analysis of polychlorinated biphenyl congener proportions in egg (red) and liver (black) samples from female plaice dosed with Aroclor 1254 (blue).

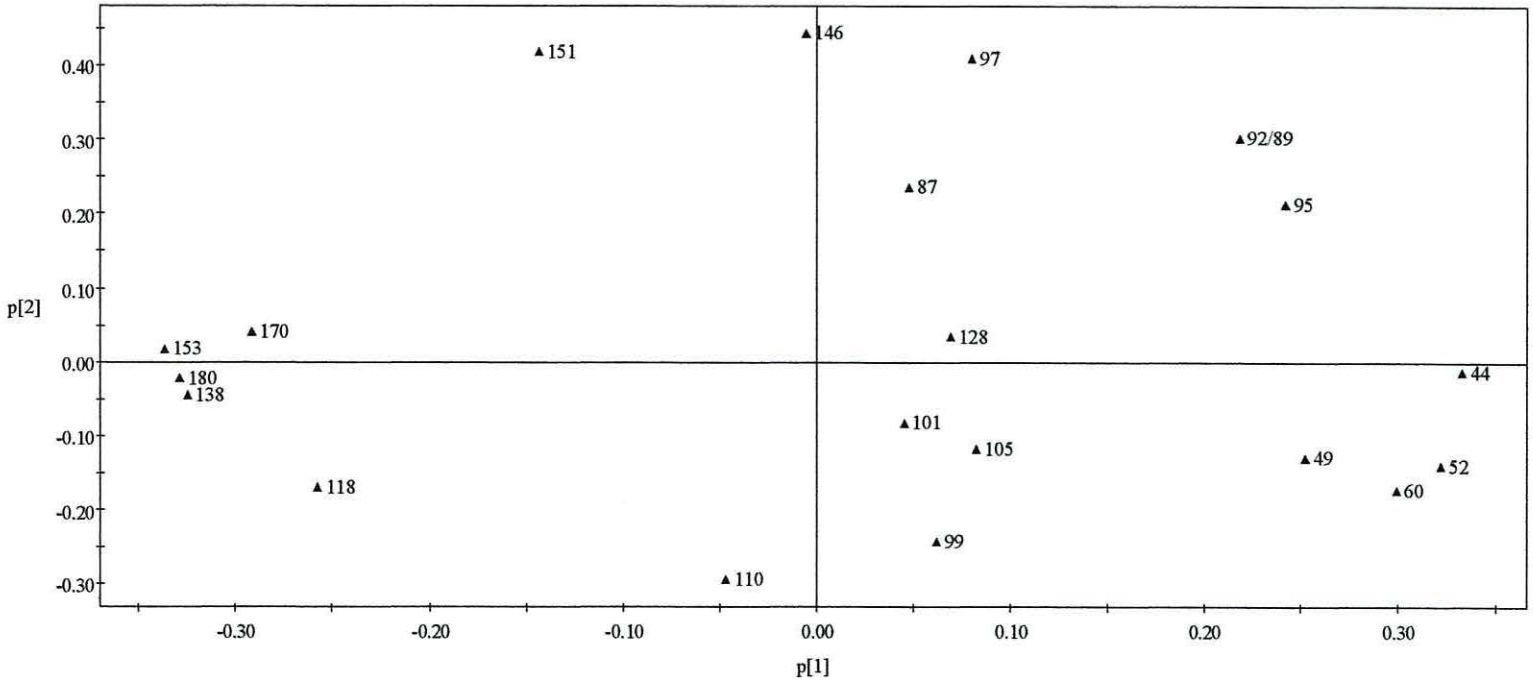


Figure 3.6 Principal component axes 1 and 2 of the polychlorinated biphenyl congener distribution among egg, liver and Aroclor 1254 samples. R^2 values indicated that axis p1 explains 37.8% of the variation and appears to be related to congener size with more highly chlorinated congeners to the left side of the plot. Axis p2 explains 20.2% of the variation.

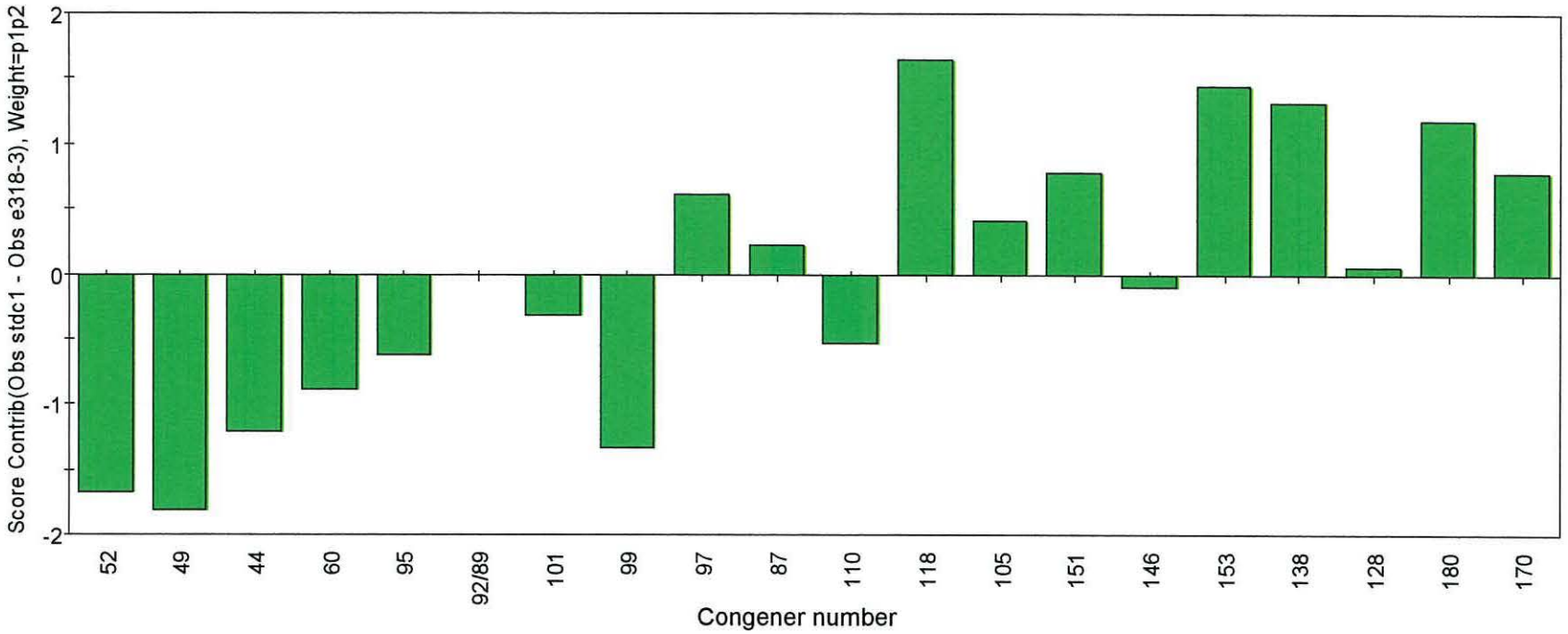


Figure 3.7 Contributions plot comparing the Aroclor 1254 standard to eggs from female 318. Bars below the *x*-axis denote higher proportions in the eggs while bars above the axis denote higher proportions in the Aroclor 1254.

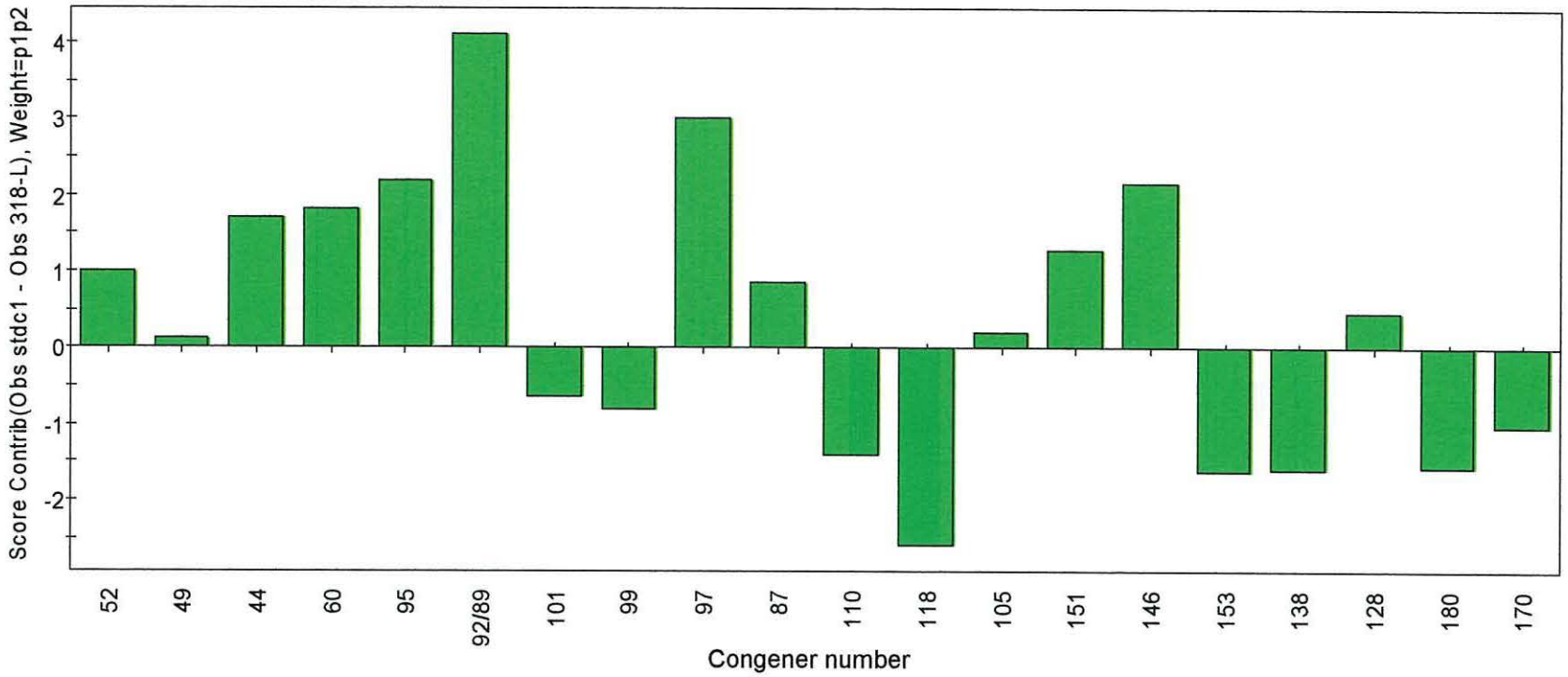


Figure 3.8 Contributions plot comparing the Aroclor 1254 standard to the liver from female 318. Bars below the *x*-axis denote higher proportions in the liver while bars above the axis denote higher proportions in the Aroclor 1254.

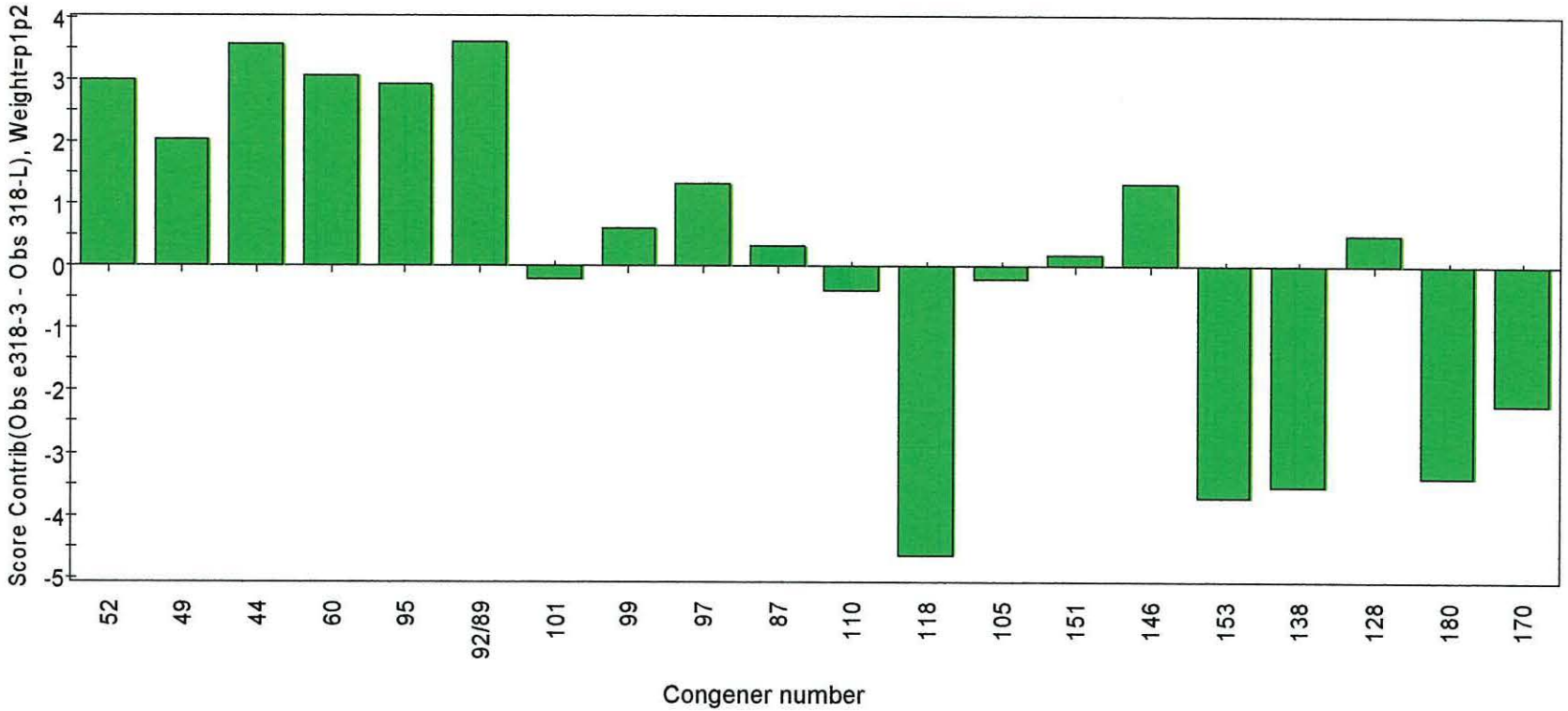


Figure 3.9 Contributions plot comparing the liver with the eggs from female no. 318. Bars below the *x*-axis denote higher proportions in the liver while bars above the axis denote higher proportions in the eggs.

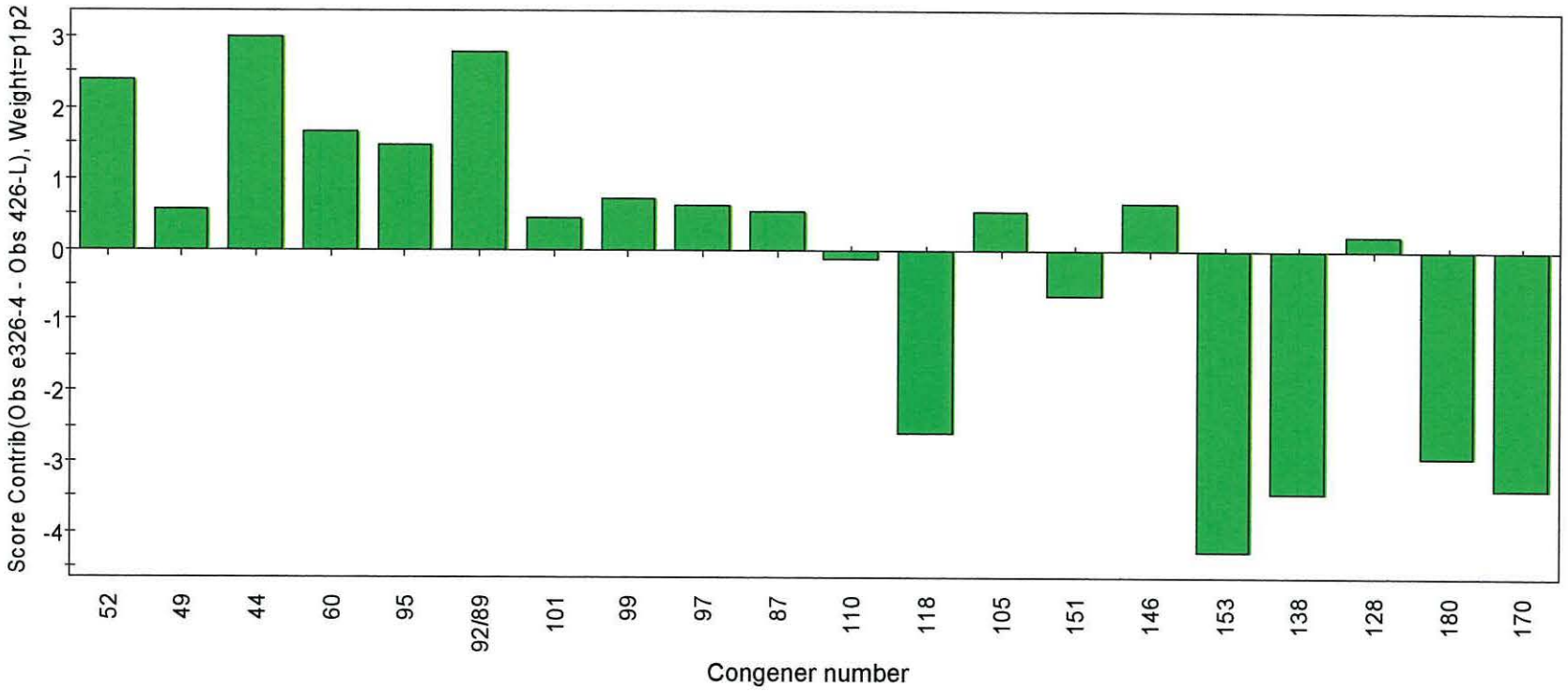


Figure 3.10 Contributions plot comparing the eggs from female no. 326 to the liver from female no. 426. Bars below the *x*-axis denote higher proportions in the liver while bars above the axis denote higher proportions in the eggs.

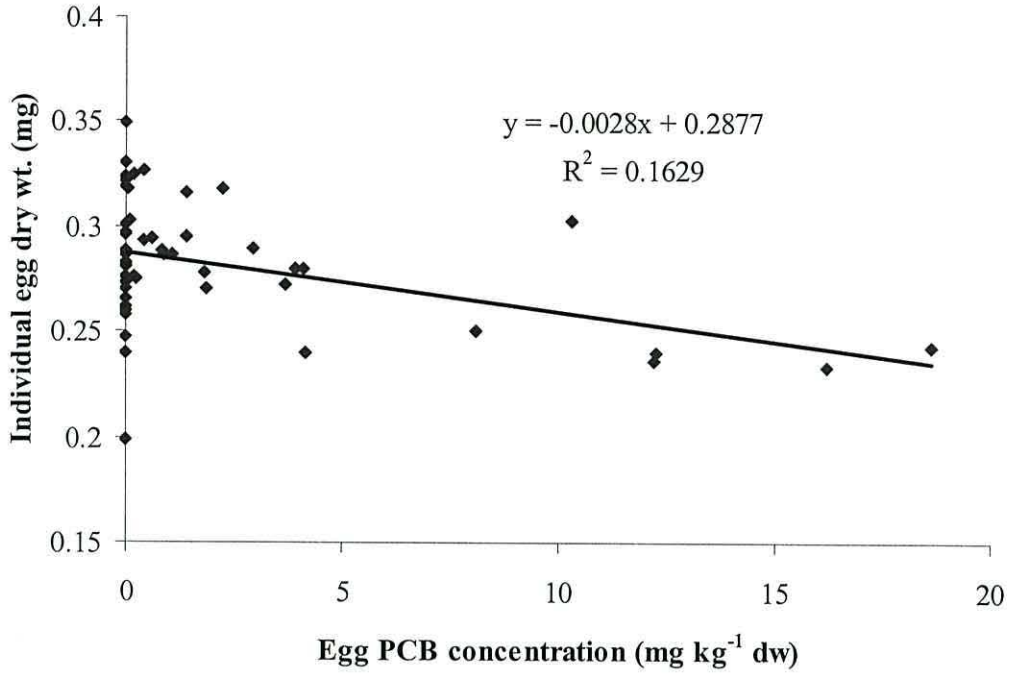


Figure 3.11 The relationship between individual egg dry weight (mg) from female plaice broodstock (either dosed orally with Aroclor 1254 or not dosed) and the concentration of ΣPCB(21 congeners) (mg kg^{-1} eggs dry wt) detected in the eggs from the dosed fish. The trend line shows the significant negative correlation between egg dry weight and egg PCB concentration (r = -0.404, p = 0.003, n = 54) and is described by the equation $y = -0.0028x + 0.2877$.

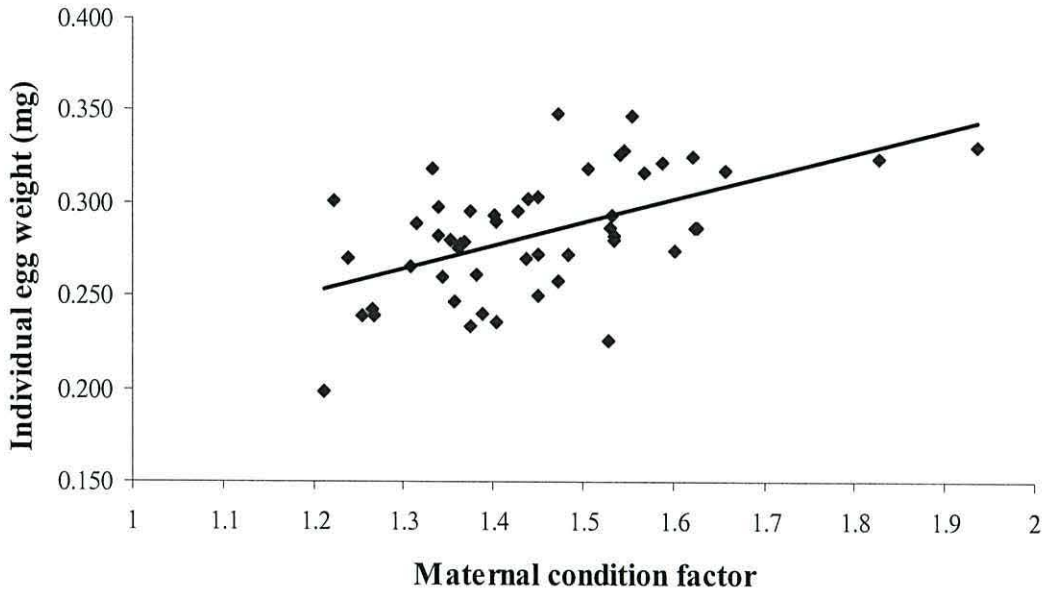


Figure 3.12 The relationship between individual egg dry weight (mg) from female plaice broodstock (either dosed orally with Aroclor 1254 or not dosed) and the maternal condition factor (Condition factor = 100 x (weight/length^3)). The trend line shows the significant positive correlation between egg dry weight and maternal condition factor (r = 0.548, p < 0.001, n = 54) and is described by the equation $y = 0.1238x + 0.104$.

The egg weight data met the requirements of the ANCOVA (Anderson-Darling, A = 0.229, $p = 0.798$; Bartlett's, T = 1.029, $p = 0.794$). Table 3.14 gives the results of the ANCOVA on egg dry weight. It demonstrates that is the present study the PCB concentrations did not have a significant effect on egg dry weight ($p = 0.510$) whereas the maternal condition factor was likely to have a significant effect ($p = 0.025$). No interaction existed between the PCB treatments and the maternal condition factor ($p = 0.592$) meaning maternal condition factor had a similar influence over egg weight across treatments.

The number of eggs per gram female per spawn was estimated each time a fish was stripped of eggs. Following chemical analysis of the spawns it was possible to analyse data based on the four PCB concentration groups (0, 0-1, 1-10, >10 mg kg^{-1} eggs, as described above) for the spawns analysed and test for differences in female fecundity (Fig. 3.13). There appeared to be a decrease in mean egg production at low PCB concentrations followed by an increase but the differences were not significant. Data did not meet the assumptions of ANOVA (Anderson-Darling test for normality, $p < 0.05$; Bartlett's homogeneity of variance, $p < 0.05$) so the non-parametric Kruskal-Wallis test was used. Consequently, the influence of maternal condition factor could not be investigated as above, however, no significant differences existed in the median number of eggs produced per gram female body weight across the PCB concentration groups (H = 7.01, df = 3, $p = 0.072$, n = 54) indicating a trend, but no significant effects of Aroclor 1254 on female egg production. Finally the product of the number of eggs produced per gram female body weight and the dry egg weights for each individual spawn were compared based on the four PCB concentration groups (0, 0-1, 1-10, >10 mg kg^{-1} eggs, as described above) using the Kruskal-Wallis test but no significant differences were detected (H = 0.52, df = 3, $p = 0.915$, n = 54). However, the product of the number of eggs produced per gram female body weight and egg dry weight was a subjective parameter for measurement when collecting eggs by hand-stripping because a conscious decision was made to spawn the females rather than allowing them to spawn naturally, thus making it difficult to draw too many conclusions regarding egg production. Nevertheless, the pattern of Fig. 3.13 hinted at the production of fewer eggs from the females with the lowest PCB concentrations.

Table 3.14 Analysis of covariance applied to the egg weight data across experiments in 2004 and 2005 using maternal condition factor (MCF) as the covariate and PCB concentration class as the treatment levels.

Source	df	Seq SS	Adj SS	Adj MS	F	*p*
PCB conc. class	3	0.0134347	0.0016685	0.0005562	0.78	0.510
MCF	1	0.0083793	0.0038566	0.0038566	5.42	0.025
PCB conc. class * MCF	3	0.0013691	0.0013691	0.0004564	0.64	0.592
Error	43	0.0305732	0.0305732	0.0007110		
Total	50	0.0537564				
Term	Coef	SE Coef	T	*p*		
Constant	0.09072	0.08044	1.13	0.266		
MCF	0.13366	0.05739	2.33	0.025		
PCB conc. class * MCF	-0.00650		-0.10	0.920		

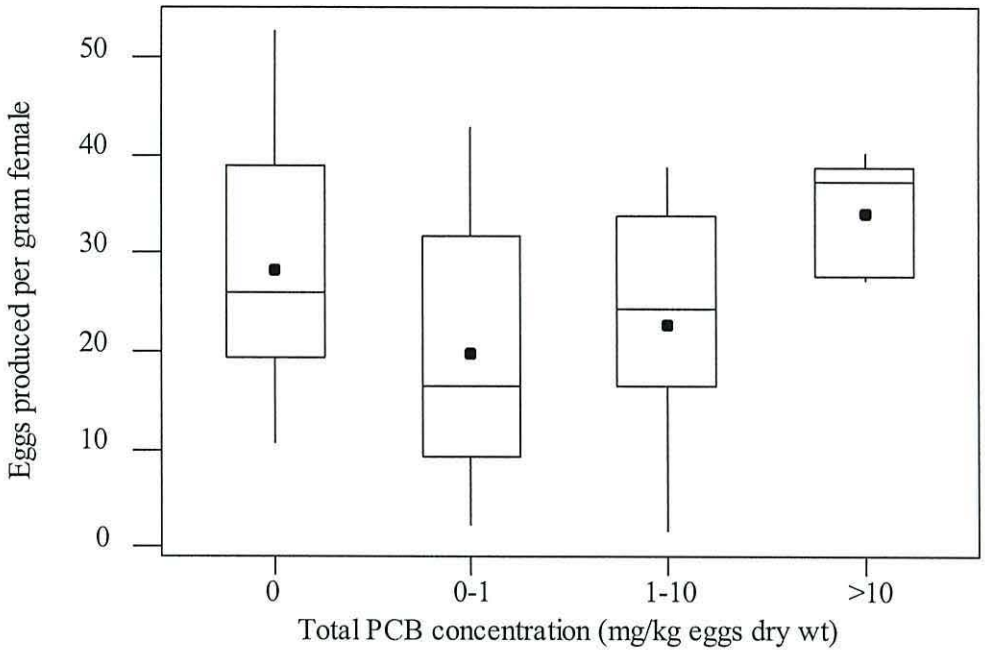


Figure 3.13 Boxplot showing the eggs produced per gram female from each spawn from female plaice (either dosed with the commercial PCB mixture Aroclor 1254 or control, undosed fish) with increasing PCB concentration (mg kg^{-1} eggs dry wt). 0, n = 24; 0-1, n = 12; 1-10, n = 13; >10, n = 5. The boxes represent the median and the interquartile ranges and the means are shown with black dots.

3.5 Discussion

3.5.1 Quality control

Quality control is important in chemical analyses in order to provide confidence in the concentrations reported. One method of quality control is to run at least one sample in duplicate per sample batch to monitor variation in the sensitivity of the analytical equipment. As stated previously, in this study three replicates of the Aroclor standard were run with each sample batch and could be monitored for such variation.

The coefficients of variation for the ICES 6 congeners in the standards run with these samples (Table 3.1) are comparable to or better than those reported as acceptable by de Boer et al. (2001) for variation in PBDE congener concentrations in their inter-laboratory study. Accuracy of ±20% CV is regarded as typical for analysis of standard solutions in GC/MS (Hites, 1997) so the CVs reported here fall well within this expectation within each sample batch and only just exceed it in some cases when each set of analyses are examined together (Table 3.1). Although there are only two or three analyses of Aroclor 1254 per sample batch, the statistics provide a view of the consistency achieved in this study of obtaining results well within the same order of magnitude within each sample run. The CVs for the vast majority of the ICES 6 congeners were well below 20% within each batch and below 30% overall for each set of analyses completed. These CVs are in the same range reported as "very good" by de Boer et al. (2001) for PBDE congeners. CB-52 consistently had the higher CV in comparison to the other congeners though the reasons for this are unclear.

The two main types of quality control that exist in chemical analyses such as those carried out here are qualitative and quantitative. The former was achieved in a relatively simple manner by running an Aroclor 1254 standard at the start, middle and end of each sample batch and using the retention times of the congener peaks identified on those chromatograms to provide accurate identification of congener peaks in both the environmental and laboratory samples. The quantitative quality control was a more technical procedure and required the calculation of two values for every congener identified in each sample batch. These two values were the limit of

detection (LD) and the practical quantification limit (PQL) and are regarded by IUPAC as fundamental performance characteristics to be used in all chemical measurement procedures. Terminology in chemical analyses regarding detection limits has not always been adopted universally and often requires clarification (Currie, 1999). Therefore, in this study, the LD applies to the minimum detectable value of each PCB congener analysed whilst PQL refers to the minimum quantifiable value for which there can be confidence in the reported values.

Various methods have been described for calculating the LD for any given compound in an analysis and are based on the analysis of the blank samples run alongside the experimental samples. Currie (1999) describes the LD as:

$$\mathrm{LD} = 2t_{1-\alpha,\upsilon}\sigma_0$$

calculated using the non-central-t distribution at $\alpha = 0.05$ with υ degrees of freedom and multiplying by the standard deviation of the concentration measured (σ_0). However, this relies on a large number of blanks being analysed (n > 7) (Rong, 2002) in order to obtain a high degree of freedom and lower the t-value used for the calculation. In this study, only three blanks were run with every ten samples and three Aroclor 1254 standards before the GC/MS required cleaning and re-tuning. It has also already been stated that in this study there was a problem with carry-over of the Aroclor 1254 standards within the GC/MS equipment through to the subsequent samples, including the blanks (see sections 2.4.1, 2.5 and Fig. 2.5). It was possible to confirm that the contamination was occurring here and not during any stages of sample preparation because OCN was also detected in the blank samples that were prepared by adding stock *n*-hexane directly to the autosampler vials. Therefore, given the high and variable congener peak values in the blanks (see section 2.4.1) coupled with the high t-values that would need to be employed, use of the suggested equation above produced unreasonably high LDs. This put an excessive burden on the measurement process capabilities, increasing the probability of Type II errors, when in reality it was clear that the PCBs were present in high quantities in many of the samples.

The LD was calculated for each congener in each sample batch using the following equation

$$LD = \text{Mean concentration in blank} + 3\sigma$$

This produced LD values that were on average approximately double the highest concentration detected in the blank samples that had been carried over from the Aroclor 1254 standard and approximately four times the standard deviation of the blanks. For example, for the data from the preliminary experiment in 2003, for CB-153 in sample batch "G" the highest concentration detected in the blank was 17.2μg l^{-1}, mean = 9.5μg l^{-1}, SD = 6.7 and the LD = 29.6μg l^{-1}. If the original equation had been used the LD would have been 57.9μg l^{-1}. Again, it should be stated that although the concentrations are expressed as μg l^{-1} the actual quantities detected were on a ng ml^{-1} basis and the former is used only to allow easy comparison to values in other studies. Although the latter LD value is twice that calculated using the mean + 3σ, our value is still very conservative considering that subsequent Aroclor 1254 carry-over from the blank to the samples being analysed was either less than or equal to the maximum quantity carried over from the standard to the blank when examining data for control laboratory samples. Although only three analyses of the Aroclor 1254 standard were completed per sample batch, the individual congener concentrations met requirements of a normal distribution (Anderson-Darling, $p > 0.05$). In a perfect normal distribution, mean $\pm 3\sigma$ should contain 99.73% of the observations (Dytham, 1999). Therefore, this method of calculating the LD should give great confidence in reducing the possibility of reporting a compound as present when in fact it is not (Type I errors). At the same time however, even with this method of calculating the LDs, the Aroclor carry-over has increased the magnitude of the LD to such a degree that the detection of very low concentrations (ca. <100-200μg kg^{-1}) was difficult and potentially increased the likelihood of Type II errors (reporting a compound as absent when it is present). The carry-over of the Aroclor 1254 standard placed this out of our control. Fig. 3.1 shows the latter point perfectly whereby PCBs are likely to be present in samples e423-2 and e424-2 (the fish were dosed) but the concentrations fall below the LD. When positive values are obtained <LD those concentrations are still reported so as to avoid unnecessary loss of data.

The PQL can be defined as:

$$PQL = k_Q \sigma_Q$$

Where: σ_Q is the standard deviation at that point and k_Q is the multiplier for which the IUPAC default value is 10 although it can be lower if taken as the reciprocal value of the relative standard deviation (CV) (Currie, 1999). If the highly conservative default value were employed in this study with the high degree of carry-over of Aroclor 1254, the PQL values would exceed many of the concentrations reported, even for the laboratory studies. Even if k_Q were calculated as 1/CV then the PQLs in this study would still be 3-10 times the LD. Rong (2002) states that the PQL can be defined as two to ten times the value of the LD depending on the skill of the analyst, quality of the instrumentation used and the sample objectives. In this study, the lower value of 2 was used given that the procedural blank showed no evidence of PCB contamination from the sample preparation techniques and apparatus and because the LDs were already so high compared to other studies (see section 2.5). In addition, because the laboratory samples were known to contain PCBs there is confidence in the detection of the congeners in these samples and to describe those congeners as present in "trace" quantities would be a misconception given the high doses used. The concentrations in the environmental samples were so low that even using the lower value of 2 x LD to calculate the PQLs, the majority of concentrations reported here still fall into the classification of "trace" contaminants as they would if a value of 10 x LD were used. Figure 3.1 demonstrates the validity of the methods used to calculate the LDs and PQLs whereby livers from undosed control fish and eggs from those given a low dose of Aroclor 1254 are regarded as "not detected" whereas livers from fish given high doses of Aroclor 1254 exhibit valid concentrations.

Although concentrations reported for the laboratory experiments may fall below the PQL or LD they remain reported in order to avoid unnecessary loss of information that can still be used to give an estimate and degree of certainty of concentrations where appropriate (Currie, 1999). Similarly, it should be noted that samples with

PCB concentrations reported as "not detected" do not necessarily contain "zero" PCBs, simply that with the methods used, none could be detected with confidence.

Although one control liver sample in Fig. 3.1 is close to the LD, the control samples are reported as "not detected" because some common sense is required to interpret these results. Egg and liver samples from undosed, control fish could not be expected to show such perfect matches to the same Aroclor 1254 standard used in this study that were also detected in the blank samples (see Fig. 2.5) unless the fish had been exposed, which they were not. Concentrations <LD are reported for those observed in the samples from dosed fish purely to avoid too much loss of information and Type II errors as suggested by Currie (1999). Most data from these fish falls into the category of "trace" meaning confidence can be taken in its presence. To report the occasional and randomly fluctuating concentrations <LD for the control fish would simply be misleading and lead to increased Type I errors. To give a further example we can take the data for the control fish number 146. In this fish, the liver concentration of CB-153 detected (0.005 $\mu g\ ml^{-1}$) was half the LD (0.01$\mu g\ ml^{-1}$). In the first spawn from this fish, following subtraction of the mean blank value for CB-153 a negative result was obtained (-0.024) yet there was obvious carry over of Aroclor 1254 to the third spawn where the concentration of CB-153 detected was 0.124$\mu g\ ml^{-1}$, which even exceeded the PQL for that particular sample batch. To suggest these PCBs in the 3rd spawn had actually been derived from that female is simply wrong and the GC equipment is deemed responsible. The application of this "common-sense" approach to the control fish samples is the only time it is applied to the chemical data. The field data in chapter 2 do represent the actual concentrations determined and where no values are recorded in Table 2.6 it is the case that no congener peaks were observed.

The internal-matrix OCN spikes were designed to assess recovery rates of OCN from the samples. Comparison to the OCN in the external Aroclor/OCN standards indicated highly variable recovery rates even within the same sample batches and from the same tissue types i.e. muscle (mean = 36.4%, SD = 25.1%, n = 54, range = 5.2 – 102.2%) or liver tissue (mean = 93.6%, SD = 78.3%, n = 31, range = 6.7 – 315.9%). The same extraction methods were used for every sample analysed, the results from laboratory fish were consistent with their dosing regime, even across different years and the hexane:acetone solvent and soxhlet equipment used are

regarded as having good extraction capabilities (de Boer et al., 2001). Therefore, the use of OCN as a sample-matrix spike in this study is not regarded as a good measure of extraction efficiency and further investigations would be required to address the reasons for this be they related to the GC/MS equipment or the type of sample spiked.

Even though this study experienced problems with Aroclor 1254 carry-over from the external standard within the GC/MS equipment between samples, the quality control measures employed and comparison to other studies give confidence to the way in which the data have been reported. The analytical techniques used for the laboratory experiments give satisfactory data regarding the PCB congener concentrations and proportions in the animals used. The methods mentioned above used to determine the detection and quantification of PCB congeners were employed due to the unusually high peaks observed in the blanks, which seemed to be unavoidable with the equipment used. Such high blank values are rare but not entirely unheard of and as such do not invalidate the results reported here. Indeed, other published studies have had high blanks values yet have still been accepted e.g. Mudroch et al. (1989) had blank values up to 45.6μg kg^{-1} yet their sediment concentrations were up to three orders of magnitude higher (as was the case for many laboratory samples in this study) making these blank values acceptable. With regard to the field observations, the data indicate the presence of PCBs in the orders of magnitude and locations that they are to be expected. However, the high LDs and PQLs due to the blank values have prevented confident determination of these very low environmental concentrations and further investigation at all sites sampled would be warranted with detailed analysis being undertaken with alternative laboratory equipment.

3.5.2 PCB concentrations

The results from the preliminary experiment allowed the successful estimation of the doses to administer to the female broodstock for the main experiments. The concentrations detected in the livers in the preliminary experiment (Table 3.2) were an order of magnitude greater than those detected in dab taken from around the UK coast (NMMP, 1998a). The concentrations in muscle were 1-2 orders of magnitude greater than concentrations found in plaice muscle in Liverpool Bay by Leah et al. (1997).

Therefore, by using broodstock that were at least an order of magnitude heavier together with a range of Aroclor 1254 doses, it was possible to obtain a range of PCB body burdens in the broodstock that were environmentally realistic. The PCB concentrations obtained in the dosing experiments varied from fish to fish (Tables 3.2 and 3.3). Variation in contaminant uptake is not unheard of and in the study by Matta et al. (1998), both low- and high-dosed fish accumulated similar quantities of PCBs whilst the medium-dosed fish accumulated least.

Conversion of the broodstock liver concentrations to a wet weight basis was not possible, as the livers were not weighed before freeze-drying took place. Converting from dry weight to wet weight would result in a decrease in the reported concentration. As many of the concentrations already fall within the range of 37 – 1200μg kg^{-1} wet wt reported in CEFAS surveys between 1984 and 1988 (see section 2.5.3 (CEFAS, 1987; CEFAS, 1990), the liver concentrations obtained in the broodstock experiments (Table 3.3) are relevant concentrations to work with even if environmental concentrations may have fallen since those surveys were undertaken. Muscle ΣPCB (21 congeners) concentrations were not measured following the broodstock experiments due to both the time and costs of the analytical processes involved and the fact that most interest was in the eggs and larvae as opposed to the broodstock. The PCB concentrations reported in the plaice eggs in this study (Table 3.3) were also favourable in comparison to the mean concentrations of PCBs in dab eggs of 239μg kg^{-1} dry wt in large fish and 614μg kg^{-1} dry wt in small fish reported by Fonds et al. (1995) using the same dosing techniques with dab, although the fish were only dosed three times compared to the five doses administered in these experiments.

The lowest laboratory concentrations reported here for the eggs and some of the livers (Table 3.3) are in the same order of magnitude as the trace concentrations reported for the juvenile fish sampled from the environment (Table 2.6). Although the tissue types analysed are different, when compared with environmental concentrations reported previously it should be noted again that the concentrations achieved in this study are environmentally realistic. The concentrations of PCBs in the eggs in this study can also be compared to concentrations detected in other fish species sampled from various field locations (Table 3.15). Comparison of Table 3.3 and Table 3.15 shows a

good overlap of the concentrations reported in this study and the relevance of the subsequent results reported in this chapter and chapters 4 and 5.

Table 3.15 A summary of environmental PCB concentrations reported in various species of fish eggs from international locations. Concentrations are expressed as μg kg^{-1} wet wt. (mean ±SD) unless otherwise stated. Ranges of the data are given where available.

Author	Location	Species	Egg PCB concentration
Hogan & Brauhn (1975)	Hatchery reared, USA	Rainbow trout (*Oncorhynchus mykiss*)	995 ±949 n = 6 range = 31 - 2800
Niimi (1983)	Lake Ontario, North America Lake Ontario, North America	Rainbow trout (*Oncorhynchus mykiss*) Smallmouth bass (*Micropterus dolomieui*)	2050 ±700 n = 9 2862 ±1548 n = 8
Monod (1985)	Lake Geneva, Switzerland	Arctic charr (*Salvelinus alpinus*)	211 ±97 n = 18 range = 100 – 310
Hansen et al. (1985)	German Baltic Sea coast	Baltic herring (*Clupea harengus*)	56.6 ±35.2 n = 63 range = 19 – 241
Black et al. (1988)	Narragansett Bay, USA	Winter flounder (*Pseudopleuronectes americanus*)	193 ±10.14 n = 9 and 7084 ±385 n = 8
Fisk & Johnston (1998)	Lake Superior, North America Lake Manitoba, North America	Walleye (*Stizostedion vitreum*) Walleye (*Stizostedion vitreum*)	240 ±24 n = 10 9.2 ±0.83 n = 19

3.5.3 Multivariate analyses

The congener proportions in the different body tissues sampled and highlighted by the Primer analyses (Fig. 3.4 and Table 3.4) can be used to investigate differences in the congener-specific tissue distributions and how different properties of individual PCB congeners influence their transport into the body organs of the plaice used in these experiments. The ANOSIM results indicated significant differences in congener profiles between body tissues in both the preliminary and the broodstock experiments. However, the results of the pairwise comparisons on individual congeners following the SIMPER analysis showed up relatively few significant results from the preliminary experiment. This is likely to be due to the small sample sizes used in that experiment and the low significance level following Bonferroni correction. Indeed, had the Bonferroni correction not been employed a further five comparisons would have been significant at the 5% level (Table 3.12). Indeed, the power analyses applied to the data (section 3.4.4) suggested many more significant results would have been detected had the sample size been at least double what it was. Nevertheless, the data followed a very similar pattern to that from the main broodstock experiment in which clear patterns can be observed (Table 3.4 and 3.13). Table 3.13 shows the general pattern well. In the liver, proportions of the lower chlorinated congeners are generally lower than those in the Aroclor 1254 whilst there is a general increase in the proportions of higher chlorinated congeners. In contrast, the eggs show elevated proportions of tetra-CBs whilst the proportions of higher chlorinated congeners generally show lower proportions than the liver and the original Aroclor 1254 mixture.

CB-52 is one of the tetra-CBs in the Aroclor 1254 mixture and therefore one of the least chlorinated and smallest congeners present. It is therefore not surprising that the proportion of CB-52 in the eggs is elevated in comparison to the liver and Aroclor 1254. CB-52 is likely to be more mobile than higher chlorinated congeners and likely to enter the bloodstream and thus other body organs such as the ovaries more readily.

The only other congeners of those subjected to the pairwise t-test comparisons that showed higher proportions in the eggs than in either the liver or Aroclor 1254 were CB-60 (higher than liver and Aroclor 1254) and CB-110 (higher than Aroclor 1254). CB-60 is a tetra-CB and may be expected to behave in a similar way to CB-52 but CB-110 was the only penta-CB analysed to show an elevated proportion in the eggs compared to Aroclor 1254. These congeners were chosen for analysis because they are both mono-ortho-substituted PCBs, which are amongst the most planar and biologically active forms of PCB with the exception of non-ortho substituted PCBs (see section 1.2.1).

CB-151 is a tri-ortho-substituted CB and the most ortho-substituted and therefore one of the least planar congeners detected in this study. Therefore it was deemed appropriate to carry out t-tests for this congener and compare the results to those for the more planar forms (CBs 60 and 110). The proportion of this in the eggs and liver was significantly less than in Aroclor 1254 indicating that a lower proportion had been absorbed compared to the other congeners. No significant differences existed between the proportions of CB-151 in eggs compared to liver samples. There were no other congeners analysed that exhibited a significantly lower proportion in the liver compared to the Aroclor 1254 and this could be an indication of the way in which congener structure can influence the bioaccumulation and possibly biological activity of specific PCBs.

Congeners 118, 153, 138 and 180 all had significantly higher proportions in the liver than the eggs and the Aroclor 1254 mixture. This result is expected as all these congeners bioaccumulate readily, for which reason they are included in the ICES 7 list of PCBs that should at the very least be included in environmental analyses. The proportions of these four congeners are higher in the liver than the egg samples and again, this may be due to the size of the molecules and increasing lipophilicity making them less likely to be transported away from the fatty tissue of the liver to the ovaries. CB-101 showed no significant differences in proportions between any of the samples analysed. CB-101 has a solubility ($10\mu g\ l^{-1}$) that is at least an order of magnitude higher than those of all the remaining congeners with a higher IUPAC number analysed in this study (Lang, 1992). Whilst CB-101 is highly bioaccumulative and included in the ICES 7 congeners, this higher solubility might result in a relatively

higher biological mobility compared to other penta- to octa-CBs thus allowing a more uniform deposition across body tissues and/or greater depuration rates from the fish into the surrounding water via excretion/egestion.

Here an attempt has been made to link the physical properties of the PCB congeners to possible reasons for the differences observed in congener proportions between tissue types. Whilst it is feasible that either the more soluble smaller congeners might enter the bloodstream more readily than others and/or the larger, more lipophilic congeners might remain in the liver tissue it should be remembered that the data are proportional. As the proportion of one congener increases or decreases for any particular reason, the proportions of the others must also change in the opposite direction, i.e. as one goes up, the other must come down, an effect known as "closure" (Johansson et al., 1984). Therefore, it is difficult to give a specific mechanism causing these differences in congener profiles as one or more processes may be causing the observed effects. However, by plotting the contribution values from Fig. 3.9 against the log K_{ow} values for each congener (obtained from the Beilstein Chemical Database) that provide an indication of solubility, a significant relationship was detected between this chemical property and the biological partitioning of congeners ($r = -0.759$, $p < 0.001$, $n = 18$) (Fig. 3.14).

CBs-52, 105, 118 and 138 belong to the group of PCB congeners regarded as most toxic due to their partial planarity (see section 1.2.1) (Wilson-Yang et al., 1991). The transfer of PCBs to the ovaries during oogenesis is regarded as an efficient, short-term method of contaminant depuration for female fish (Weis & Weis, 1989). Of these four congeners mentioned here, only the proportion of CB-52 and CB-105 increases in the eggs compared to the liver (0.07 to 0.04 for CB-52 and 0.05 to 0.04 for CB-105) leaving the adult female with a higher proportion of CB-118 and CB-138 in the liver compared to the eggs. From the embryo/larval point of view this is beneficial as the proportion of the most "toxic" congeners needing to be depurated or metabolised in the critical early stages of development is reduced in comparison to those to which the female is exposed.

Niimi and Oliver (1983) examined the biological half-lives of PCBs in rainbow trout. They found that the whole body half-lives of the more highly chlorinated congeners

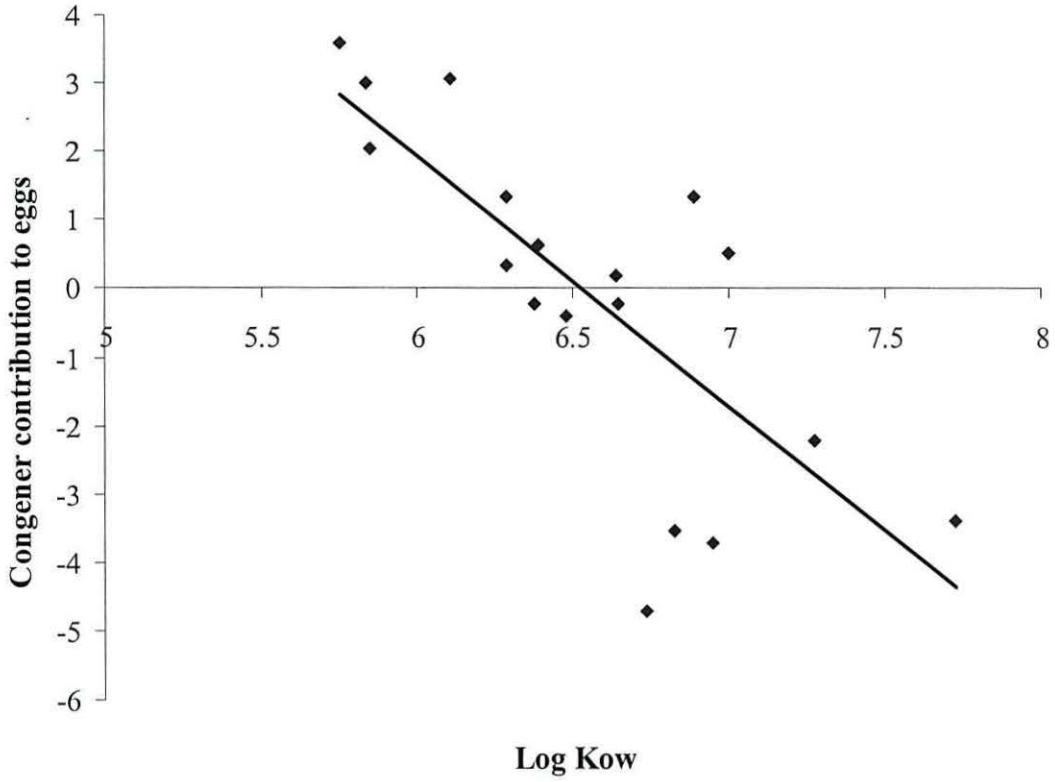


Figure 3.14 Scatter plot of the contribution values from Fig. 3.9 against the log K_{ow} values for each congener (obtained from the Beilstein Chemical Database) for a female plaice (*P.platessa*) dosed orally with Aroclor 1254 at 4.76mg kg^{-1} fish $month^{-1}$. Points above the *x*-axis denote greater PCB congener proportions in the eggs whilst those below denote greater proportions in the liver. A significant relationship was detected between the log K_{ow} values and the contribution values ($r = -0.759$, $p < 0.001$, $n = 18$). The trend line is described by the equation $y = -3.648x + 23.798$.

were greater in comparison to lower chlorinated congeners and they appeared harder to eliminate. This study goes some way to supporting that finding given the patterns of congener distributions observed between the livers and the eggs from the plaice broodstock (Tables 3.9 – 3.11). In the study by Fonds et al. (1995) where dab where exposed to the commercial PCB mixture Clophen A40, multivariate analysis of tissue samples was not completed. However, the mean percentage contribution of congeners detected in eggs in fish from different exposure treatments was listed. In the same way in this study that the lower chlorinated congeners dominated in the eggs, so too did congeners 28, 31, 44 and 52 in the study of Fonds et al. (1995). The way in which the structure of PCB congeners influences their bioaccumulation and subsequent fate inside the host organism is clearly demonstrated in the present study and the work of Niimi & Oliver (1983) and Fonds et al. (1995).

Multivariate analyses have been used in environmental studies to investigate differences in contaminant profiles between individuals and species. Zitko (1989) demonstrated differences in PCB profiles between porpoise body organs using data from Duinker et al. (1988) and demonstrated the simplicity of these methods in displaying clear visual patterns of congener distribution rather than the use of multiple graphs and/or large tables of individual congener concentrations. In contrast, Monosson et al. (2003) examined PCB congener distribution both between field sampling sites and between body organs within sites in mummichogs (*Fundulus heteroclitus*), and reported congener patterns as different between sampling sites but similar between body organs at each sampling site.

Additional studies have examined the congener-specific tissue distribution of PCBs in other animals and humans and can be compared to the results of this study. Kania-Korwell et al. (2005) examined the congener-specific tissue distribution of Aroclor 1254 in rats. As with this study (Tables 3.8-3.10), CBs 118, 138 and 153 all showed an increase in proportion in the rat body tissues compared to the original Aroclor 1254 mixture. Kania-Korwell et al. (2005) also noted that CBs 52, 95 and 110 decreased in proportion compared to the original mixture. This was true for CBs 52 and 95 in the plaice livers in this study but there was an overall increase in the proportion of CB-110 compared to the Aroclor 1254 standard. The same reasons were suggested by Kania-Korwell et al. (2005) for the observed differences in congener behaviour with

regard to the degree of chlorination on the CB molecule, whereby the lower chlorinated congeners can be metabolised and/or eliminated from the animals far more readily than those with a higher degree of chlorination, which the animals may not even be able to metabolise. Gonad tissue was not analysed by Kania-Korwell et al. (2005) in that study so it is not possible to compare whether the same patterns occurred in rat gonads.

DeCaprio et al. (2005) examined the blood serum PCB congener levels in a native North American human population. As with the aforementioned study, proportions of CBs 118, 138 and 153 increased in comparison to the commercial mixture Aroclor 1262, whereas the relative proportions of CBs 95 and 110 decreased. CB-52 was not detected in the serum or the commercial mixture. The results from this study and those by DeCaprio et al. (2005) and Kania-Korwell et al. (2005) highlight the similar ways across different species in which PCB congeners accumulate which, appear to be linked closely to the degree of chlorination and associated physicochemical properties such as solubility. The use of multivariate statistics such as those employed here can provide valuable information in toxicological studies for examining the kinetics of specific congeners in relation to uptake in either field or laboratory situations.

3.5.4 Effects on spawning

The chemical data presented in Table 3.3 demonstrates that spawning is an efficient method of PCB depuration for the female broodstock with PCB concentrations in the eggs often exceeding those in the livers analysed at the end of the spawning period. This has also been reported in wild-caught winter flounder (Johnson et al., 1992), whereby liver PCB concentrations decreased to levels below those detected in the ovaries during the spawning season as PCBs were likely to be transported out of the livers with the production of the egg protein vitellogenin (Ungerer & Thomas, 1996) (see section 1.5.2). However, this method of depuration appeared to have minimal impacts on egg production in this study.

Whilst no significant differences were detected in the numbers of eggs produced per gram body weight by the female broodstock dosed with different amounts of Aroclor 1254 (Fig. 3.13), this result should be treated tentatively given that the fish were stripped of eggs when judged ripe and not allowed to spawn naturally. The pattern in the data in Fig. 3.13 hints at that of a typical dose-response curve observed when investigating endocrine disrupting chemicals (see section 1.6.1) (Colborn et al., 1996; Calabrese & Baldwin, 2003) and further investigations allowing the fish to spawn naturally in individual tanks would confirm whether or not egg production was unaffected. Orn et al. (1998) reported a reduction in egg production of zebrafish dosed with a mixture of 20 PCB congeners though there were no differences in hatching frequency or developmental time. These latter parameters for this study are addressed in the following chapter. Holm et al. (1993) reported no differences in spawn success or in the number of eggs laid per gram female when three-spined sticklebacks were dosed with Clophen A50 resulting in whole body concentrations "several orders of magnitude higher than those found in the Baltic" (Jansson et al., 1993). Westerlund et al. (2000) also found no differences in the number of eggs produced per female zebrafish following dosing with eight individual PCB congeners though tissue concentrations were not measured. DeFoe et al. (1978) examined the effects of Aroclor 1248 and 1260 exposure on the fathead minnow (*Pimephales promelas*). Variation in the data failed to produce any clear trends on the effects on fecundity though significant mortality occurred in both exposures following hatch.

In this study, individual egg weight was influenced significantly by maternal condition factor even though a significant negative correlation was detected between egg dry weight and egg PCB concentration (Table 3.14, Fig. 3.11). This negative correlation could either be a result of the PCB contamination or that of the reduced number of data points at the high end of the PCB concentrations. Further replicate spawns in the high concentration range would assist in clarifying this. However, the influence of the maternal condition factor was greatest in the present study and when accounted for, the ANCOVA revealed no significant differences in egg dry weight in relation to egg PCB content (Table 3.14). Previous studies have shown maternal length and condition to influence egg size making it an important variable to consider in such investigations (Chambers & Waiwood, 1996; Huang et al., 1999; Trippel & Neil, 2004). Fisk and Johnston (1998) investigated the egg dry masses of walleye

eggs in Lake Superior and Lake Manitoba in the Great Lakes region, North America and found that eggs from Lake superior were greater in size than those from Lake Manitoba but showed no correlation with female length or age. Lake Superior contained the higher concentrations of PCBs (Table 3.15). As with any field based data it is difficult to define the exact cause of this as any number of ecological and toxicological variables could play a role.

If a reduction in egg or hatch size were observed in the field, consequences for larvae could make them susceptible to a larger range of predators for longer time periods (Houde, 2002) and a subsequent increase in mortality. Smaller larvae also might not be able to feed on such a wide range of food as larger counterparts putting them at a disadvantage regarding foraging.

3.5.5 Conclusions

The methods used for the chemical analysis and subsequent interpretations of the data have proved satisfactory for the higher concentrations used in the laboratory work reported here. When examining lower concentrations at the level of the low dosed plaice broodstock and the low concentrations detected in the field samples in chapter 2, a measure of caution needs to be applied regarding the precise concentrations reported. Further investigation into the degree of contamination at the low levels and at the concentrations detected at the field sites in chapter 2 would be best carried out after a full review of the issues encountered with the high limits of detection involved.

As PCBs have been shown to compartmentalise in different proportions in different sample matrices, it has been demonstrated that this is also the case within fish body tissues. Though the impacts on female plaice spawning capability seem to be minimal, the subsequent impacts on the larvae must be addressed before conclusions regarding the impacts of contamination can be reached. These issues are addressed in chapter 4.

Chapter 4

Effects of maternal transfer of Aroclor 1254 on survival, growth and behaviour of plaice, *Pleuronectes platessa*, larvae

4.1 Abstract

Aquatic organisms are exposed to a wide range of xenobiotics in their environment, which often includes the polychlorinated biphenyls. Maternal transfer of sub-lethal body burdens to the ovaries during vitellogenesis is one method of depurating contaminants from the body. Whilst this is an efficient method of short-term decontamination for the parent, it might impair the survival, growth and behavioural performance of offspring, with subsequent long-term implications for recruitment and population and community ecology. Eggs from European plaice (*Pleuronectes platessa*) broodstock dosed in the laboratory with the commercial PCB mixture Aroclor 1254 (low, 0.1-0.175mg kg^{-1} fish $month^{-1}$; medium, 1.0-1.75mg kg^{-1} fish $month^{-1}$; high, 2.0-3.5mg kg^{-1} fish $month^{-1}$) were hand-stripped and fertilized. Egg survival through to hatch was unaffected by parental contamination but was significantly reduced post-hatch until first feeding in larvae from eggs with the highest PCB concentrations. Size at hatch was reduced significantly in larvae spawned from medium- and high-dosed female broodstock compared to control larvae but growth rates until metamorphosis did not differ significantly between treatments. Larvae were also assessed for routine swimming activity (n = 30 per spawn) and response to a vibratory stimulus using video recordings. A significant reduction in routine swimming activity within a 30-second time period was observed in medium- and high-dosed larvae at hatch and in low- and high-dosed larvae at first-feeding compared to control larvae. No significant differences were detected in response to the vibratory stimulus between treatments. In the wild, reduced swimming performance and impaired predator avoidance together with a reduction in size could leave a larva susceptible to a wider range of predators for a longer time. Concentrations of PCBs detected in the eggs from the spawns used to rear larvae were at or near to those reported in field-caught fish. Results of this study highlight the potential ecological impacts of such contamination to exposed populations.

4.2 Introduction

The previous chapter demonstrated the efficient means by which depuration of PCBs occurs in female plaice during maturation. This has also been demonstrated in the field in species such as winter flounder (*Pseudopleuronectes americanus*) (Black et al., 1988), Baltic flounder (*Platichthys flesus*) (von Westernhagen et al., 1981), North Sea whiting (von Westernhagen et al., 1989) and Arctic charr (*Salvelinus alpinus*) (Monod, 1985). Depuration occurs during gonadal recrudescence and organochlorines such as PCBs and DDT can be transported to the ovaries with the egg protein vitellogenin (Ungerer & Thomas, 1996). Whilst this is an efficient method of depuration for the adult female fish, there is a risk of transfer of xenobiotics onto the progeny. Early stages of development in all organisms are extremely sensitive to physiological disruption given the complex changes that occur at this time, especially with regard to hormone concentrations (Colborn et al., 1993; Birnbaum, 1995). Disruption or hindrance of development may not always lead directly to obvious effects such as increased mortality but may manifest as more subtle impacts on size, metabolism, the sensory system or behaviour that could have an indirect influence on survival or performance of individuals at the time (Faulk et al., 1999; McCarthy et al., 2003; Alvarez et al., 2006). Modelling approaches that use probabilities of prey capture and/or predator avoidance success indicate that inhibition of these skills might affect future recruitment to the adult population (Rose et al., 2003; Alvarez et al., 2006).

The use of behaviour in toxicological studies is being used with increasing regularity in many species such as fish and birds (Blaxter & Ten Hallers-Tjabbes, 1992; Peakall, 1996; Jones & Reynolds, 1997; Bustnes et al., 2005). Two recent reviews by Clotfelter et al. (2004) and Zala & Penn (2004) outline the diversity of the traits studied including social interactions, reproductive and courtship behaviour, foraging activity and antipredator responses to name a few. Alterations in fundamental behaviours such as foraging or antipredator responses could have considerable repercussions for individuals, populations or communities (Weis et al., 1999; Rose et al., 2003; Clotfelter et al., 2004; Alvarez et al., 2006). Larvae of many fish species have extremely high mortality rates compared to later life stages (Houde, 2002; Jones,

2002), and any changes in behaviour, reduced foraging activity or increased predation risk can have impacts on future populations. Those behaviours that allow successful predator evasion and foraging can be described as larval survival skills (McCarthy et al., 2003).

As larvae grow, so their sensory systems develop and allow them to better detect predators by mechano- or chemoreception and ultimately by sight (Fuiman & Magurran, 1994). The development of these systems and associated organs will be under the complex and tight control of the endocrine system, which is open to disruption by xenobiotics acting as hormone agonists or antagonists (Colborn et al., 1993). A delay in, or disruption of, the development of these systems by compounds such as PCBs, DDT or MeHg can compromise the ability of larval fish to either respond to predator attacks or encounter and detect food in their environment (Faulk et al., 1999; Zhou et al., 2001; McCarthy et al., 2003; Alvarez et al., 2006). Even a reduction in overall body size can result in individuals being susceptible to predation for a longer period of time compared to larger counterparts since mortality is strongly size-dependent (Houde, 2002).

Plaice embryos and larvae undergo major changes early in ontogeny, both physical and physiological (Wimpenny, 1953; Ehrlich et al., 1976; Hodgson & George, 1998). The measurement of only one parameter, be it physical, behavioural or physiological is less likely to provide a conclusive indication of performance under sub-optimal conditions compared to a study based on multiple assessments (Fuiman, 2002). This study aimed to examine the impacts of maternal PCB transfer on the development and successful hatch of plaice eggs and the survival, growth and behaviour of plaice larvae across a range of environmentally realistic PCB concentrations. Measurements of survival relative to control fish will provide an indication of PCB concentrations having lethal effects. Surviving offspring may not necessarily be unaffected by inherited contamination and assessments of growth and behaviour can be used to provide information on changes that occur below lethal PCB concentrations compared to control fish, potentially acting as a more sensitive indication of pollutant-related stress (Little & Finger, 1990; Faulk et al., 1999).

4.3 Methods

The female plaice broodstock dosed with Aroclor 1254 as described in section 3.3.4 were hand-stripped for eggs during the spawning season using the methods described below. Eggs were fertilised with milt from a male plaice selected at random from a pool of males (n = 6) and the larvae from different females and dosing regimes were compared for differences in size at hatch, growth rates and behavioural performance in response to maternal Aroclor 1254 exposure.

4.3.1 Fish reproduction and larval rearing

The methods use to spawn the fish and rear the eggs and larvae were similar to those used by Hiddink (1997) to spawn and rear larval plaice and judged to be appropriate for this study. A sub-sample of eggs (20 to 30g wet wt.) was retained from each female spawn, rinsed in seawater and placed in a 30ml hexane-washed, glass vial and frozen at –20°C prior to analysis of PCB content by GC/MS. Approximately 1g of eggs per spawn were retained and counted within a few minutes of spawning to determine the number of eggs g^{-1} wet wt $spawn^{-1}$. These eggs were then rinsed in filtered seawater over a 0.5mm mesh to remove ovarian fluid, excess water was absorbed using paper towelling underneath the mesh before drying the eggs in an oven at 60°C for 72-hours to determine the egg dry weights (Fisk & Johnston, 1998).

Plaice will spawn naturally in large tanks (Baynes, CEFAS, pers. comm.) but were hand-stripped in this study because it was necessary to know from which females the larvae originated. When females were judged ripe, by visible swelling, they were removed from the holding tanks and anaesthetised lightly in 2-phenoxyethanol (see section 3.3.1 and 3.3.3). Total length and weight were recorded and fish were identified by the Alcian blue markings. Eggs were hand-stripped from the fish and collected in a clean glass beaker and weighed. Male fish were also stripped for sperm, which was added immediately to the eggs. Eggs and sperm were mixed with approximately 100ml seawater and allowed to stand for 20-minutes. They were then transferred to a new 1l beaker with seawater at a salinity of 35-36 psu. At this salinity

healthy eggs will float and dead eggs will sink to the bottom (Hiddink, 1997). Spawning occurred between January and April in both 2004 and 2005.

Healthy eggs were transferred to 8l tanks at 9°C ±1°C situated in a water bath containing seawater to mimic ambient field temperatures in an air-conditioned room. Water entering the tanks came from the same source as that for the broodstock tanks but was also passed through a 5μm filter and drip fed into the tanks. The tanks were treated every other day with 200 International Units of Penicillin (Sigma-Aldrich) and 200mg Streptomycin (Sigma-Aldrich) to guard against bacterial infection. All the eggs from each spawn were maintained in the same rearing tank due to space constraints and this was accounted for by using nested ANOVAs during subsequent statistical analyses, except where Kruskal-Wallis tests were performed. Each day, dead eggs that had sunk to the bottom of the tanks were siphoned out and the water temperature was measured.

At hatch, water flow rates in the tanks were increased and maintained at approximately 4l hr^{-1} until metamorphosis and temperatures were maintained at 9°C ±1°C (measured daily) as it would take too long to grow the fish to metamorphosis at lower temperatures. Antibiotics were added to the tanks for three days following hatch but were not required after this time. Water quality was also monitored (as reported in section 3.3.3) but due to the high flow rates in each tank there was no build-up of nitrates or nitrites in any of the larval tanks at any stage of development. Rearing tanks did not contain any substratum for ease of cleaning but this was unlikely to affect larval survival or success or timing of metamorphosis (Gibson & Batty, 1990). The temperature used in the larval rearing tanks (9 ±1°C).

Plaice larvae were fed on 24-hour old *Artemia salina* nauplii (Vin Châu Gold, College of Fisheries & Aquaculture, Can Tho University, Vietnam) from 6-days post-hatch (dph) until they reached metamorphosis. *Artemia* were hatched from cysts at 30°C in conditions of constant light in 2l round-bottomed flasks at a room temperature above 25°C under vigorous aeration. At 14-dph, larvae were fed *Artemia* enriched with an ICES fish oil mixture (Laboratory of Aquaculture and Artemia Reference Centre, University of Ghent, Belgium). *Artemia* were added to the tanks twice daily at a density of approximately 5000 *Artemia* l^{-1}. Unpredated *Artemia* either died and

collected on the bottom of the tank and were siphoned out daily or passed through the mesh on the outflow pipes.

4.3.2 Larval survival

When the fertilised eggs were transferred to the 8l tanks, replicate sub-samples (n = 3) of 120 eggs from the same spawn were placed into 1l beakers in an adjacent water bath at the same temperature as that of the 8l tanks. Water in these beakers was treated in the same way with antibiotics as outlined above. Each day, dead eggs were removed from each replicate beaker and counted. 50% of the water was changed every other day. For each spawn, hatch date, defined as the day when more than 50% of eggs in each beaker had hatched, was recorded. Egg and larval survival was monitored through hatch until the larvae were 8-days old; the time when the yolk sac is completely absorbed at 9 ±1°C (Wimpenny, 1953). Larval survival in the 1l beakers was not monitored past this stage due to space constraints and Home Office regulations that limited the numbers of procedural animals that could be used each year. The proportion of larvae surviving in each beaker was calculated for each day post-fertilisation and arcsine transformed before statistical analysis.

4.3.3 Larval growth

Within 24-hours of 50% hatch by which time almost all of the eggs had hatched, and every week thereafter until metamorphosis, 20 larvae were removed from each 8l tank and photographed using a Nikon Coolpix 4500 digital camera (Plates I - VII) attached to a light microscope (WILD, M3Z). A graticule slide was used to provide a scale for the pictures, which were then analysed using ImageJ version 1.32j image analysis software. Over 3000 images were analysed, covering the spawns from the 2004 and 2005 experiments. The body length of each larva was measured from the tip of the snout to the end of the notochord in flexion larvae. When notochord flexion occurred between the 3rd and 4th weeks of growth, standard length measurements were taken from the tip of the snout to the posterior hypural margin (Berra & Neira, 2003).

Since a single assessment of length was conducted for each larva it was necessary to check the repeatability (Lessells & Boag, 1987) of the measurements of length to ensure that they were accurate and truly representative of the actual length of the plaice larvae. Repeatability, r, is given by:

$$r = s^2_A/(s^2 + s^2_A)$$

where s^2_A is the among-groups variance component and s^2 is the within-group variance component (Lessells & Boag, 1987). The closer the value of r to 1, the more repeatable the results are. The variance components can be calculated from the mean squares values resulting from the ANOVA as:

$$s^2 = MS_w$$

and

$$s^2_A = (MS_A - MS_w)/n_0$$

where MS_A is the among groups mean squares, MS_w is the within groups mean squares and n_0 is a coefficient related to the sample size per group in the ANOVA; as long as a balanced design is used, $n_0 = n$ (Lessells & Boag, 1987).

Prior to measuring fish from all spawns, one photograph of a newly hatched larva was picked at random from each of 10 different female spawns. These photographs were each measured to the nearest 0.01mm five times over a period of one week in order that the recorder did not remember exactly where the measurements of each fish were taken on each photograph. The photographs measured included larvae that were in both straight and curved positions on the microscope slide to account for different body positions. ANOVA of the results (Table 4.1) provided the data to carry out the test for repeatability.

Table 4.1 Analysis of variance of five repeated measures of the standard lengths of 10 plaice larvae selected at random from 10 different female spawns.

Source of variation	df	Sums of squares	Mean squares	F ratio	*p*
Larva no. (between groups)	9	6.688722	0.743191	6406.822	<0.001
Error (within groups)	40	0.00464	0.000116		
Total	49	6.693362			

The *r*-value was calculated:

$$r = 0.148615/(0.000116 + 0.148615) = 0.999$$

and showed the measurements to be nearly identical. Therefore, it was deemed very acceptable to measure each larva only once.

Differences in larval standard length were investigated at hatch (11-dpf) and metamorphosis (42-dph). Data at 7- and 28-dph were limited due to reduced sampling at these times and therefore no analysis was carried out on standard lengths at these times.

4.3.4 Behaviour

In order to assess the behavioural performance of the larvae, three replicates of 20 larvae from each spawn were assessed for differences in routine swimming behaviour and response to a startle stimulus. These assessments were completed within 24-hours of 100% hatch, 7-days later at first feeding and again at 42-days old at metamorphosis.

A startle stimulus was produced by tapping a remotely controlled metal hammer against a metal post 50mm below its highest point (Fig. 4.1). On contact with the post, an electrical circuit was closed causing a red light emitting diode (LED) to light up out of view of the fish larvae marking the start of the stimulus. The system used was similar to that used by Faulk et al. (1999), McCarthy et al. (2003) and Alvarez et al. (2006). For each assessment of behaviour, a petri dish containing ice was first

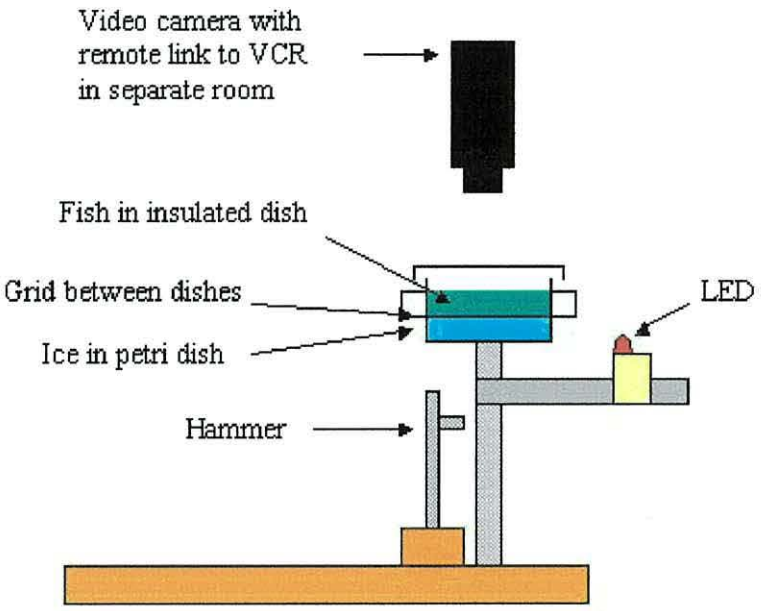


Figure 4.1 Diagram of the apparatus used to deliver a vibratory stimulus and video equipment used to record the routine swimming and startle response behaviour of plaice larvae (not to scale). The hammer was on a pivot and activated by use of a remote electrical switch.

placed on top of the post followed by a piece of white paper with a 20mm grid. Larvae were placed in a petri dish (81mm diameter; approximate volume 50ml; insulated with polystyrene around the sides) on top of the grid and left to acclimatise for five minutes before filming. The combination of ice and polystyrene prevented the water temperature in the dish from changing by no more than 0.5°C above 10°C during the 8-9 minutes that the fish were in the petri dish. These assessments were carried out in a Home Office approved procedures room adjacent to the larval aquarium and cooled by air conditioning units.

Following acclimation, the routine swimming activity of the larvae was filmed for three minutes using a camera positioned above the dish. Recordings were made using a black and white video camera (Geprüffe Sicherheit) connected to a video recorder (JVC S-VHS HR-S5967) housed in a separate room. After three minutes, the hammer was triggered and the larval response recorded. The strike of the hammer against the pole supporting the behavioural arena was designed to send a small vibration through the water to mimic the vibration a larvae might detect as a predator approaches it (Fuiman & Magurran, 1994). In preliminary studies a high-speed NAC400 video camera (loaned by the BBSRC) was used to record larval behaviour but the recording quality was too poor to allow accurate determination of individual larvae and its use was discontinued.

On playback of the videotapes the routine swimming behaviour was assessed by selecting a random 30-second time period within each of the three-minute recording sets (McCarthy et al., 2003) and counting the number of gridlines crossed by a larva during those 30-seconds. Startle response was assessed by counting the number of larvae responding within 10 video frames of illumination of the LED. Each frame of the videotape represented 40 milliseconds (ms). The eyes of larvae showed up clearly against the grid when the videos were played back. Larvae were judged to have responded to the stimulus if their eyes were seen to move away rapidly from the pixels on the television screen at the point of hammer contact (LED illuminated) within 10 video frames (400ms). The number of frames taken for the response to occur was also recorded as a measure of response latency. The merit of this last measurement is discussed further in section 4.5 with respect to the duration of one video frame and the expected larval response time.

4.3.5 Statistical analyses

Minitab version 13.2 was used for most statistical analyses completed in this chapter. Nested ANOVA tests were applied to the data for egg and larval survival, larval lengths and behavioural performances. Nested ANOVAs were used because the larvae originated from females assigned at random to each treatment tank (Latchford & Whitaker pers. comm.). When assumptions of ANOVA were not met (Anderson-Darling normality test, $p > 0.05$; Bartlett's homogeneity of variance test, $p > 0.05$) the non-parametric Kruskal-Wallis test was used as an alternative. ANCOVA was used to test for differences in the growth rates of larvae from different females and treatments. Tukey's Honestly Significant Difference (HSD) was used for pairwise comparisons of treatment groups following significant ANOVA results. SPSS version 12.0 was used to fit the egg and larval survival curves and calculate instantaneous daily mortality rates (Z).

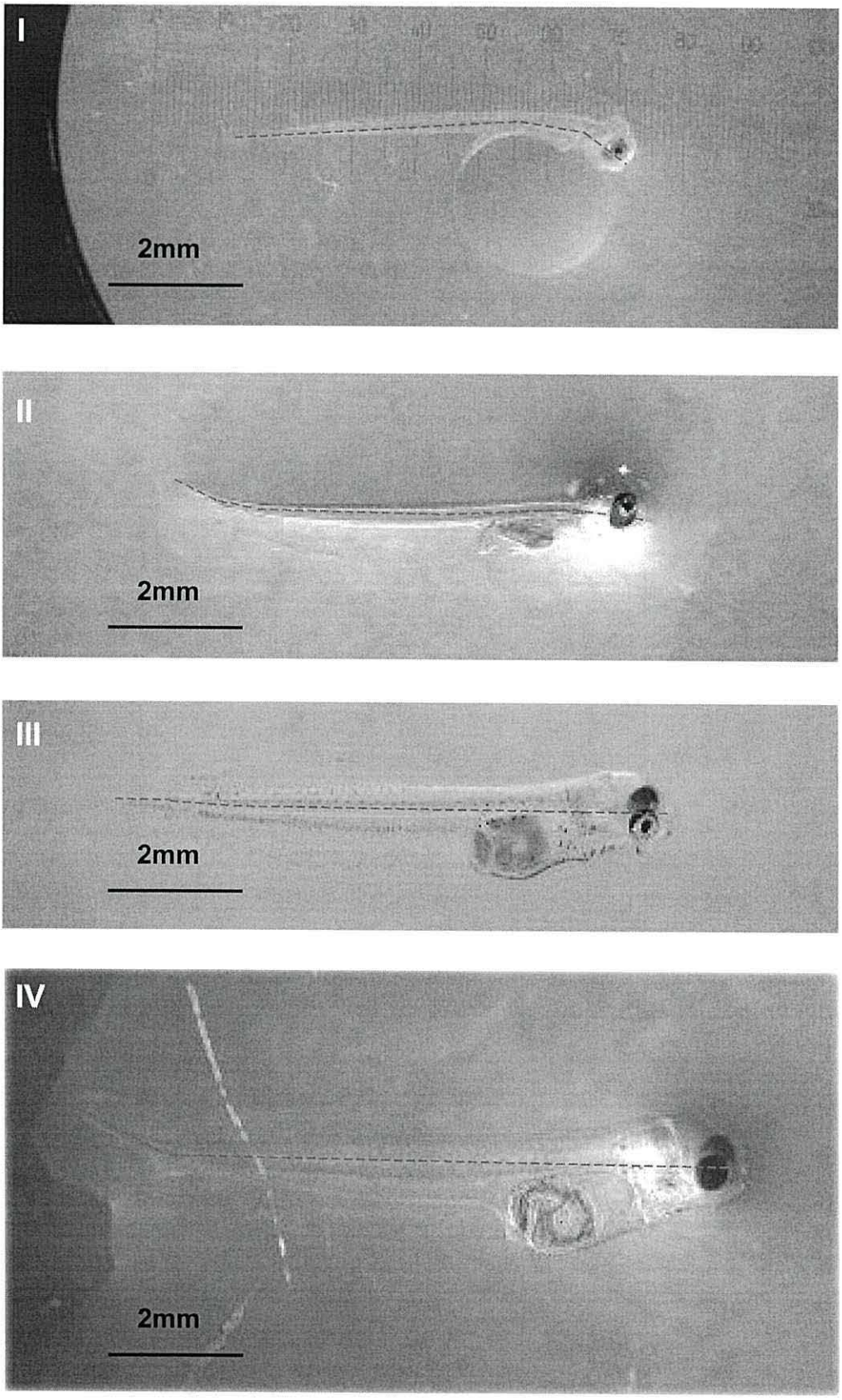
4.4 Results

Across all the spawns from the experiments in both 2004 and 2005, >50% hatch occurred at 11-days post fertilisation (dpf) (Plate I) at temperatures between 9 and 10°C. Larvae began to feed at 6-dph (Plate II) and >50% metamorphosis was complete at 42-dph in all spawns (Plate VII). Larvae from each spawn could not always be assessed for survival, growth and behaviour due to unforeseen circumstances arising such as small spawns restricting the numbers of fish that could be used or heavy mortality in the larval rearing tanks. Table 4.2 summarises the female broodstock that spawned in each treatment and the data collected from the larvae obtained from each of these spawns. The reason for the occasional heavy mortality was either unclear or it was caused by fungal infections of eggs in the rearing tanks. In such cases, these spawns were not used for data collection.

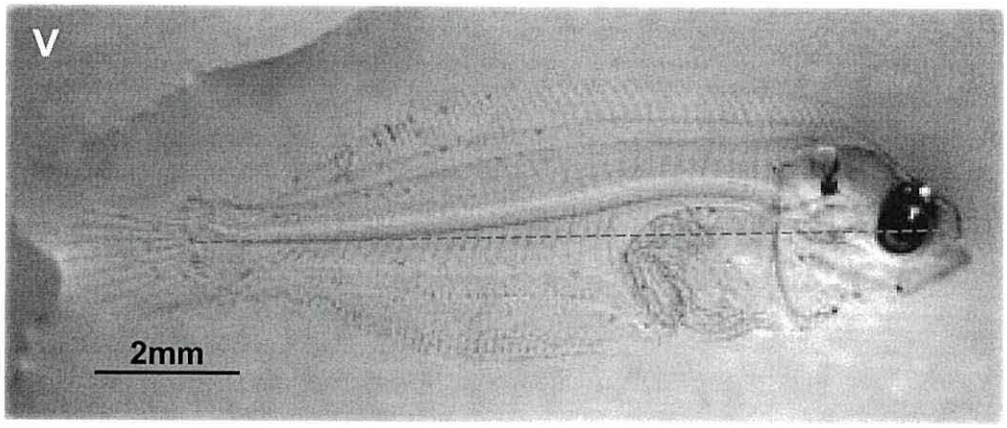
4.4.1 Broodstock and larval water temperatures

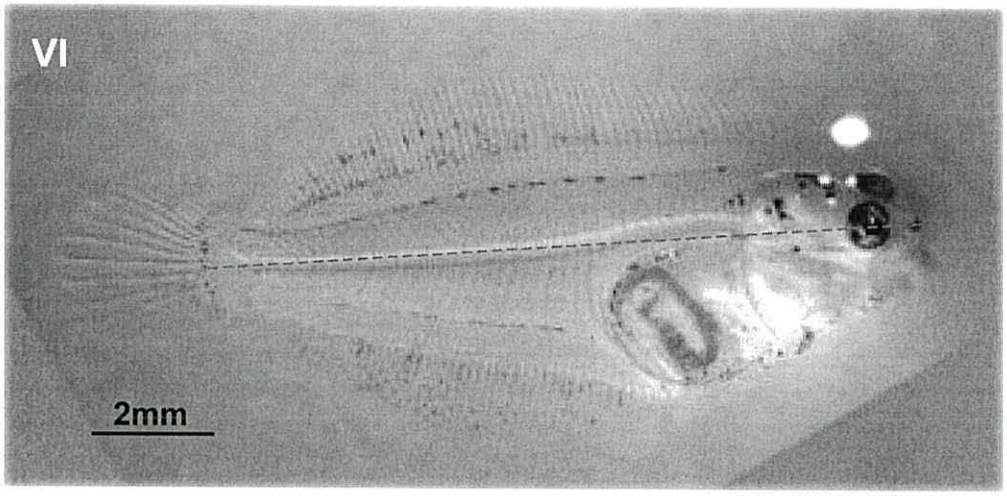
The broodstock water temperatures were lower during the 2005 (5.5 - 10°C, mean = 7.7) spawning period compared to 2004 (8 - 10°C, mean = 8.7) although in both years fish spawned successfully over the same time period (Fig. 4.2). Some fish continued to spawn past the final collections of eggs in both years but sufficient spawns had already been collected and further samples were not required from these particular fish. There was no apparent difference in the timing of spawning activity between control or treatment fish in either year.

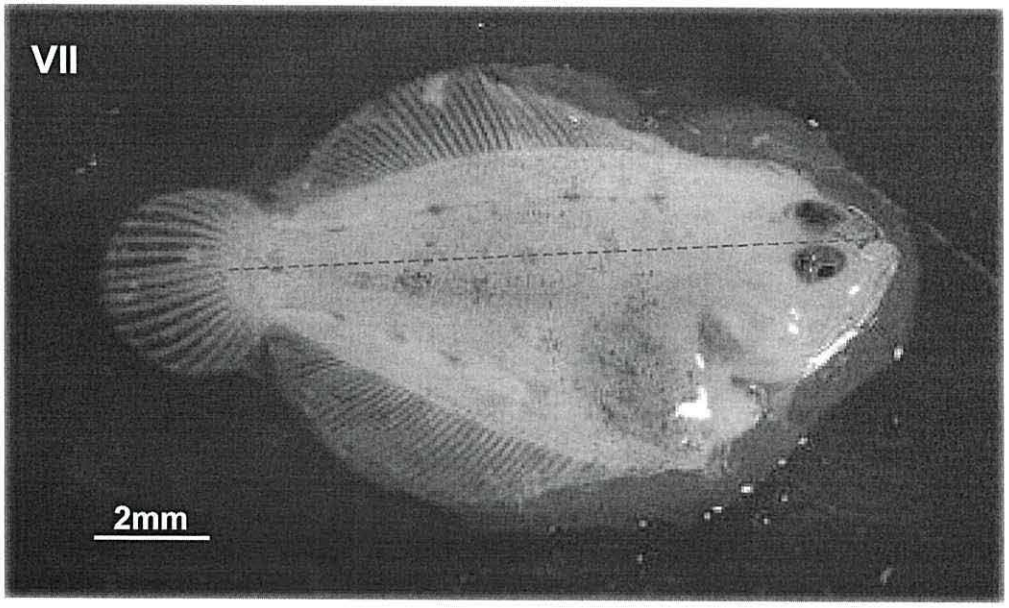
Fluctuations in the water temperatures for the egg survival experiment and larval rearing were small (Fig. 4.3). The increase in temperature by approximately 0.5°C toward the end of the 2004 experiment was due to an increase in ambient temperature. There were no noticeable changes in larval mortality or growth in larvae during this period. The peak in temperature during January 2005 occurred just as larval rearing began but no problems with egg survival were observed. Unfortunately in 2004, only the mean water temperatures were retained from each of the 125-days of the spawning period, for each set of rearing tanks within each treatment group. Temperature data



Plates I – IV Examples of the digital photographs used to measure the length of plaice (*Pleuronectes platessa*) larvae at hatch (11-days post fertilisation) (I) and at ages 7-days (II), 14-days (III) and 21-days (IV) post hatch. The red, dashed lines indicate the body length measurements recorded. Scales are approximate.





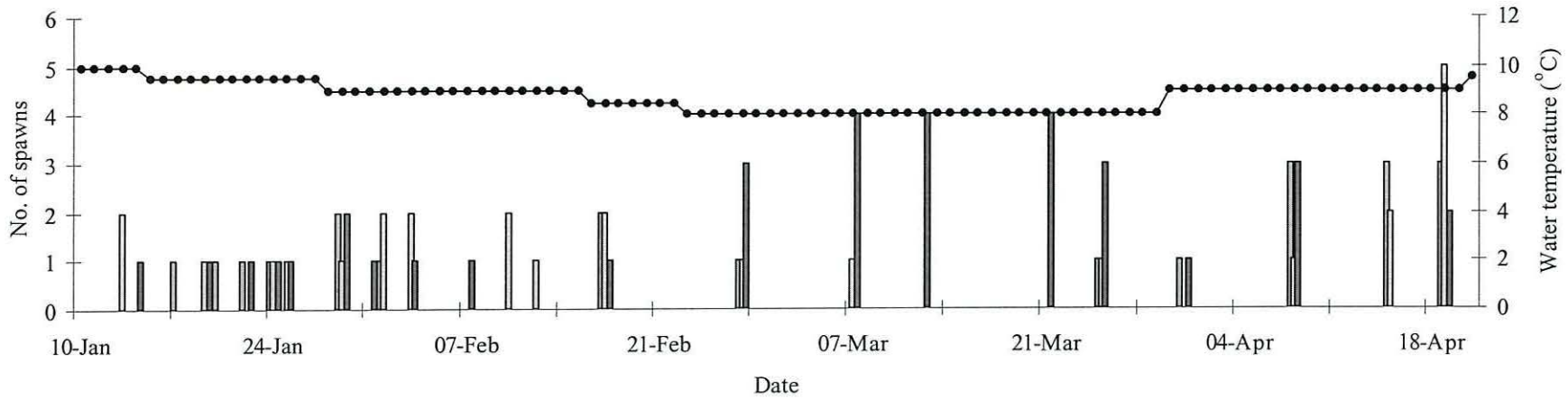


Plates V – VII Examples of the digital photographs use to measure the length of plaice (*Pleuronectes platessa*) larvae and at ages 28-days (V), 35-days (VI) and 42-days (VII) post hatch. The red, dashed lines indicate the body lengths recorded. Scales are approximate.

Table 4.2 Details of the numbers of female fish spawned within each treatment in 2004 and 2005 and the assessments made on larvae from those spawns. The colours denote the egg PCB concentrations detected (Table 3.3): red (high >1.0mg kg^{-1} dry wt.), orange (medium 0.2-1.0mg kg^{-1} dry wt.), yellow (low <0.2mg kg^{-1} dry wt.), green (undosed larvae). Blank spaces indicate data that were not collected due to low numbers of fish. H = hatch, ff = first-feeding.

Year	Dose group	Maternal ID code	Larval Assessments			Comments
			Behaviour	Growth	Survival	
2004	Medium	210				
		213				Small spawn, no survival data collected
		225				High rearing tank mortality
		228	H & ff only	H only		High rearing tank mortality
		247				First to spawn before other apparatus ready
		256				
		257				High rearing tank mortality
		278				Small spawn, no survival data collected
	High	318				
		324				High rearing tank mortality
		326				
		334				High rearing tank mortality
		336		H only		High rearing tank mortality
		347				
		358				
		368		H only		High rearing tank mortality
	Control	112				
		118				
		125				
		126	H only			High rearing tank mortality
		127				Small spawn, no survival data collected
		136				High rearing tank mortality
		146				
2005	Low	423				
		424				
		426				
		427				
		434				
	Control	514				
		517				
		523				High rearing tank mortality
		527				
		528		H only		High rearing tank mortality

A - 2004



B – 2005

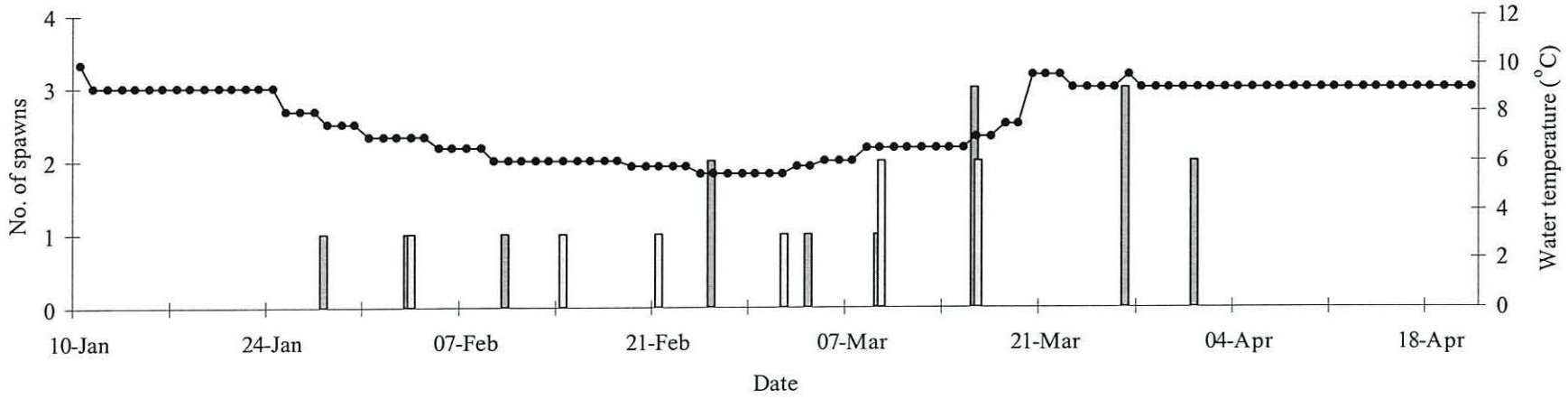
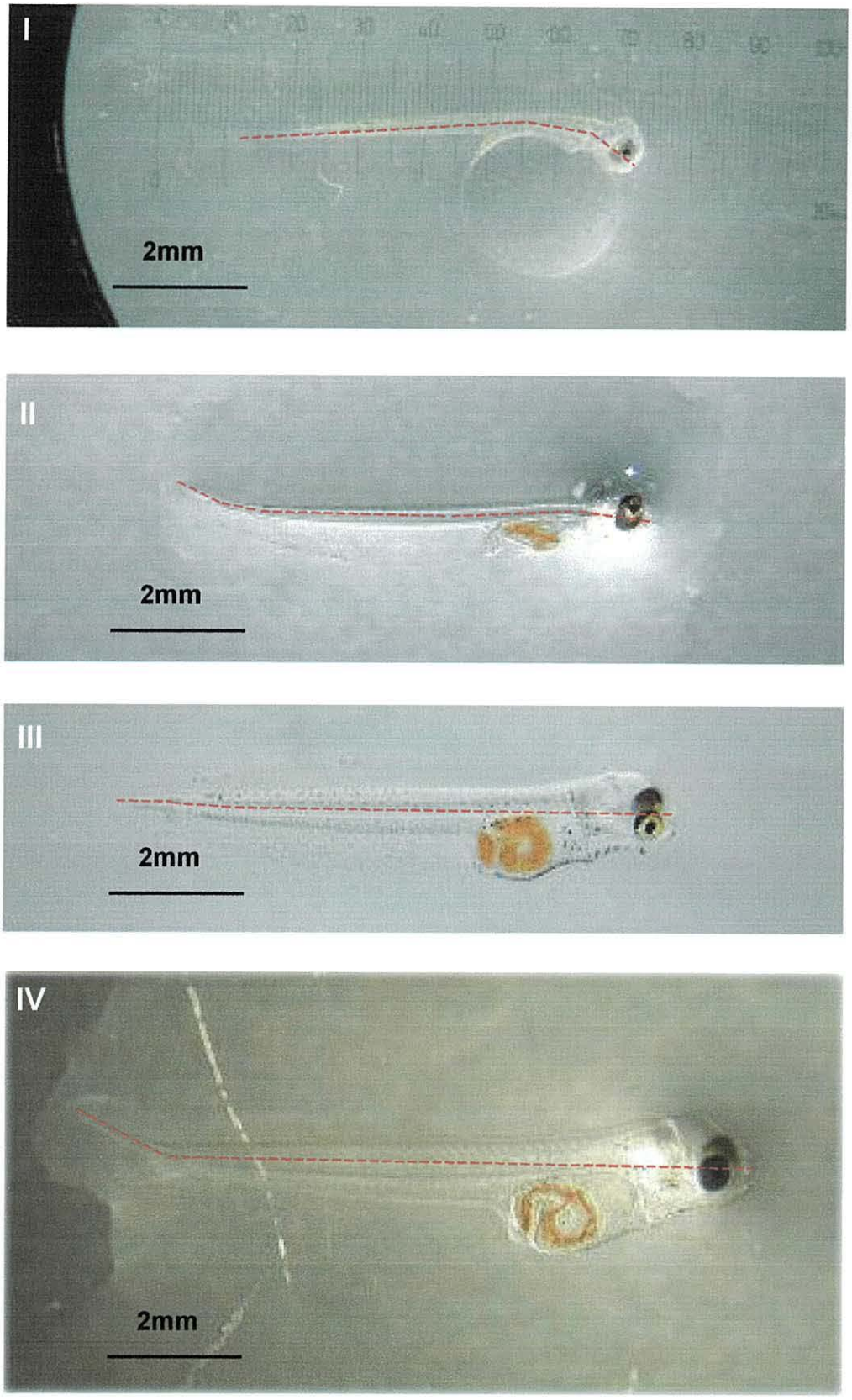
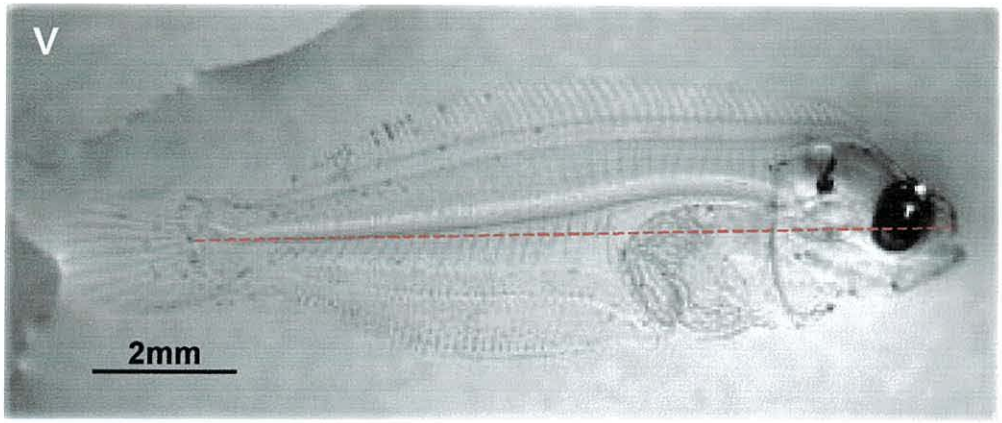
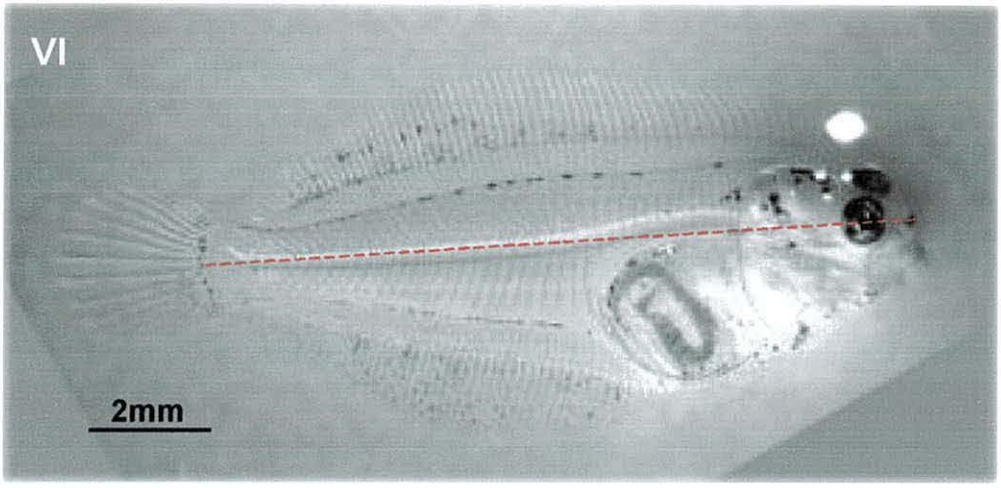


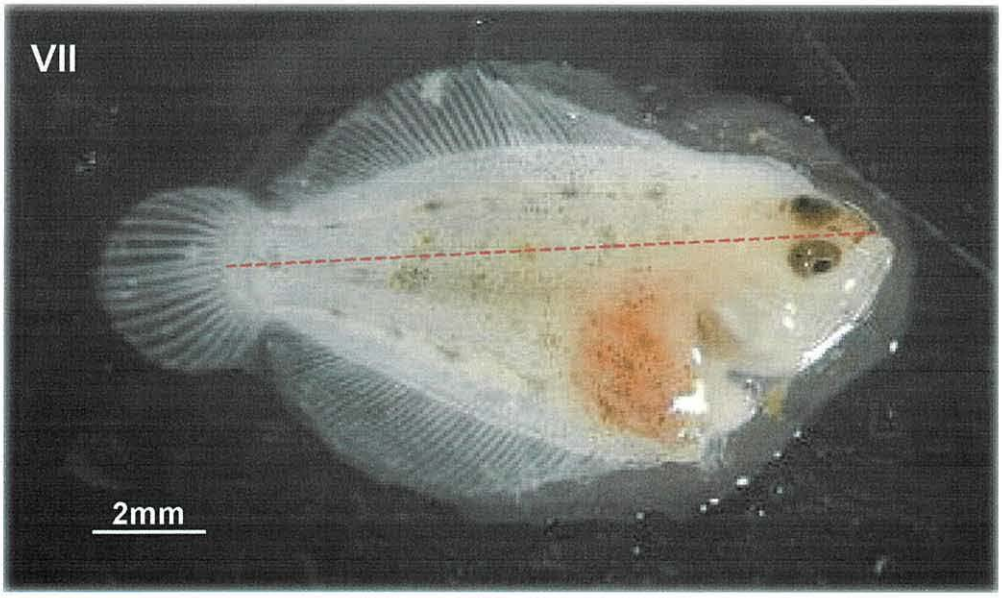
Figure 4.2 Water temperatures (°C) of the broodstock tanks and spawning frequency of the female plaice (*Pleuronectes platessa*) broodstock in (A) 2004 and (B) 2005. Undosed control fish (green bars) and fish dosed with the commercial PCB mixture, Aroclor 1254 - low dosed fish (yellow bars, 0.1-0.175mg month^{-1}), medium dosed fish (orange bars, 1.0-1.75mg month^{-1}) and high dosed fish (red bars, 2.0-3.75mg month^{-1}).



Plates I – IV Examples of the digital photographs used to measure the length of plaice (*Pleuronectes platessa*) larvae at hatch (11-days post fertilisation) (I) and at ages 7-days (II), 14-days (III) and 21-days (IV) post hatch. The red, dashed lines indicate the body length measurements recorded. Scales are approximate.





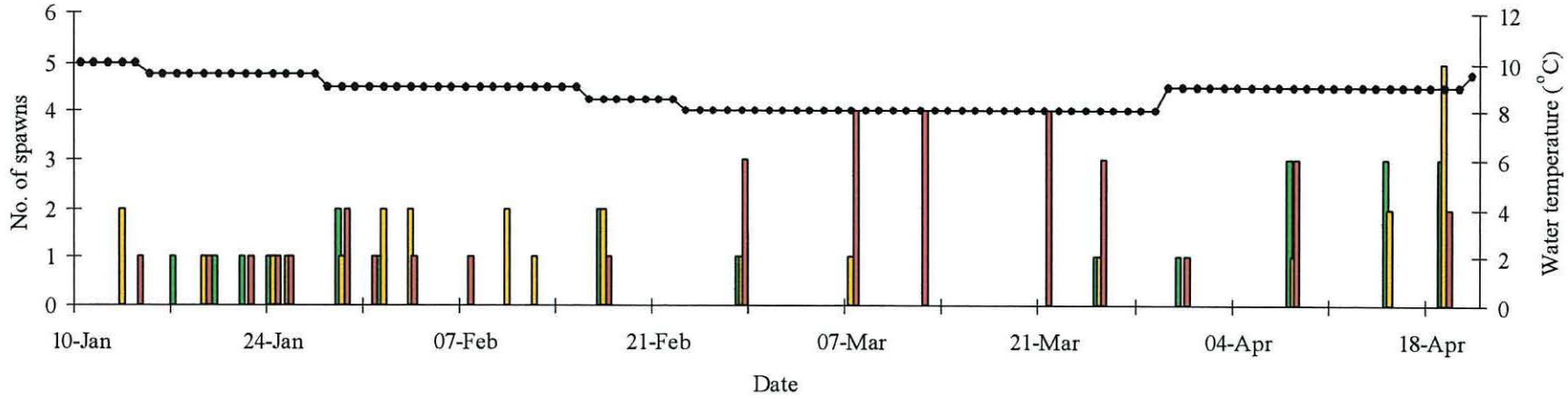


Plates V – VII Examples of the digital photographs use to measure the length of plaice (*Pleuronectes platessa*) larvae and at ages 28-days (V), 35-days (VI) and 42-days (VII) post hatch. The red, dashed lines indicate the body lengths recorded. Scales are approximate.

Table 4.2 Details of the numbers of female fish spawned within each treatment in 2004 and 2005 and the assessments made on larvae from those spawns. The colours denote the egg PCB concentrations detected (Table 3.3): red (high >1.0mg kg^{-1} dry wt.), orange (medium 0.2-1.0mg kg^{-1} dry wt.), yellow (low <0.2mg kg^{-1} dry wt.), green (undosed larvae). Blank spaces indicate data that were not collected due to low numbers of fish. H = hatch, ff = first-feeding.

Year	Dose group	Maternal ID code	Larval Assessments			Comments
			Behaviour	Growth	Survival	
2004	Medium	210				
		213				Small spawn, no survival data collected
		225				High rearing tank mortality
		228	H & ff only	H only		High rearing tank mortality
		247				First to spawn before other apparatus ready
		256				
		257				High rearing tank mortality
		278				Small spawn, no survival data collected
	High	318				
		324				High rearing tank mortality
		326				
		334				High rearing tank mortality
		336		H only		High rearing tank mortality
		347				
		358				
		368		H only		High rearing tank mortality
	Control	112				
		118				
		125				
		126	H only			High rearing tank mortality
		127				Small spawn, no survival data collected
		136				High rearing tank mortality
		146				
2005	Low	423				
		424				
		426				
		427				
		434				
	Control	514				
		517				
		523				High rearing tank mortality
		527				
		528		H only		High rearing tank mortality

A - 2004



B – 2005

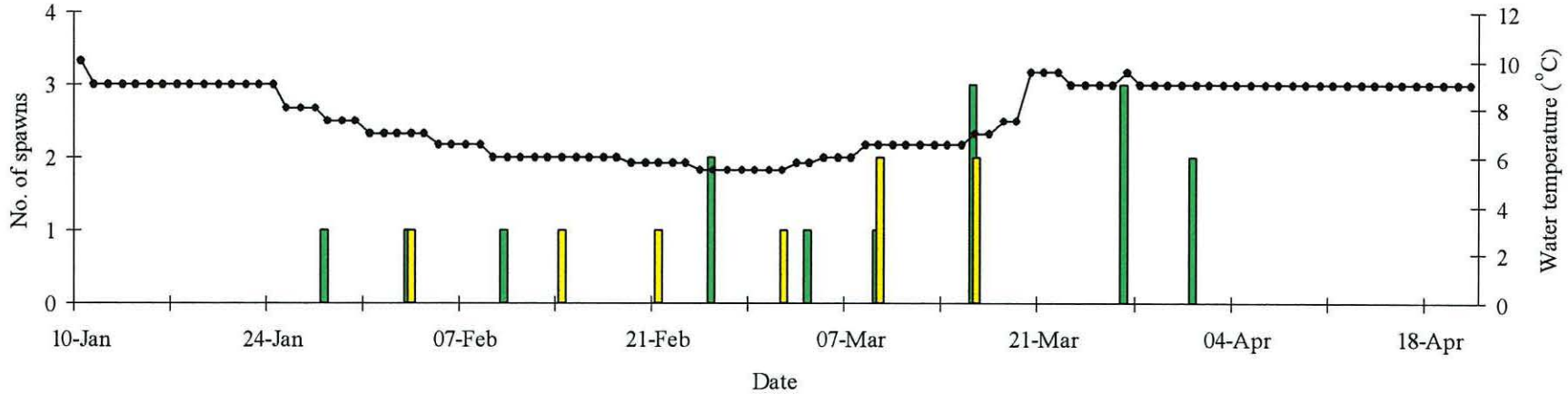
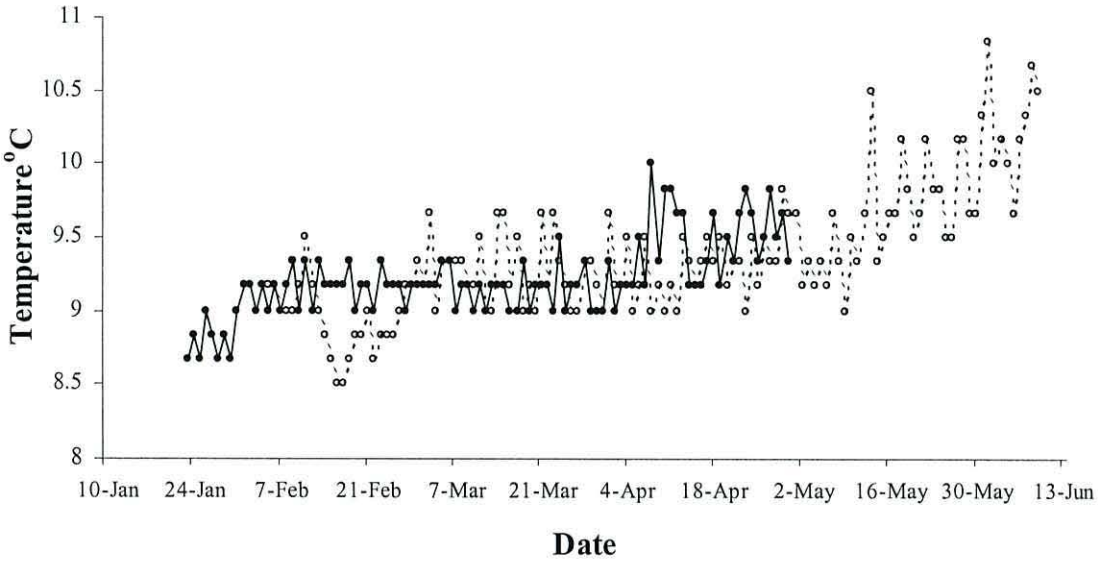


Figure 4.2 Water temperatures (°C) of the broodstock tanks and spawning frequency of the female plaice (*Pleuronectes platessa*) broodstock in (A) 2004 and (B) 2005. Undosed control fish (green bars) and fish dosed with the commercial PCB mixture, Aroclor 1254 - low dosed fish (yellow bars, 0.1-0.175mg month^{-1}), medium dosed fish (orange bars, 1.0-1.75mg month^{-1}) and high dosed fish (red bars, 2.0-3.75mg month^{-1}).

A - 2004



B - 2005

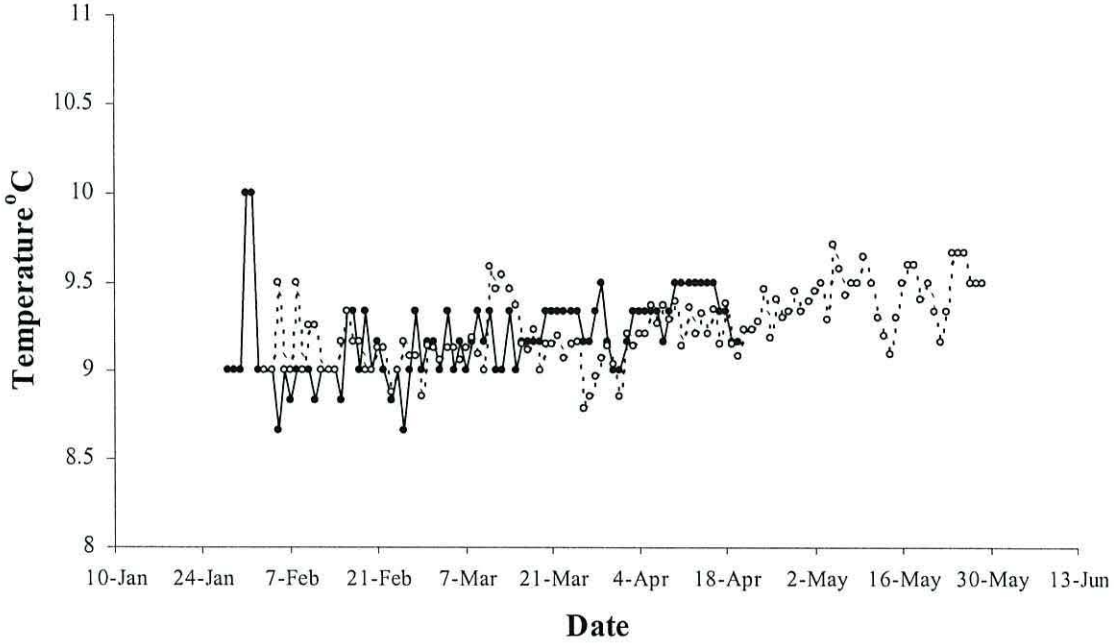


Figure 4.3 Water temperatures (°C) in the egg survival experiments (closed circles) and in the egg and larval rearing experiments (open circles, dashed lines) in (**A**) 2004 and (**B**) 2005.

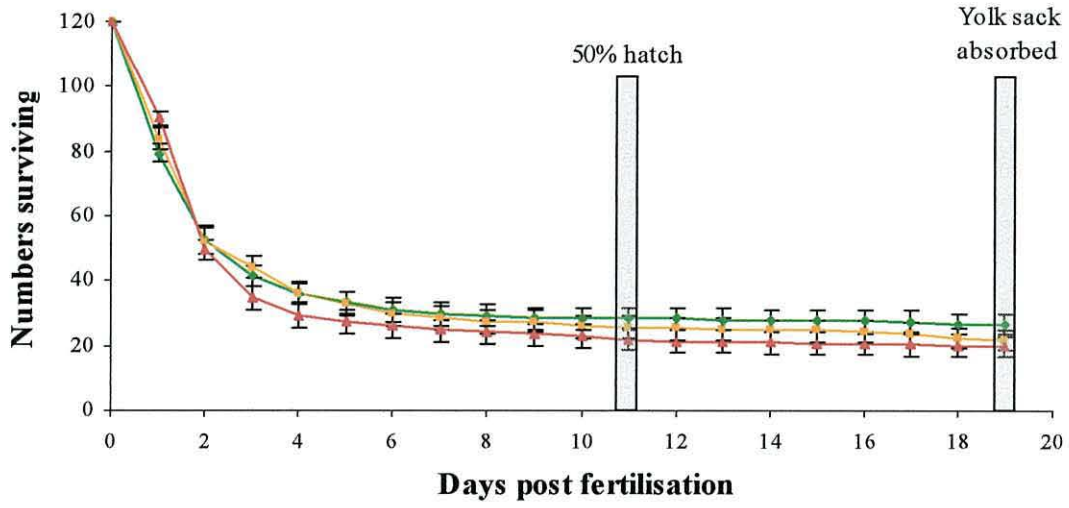
were not normally distributed (Anderson-Darling, $p < 0.001$) and pairwise comparisons of the mean daily temperatures revealed no significant differences in daily water temperatures between treatment groups throughout the rearing period in 2004 (Wilcoxon's signed ranks tests, n = 125, $p > 0.05$ in all cases). In 2005 raw tank water temperature data were retained and again, no significant differences existed between treatments (Kruskal-Wallis, H = 3.06, df = 1, n = 614, $p = 0.080$).

4.4.2 Egg and larval survival

A total of 20 spawns using 7200 eggs/larvae in 2004 and 10 spawns using 3600 eggs/larvae in 2005 were assessed for survival and grouped based on the egg PCB concentration. Across all spawns in both years there was a sharp drop in survival during the first 3-dpf before the mortality rates reduced and began to level out (Fig. 4.4 A & B). Survival between treatments was compared at 3-dpf, 11-dpf (hatch) and 18-dpf (first-feeding). The data from the 2004 experiments failed to meet the requirements of normality for the ANOVA tests and therefore non-parametric Kruskal-Wallis tests were applied to the medians of the data from 3-dpf, hatch (11-dpf) and first-feeding (18-dpf). Although there appeared to be more larvae from undosed females surviving in 2004 compared to those from dosed fish, no significant differences were detected (Table 4.3) although the mean and median numbers surviving were consistently higher for eggs and larvae from undosed broodstock than for those from dosed broodstock (Fig. 4.4 A, Table 4.3).

Whilst the Kruskal-Wallis test demonstrated there were no significant differences in mortality at various stages of larval development, it was not clear if the rates of mortality differed between the offspring of different treatment groups over the first 3-dpf when the highest mortality was observed. Data from 2004 was subjected to an ANCOVA test (Anderson-Darling, A = 0.673, $p = 0.078$; Bartlett's homogeneity of variance, T = 2.513, $p = 0.285$) using age as a covariate to test for this (Table 4.4). Table 4.4 shows the significant negative relationship of survival with age ($p < 0.001$). Significant differences in survival did exist between spawns in each treatment group ($p < 0.001$) but these could not be attributed to the different dosing treatments

A - 2004



B - 2005

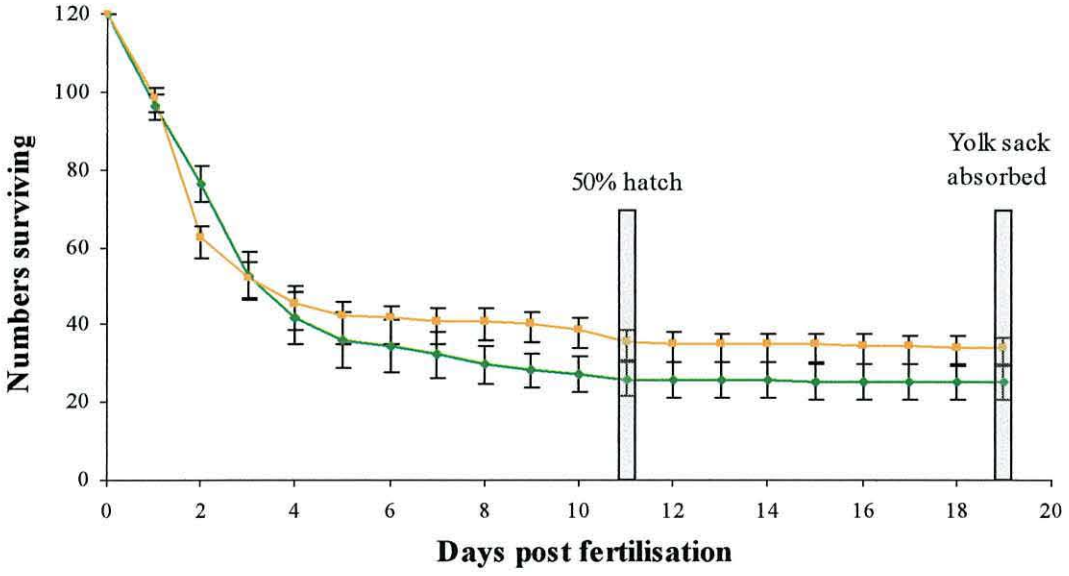


Figure 4.4 Numbers (mean ±SE) of eggs (days 0-11) and larvae (days 11-19) surviving from female plaice (*Pleuronectes platessa*) broodstock (dosed and undosed) with Aroclor 1254 in (**A**) 2004 and (**B**) 2005. (**A**) – green line = undosed; medium, yellow line = 1-1.75mg dose^{-1}; high, red line = 2-3.5mg dose^{-1}. (**B**) – green line = undosed; low, yellow line = 0.1-0.175 mg dose^{-1}.

Table 4.3 Results of Kruskal-Wallis tests on the arcsine-transformed proportional survival data for plaice eggs and larvae reared in 11 beakers in the 2004 broodstock dosing experiment.

Comparison at age (days post fertilisation)	Median proportion surviving			H	df	*p*
	Control	Medium	High			
3	0.345	0.347	0.301	0.47	2	0.790
11	0.267	0.221	0.171	3.93	2	0.140
18	0.242	0.196	0.146	5.33	2	0.070

Table 4.4 Analysis of covariance applied to the survival data for the first 3-days post fertilisation of eggs from plaice broodstock in the 2004 experiment.

Source	df	Adjusted sum of squares	Mean squares	*F*	*p*
Age	1	213089	213089	1378.68	<0.001
Treatment	2	54	27	0.17	0.841
Spawn (treatment)	16	19434	1143	7.40	<0.001
Error	207	31994	155		
Total	227				
Term	Coef	SE Coef	T	*p*	
Constant	117.291	1.406	79.90	<0.001	
Age	-27.9896	0.7538	-37.13	<0.001	

Table 4.5 Results of nested ANOVAs on survival of eggs and larvae of the European plaice from broodstock either undosed or dosed with 0.1-0.175mg Aroclor 1254 month^{-1} in the 2005 broodstock experiment.

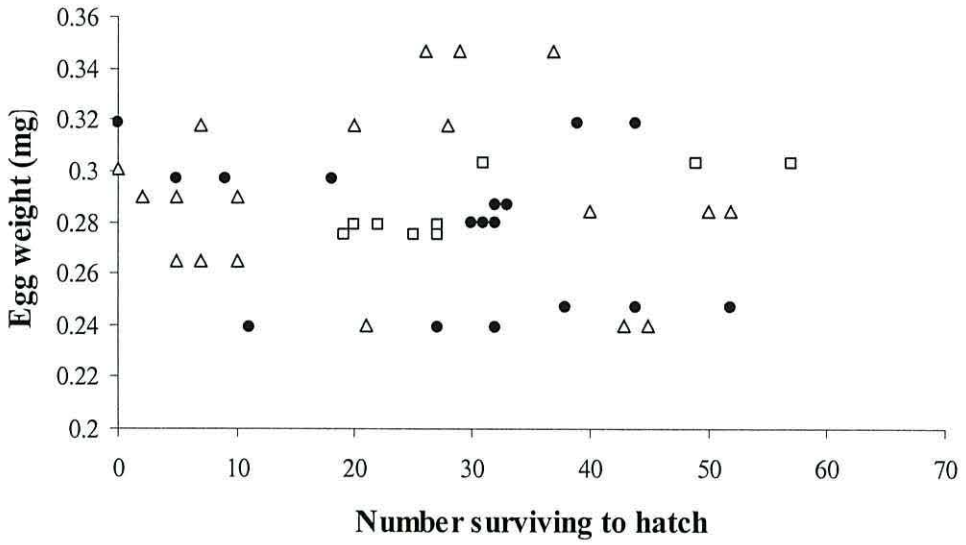
Days post fertilisation	Factor	df	Sums of squares	Mean squares	F	*p*
3	Treatment	1	0.00499	0.00499	0.97	0.336
	Spawn(treatment)	8	1.21348	0.15168	29.57	<0.001
	Error	20	0.10258	0.00513		
	Total	29	1.32105			
11	Treatment	1	0.08385	0.08385	13.54	0.001
	Spawn(treatment)	8	1.13884	0.14235	22.99	<0.001
	Error	20	0.12386	0.00619		
	Total	29	1.34655			
18	Treatment	1	0.07274	0.07274	11.11	0.003
	Spawn(treatment)	8	1.10273	0.13784	21.06	<0.001
	Error	20	0.13092	0.00655		
	Total	29	1.30639			

($p = 0.841$). There was no correlation between the number of larvae surviving to hatch and the estimated individual egg dry weight ($r = 0.127$, $p = 0.391$) (Fig. 4.5 A).

In 2005, the general pattern of mortality appeared to be the same with a rapid decline over the first three days followed by a reduction in the mortality rate (Fig. 4.4 B). However, in 2005 more individuals appeared to survive from female plaice administered a low dose of Aroclor 1254 compared to those from control fish and in this case the differences were significant. The data met the requirements for a nested ANOVA (Anderson-Darling test for normality, $p > 0.05$; Bartlett's homogeneity of variance, $p > 0.05$) at each of the three ages examined, and was analysed with female spawns nested within treatment (Table 4.5). In all cases there were significant differences in survival between spawns, demonstrating high inter-spawn variation. As with the larvae in 2004, there was no correlation between the estimated individual egg dry weight and the number of larvae hatching ($r = 0.139$, $p = 0.464$) (Fig. 4.5 B). The differences observed between treatments at 11- and 18-dpf show that the dosed larvae had better survival in this experiment. However, there was no correlation of the proportion surviving to hatch ($r = -0.037$, $p = 0.862$) or first feeding ($r = -0.061$, $p = 0.778$) with egg ΣPCB(21 congeners) concentration. Again, an ANCOVA was applied to the data (Anderson-Darling, $A = 0.287$, $p = 0.618$; Bartlett's homogeneity of variance, $T = 1.637$, $p = 0.053$) over the first 3-dpf to test for differences in the mortality rates (Table 4.6). The results mirrored those from 2004 whereby there was a significant effect of age on survival ($p < 0.001$). Significant differences in survival did exist between spawns within in each treatment group ($p < 0.001$) but these could not be attributed to the different dosing treatments ($p = 0.127$). The regression analysis showed the relationship of survival with age to be significantly negative ($p < 0.001$) (Table 4.6).

Fig. 4.6 shows the data for egg and larval survival from both years at each of the key stages analysed (3-dpf, hatch and first-feeding) for simpler presentation, in terms of mean (±SE) survival per treatment group. Attempts were made to log the data in order to straighten the survival curves (Fig. 4.4) and calculate overall mortality rates using regression. However, such transformations using natural log and $\log_{10}$ did not straighten the data and prevented analysis of this type.

A - 2004



B - 2005

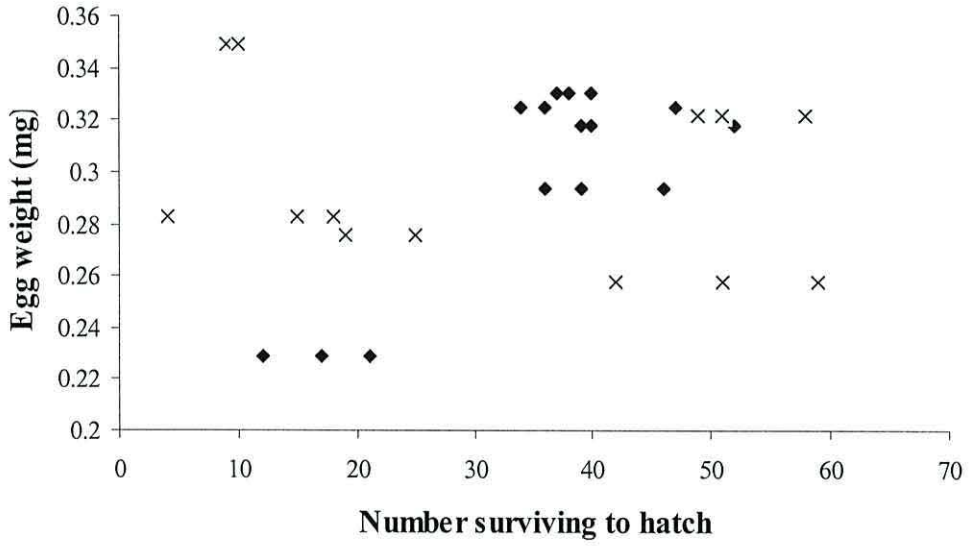


Figure 4.5 Scatter plots showing the lack of correlation between egg dry weight (mg) at fertilisation and the number of eggs surviving to hatch at 11 days post fertilisation in (**A**) 2004 ($r = 0.127$, $p = 0.391$) and (**B**) 2005 ($r = 0.139$, $p = 0.464$). Eggs were from plaice (*Pleuronectes platessa*) broodstock either undosed, control (♦) or dosed orally with Aroclor 1254: low dose 0.1-0.175 mg dose^{-1} (×), medium dose 1-1.75mg dose^{-1} (□), high dose 2-3.5mg dose^{-1} (△).

Table 4.6 Analysis of covariance applied to the survival data for the first 3-days post fertilisation of eggs from plaice broodstock in the 2005 experiment.

Source	df	Adjusted sum of squares	Mean squares	*F*	*p*
Age	1	87193	87193	660.46	<0.001
Treatment	1	311.1	311.1	2.36	0.127
Spawn (treatment)	9	14680.5	1631.2	12.36	<0.001
Error	120	15842.3	132		
Total	131				
Term	Coef	SE Coef	T	*p*	
Constant	119.190	1.676	71.12	<0.001	
Age	-22.9879	0.8945	-25.70	<0.001	

Table 4.7 Results of the nested ANOVA on the survival of plaice (*Pleuronectes platessa*) larvae post-hatch until first-feeding from broodstock either undosed (control) or dosed with 1.0-1.75mg Aroclor 1254 month^{-1} (medium) or 2.0-3.5mg Aroclor 1254 month^{-1} (high).

	Factor	df	Sums of squares	Mean squares	F	*p*
	Treatment	2	0.25594	0.12327	7.44	0.003
	Spawn(treatment)	11	0.68804	0.06255	3.77	0.003
	Error	25	0.41444	0.01658		
	Total	38	1.35842			
Pairwise comparisons	T	*p*				
Control vs…						
Medium	-3.185	0.010				
High	-3.486	0.005				
Medium vs…						
High	-0.1965	0.979				

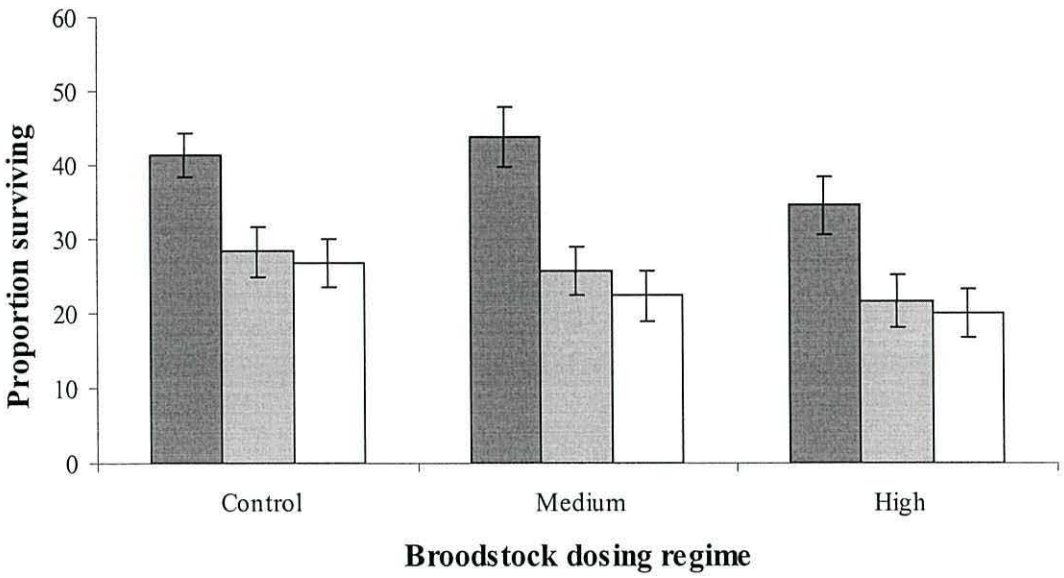
In a final analysis of survival, data for all beakers containing 20 or more larvae at hatch (11-dpf) were re-grouped according to the total egg PCB concentration. Data were selected on this basis so as only to use replicates with a reasonable sample size. The percentage survival until first feeding (18-dpf) was then calculated for each beaker. Data from 2004 met the assumptions of ANOVA whilst data from 2005 did not and was analysed using the Kruskal-Wallis test. In 2004, there were significant reductions in the percentage hatch between control larvae and larvae from eggs containing both medium and high concentrations of PCBs (Table 4.7). In 2005, no significant differences were found in the percentage survival from hatch to first-feeding between the control and low dosed larvae (KW, H = 0.40, df = 1, p = 0.529).

4.4.3 Larval growth

In 2004, a total of 15 spawns (n = 5 per treatment) were assessed for differences in growth rates and total length (Fig. 4.7 A). Growth was slow during the first week following hatch in experiments in both years and increased with the onset of feeding from 6-dph onward. Some data for 2004 were lacking for some spawns in weeks one and four due to a visit to Belgium to learn the techniques required for chapter 5 and have been omitted to avoid presenting confusing growth patterns (Fig. 4.7 A). The standard error increased on each occasion the larvae were measured. High-dosed larvae in 2004 appeared to take an extra two to three days to reach the same mean length as the larvae from the control group females. In 2005, the delay in attaining the same size as control larvae appeared to be minimal during the period the fish were measured. Table 4.8 shows the data for mean lengths (mm) at hatch and metamorphosis from all the larvae measured in the 2004 and 2005 experiments together with the ΣPCB(21 congeners) concentrations detected in the eggs. Spawns with a mean hatch length of less than 6.10mm were only observed from the female broodstock dosed with >1.0mg Aroclor 1254 per month although hatch length remained variable within all treatment groups.

The length at hatch data failed to meet the requirements for an ANCOVA test in order to account for maternal condition factor (as in section 3.4.5) (Bartlett's, T = 11.661, p = 0.003), even after $\log_{10}$ or $\log_e$ transformation of the data was attempted, so the non-

A - 2004



B - 2005

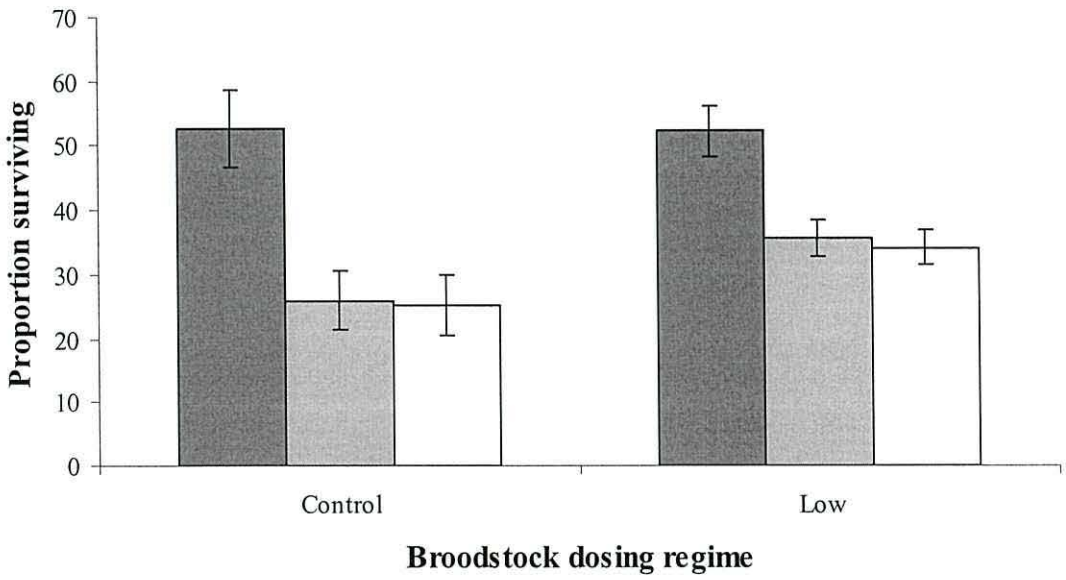
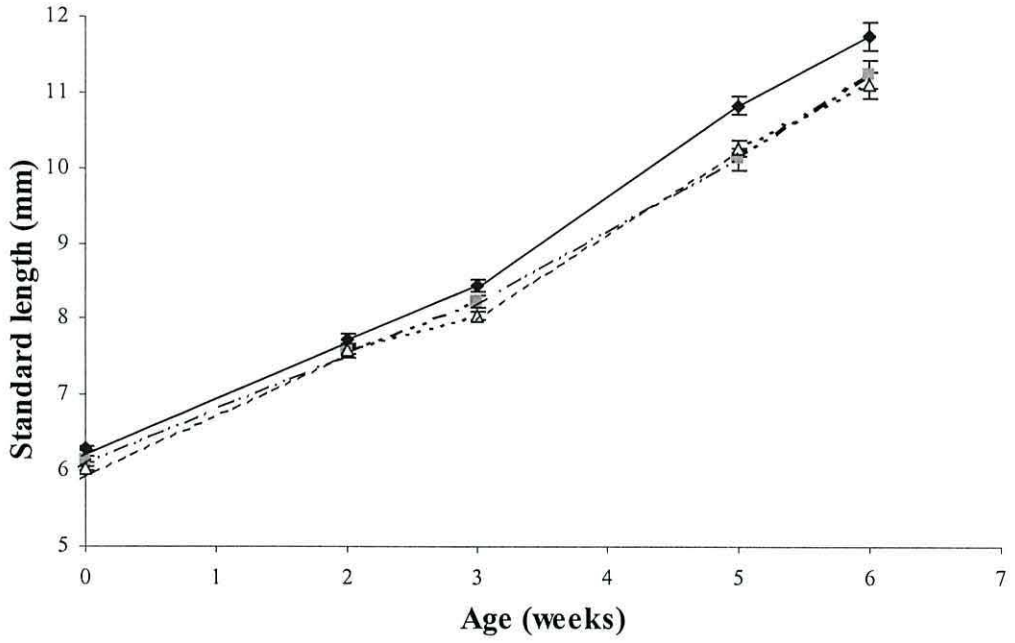


Figure 4.6 Mean numbers (± standard error) of eggs and larvae surviving from female plaice (*Pleuronectes platessa*) broodstock (dosed and undosed) with Aroclor 1254 in (**A**) 2004 and (**B**) 2005. Control = undosed; Low = 0.1-0.175mg per dose; Medium = 1-1.75 mg per dose; High = 2-3.5mg per dose. Dark bars = 3dpf; grey bars = 11dpf (hatch); white bars = 18dpf (first feeding).

A - 2004



B - 2005

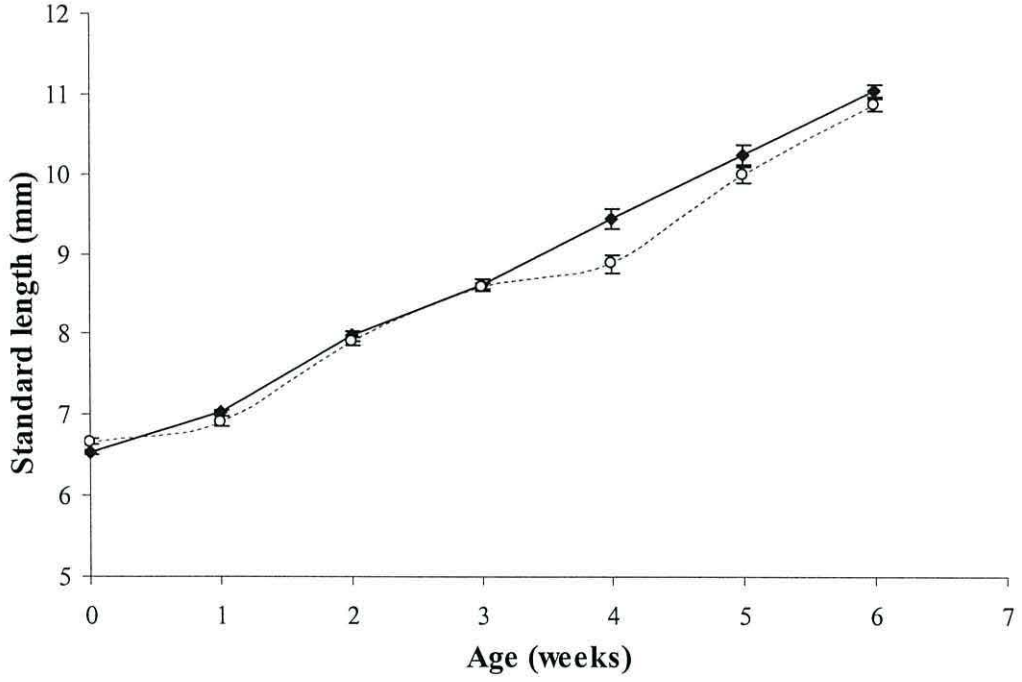


Figure 4.7 Mean standard length (± SE, mm) of plaice (*Pleuronectes platessa*) larvae following hatch in (**A**) 2004 and (**B**) 2005. Larvae originated from female broodstock dosed with different amounts of Aroclor 1254. ◆ = control, undosed; △ = high dosed (2-3.5mg month^{-1}); ■ = medium dosed (1-1.75mg month^{-1}); ○ = low dosed (0.1-0.175mg month^{-1}).

Table 4.8 Mean (±SE) body lengths (mm) at hatch and standard lengths at metamorphosis of plaice (*Pleuronectes platessa*) larvae from female broodstock either undosed or dosed with Aroclor 1254 at 0.1-0.175mg month^{-1} (low), 1-1.75mg month^{-1} (medium) or 2-3.51mg month^{-1} (high). N = 20 except where indicated (*).

Year	Aroclor 1254 dose	Spawn number	Hatch length (mm) ± SE	Metamorphosis length (mm) ± SE	Egg ΣPCB(21 congeners) μg kg^{-1} dry wt
2004	Control	112-1	6.29 ±0.05	11.20 ±0.38	nd
		118-4	6.62 ±0.03	12.35 ±0.58	nd
		125-1	6.18 ±0.04	11.46 ±0.34	nd
		127-1	6.22 ±0.05	11.55 ±0.34	nd
		146-2	6.14 ±0.03	12.12 ±0.41	nd
2005	Control	514-1	6.39 ±0.06	11.37 ±0.11	nd
		517-1	6.63 ±0.04	10.89 ±0.15	nd
		527-2	6.61 ±0.03	10.87 ±0.12	nd
		528-3	6.52 ±0.04	ns	nd
2005	Low	424-1	6.57 ±0.05	11.17 ±0.12	27.1 (trace)
		434-1	6.29 ±0.05	11.07 ±0.12	ns
		423-2	6.67 ±0.02	10.32 ±0.08	126.0 (trace)
		426-1	6.88 ±0.04	11.59 ±0.12	68.1 (trace)
		427-1	6.94 ±0.03	10.25 ±0.14	197.5 (trace)
2004	Medium	210-2	6.10 ±0.06	12.26 ±0.32	3672.7
		213-1	6.01 ±0.05	10.62 ±0.34	2359.6
		228-3	6.53 ±0.05	9.40 ±0.20* n = 7	470.5
		256II-3	6.56 ±0.05	10.71 ±0.38	906.9
		278-1	5.52 ±0.04	12.02 ±0.35	6114.5
2004	High	318-1	6.24 ±0.05	10.88 ±0.29	12308.6
		326-2	5.57 ±0.06	10.74 ±0.22	1264.4
		336-2	6.05 ±0.05	ns	ns
		347-1	6.16 ±0.05	11.42 ±0.38	2916.2
		358-1	5.81 ±0.04	11.33 ±0.47* n = 10	892.6
		368-2	6.24 ±0.06	ns	2254.6

ns = not sampled; nd = not detected

Table 4.9 Results of the Kruskal-Wallis analysis of hatch length (mm) of plaice (*Pleuronectes platessa*) larvae from female broodstock either undosed or dosed with Aroclor 1254 at doses of 1-1.75 mg month^{-1} (medium dose) or 2-3.51mg month^{-1} (high dose) in 2004.

Treatment	n	Median	Average rank	Z
Control	100	6.235	171.5	3.83
Medium dose	100	6.210	145.1	-0.06
High dose	90	6.015	117.0	-3.88
Overall	290		145.5	

H = 20.00 df = 2 $p < 0.001$
H = 20.00 df = 2 $p < 0.001$ (adjusted for ties)

parametric Kruskal-Wallis test was applied. At hatch, in 2004 there were significant differences in the median standard lengths between treatments, with the larval in the control group having the greatest median length, followed by the medium- and high-dose group larvae respectively (Table 4.9).

When the data were re-grouped according to the total egg PCB concentrations only spawns from three females accounted for larvae with a medium PCB concentration. Larvae from one of these spawns suffered high mortality and were not sampled for growth data following hatch. Consequently, the growth data for these larvae at medium PCB concentrations appears erratic because of the reduced sample size (n = 2) (Fig. 4.8).

The significant reductions in hatch length of the highest dosed larvae compared to controls remained even when the data were re-grouped according to ΣPCB(21 congeners) concentration in the eggs (Table 3.3) of 0, 0-1 and >1.0 mg kg^{-1} dry wt. (Table 4.10). However, in this case, the medium PCB concentration larvae had a higher median length than the control and the high-dosed larvae.

The data for length at metamorphosis was $\log_{10}$-transformed to meet the requirements of ANOVA (Anderson-Darling, A = 0.737, p = 0.054). A nested-ANOVA demonstrated that differences in $\log_{10}$ mean standard length between treatments remained at least until metamorphosis and also showed that offspring from different females within each treatment had high variability in size at metamorphosis (Fig. 4.7; Table 4.11).

Subsequent pairwise comparisons (Tukey's HSD) confirmed that the significant differences between groups existed between the undosed and high-dosed fish (T = -2.545, p = 0.0294) and not between the undosed and medium-dosed groups (T = -1.275, p = 0.4093) or the medium- and high-dosed groups (T = -1.205, p = 0.4502). When the data was re-grouped according to the initial egg PCB concentrations (Table 3.3) the data no longer met the requirements of ANOVA, even following $\log_{10}$ transformation so the Kruskal-Wallis test was applied to test whether the previously observed differences still remained. The control larvae still had higher median standard lengths compared to the high dosed broodstock but the difference was no

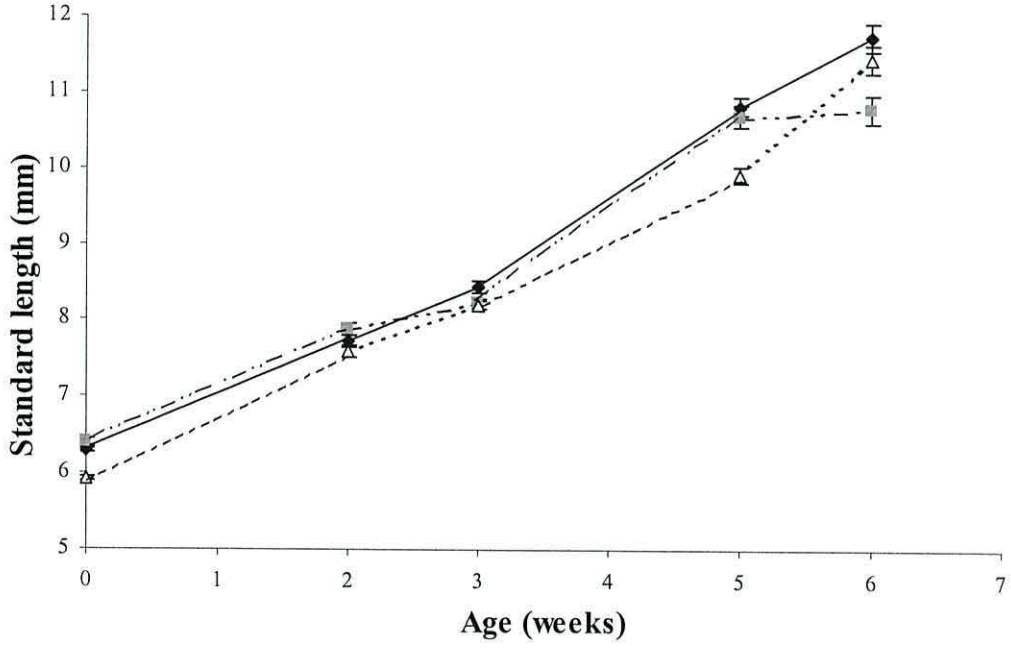


Figure 4.8 Mean standard length (± SE, mm) of plaice (*Pleuronectes platessa*) larvae following hatch in 2004. Larvae originated from eggs containing different concentrations of PCBs from females dosed orally with Aroclor 1254. ◆ = control, undosed, n = 5; ■ = medium PCB concentration (0.2-1.0 mg kg^{-1} dry wt.), n= 2; △ = high PCB concentration (>1.0mg kg^{-1} dry wt.), n = 8. Data for the larvae from eggs with medium PCB concentrations are erratic due to the small number of replicates following re-grouping by egg PCB concentration, n=2.

Table 4.10 Results of pairwise Kruskal-Wallis analysis of hatch length (mm) of plaice (*Pleuronectes platessa*) larvae from eggs classified as either control (uncontaminated) or with ΣPCB(21 congeners) concentrations of 0-1mg kg^{-1} dw (medium) or >1mg kg^{-1} dw (high).

PCB concentration	n	Median	Average rank	Z
Control vs...	100	6.235	69.0	-2.61
Medium	50	6.490	88.6	2.61
Overall	150		75.5	
H = 6.79 df = 1 $p = 0.009$				
H = 6.79 df = 1 $p = 0.009$ (adjusted for ties)				
Control vs...	100	6.235	153.0	6.14
High	140	6.005	97.3	-6.14
Overall	240			
H = 37.66 df = 1 $p < 0.001$				
H = 37.67 df = 1 $p < 0.001$ (adjusted for ties)				

Table 4.11 ANOVA table for standard lengths (mm) of plaice (*Pleuronectes platessa*) larvae at metamorphosis from broodstock undosed or dosed with 1-1.175 (medium) or 2-3.5 (high) mg Aroclor 1254 month^{-1}.

Factor	df	Sums of squares	Mean squares	F	*p*
Treatment	2	0.024761	0.012381	3.25	0.040
Spawn(treatment)	10	0.096043	0.009604	2.52	0.007
Error	247	0.940321	0.003807		
Total	259	1.061125			

Table 4.12 Results of the Kruskal-Wallis analysis of length at metamorphosis (mm) of plaice (*Pleuronectes platessa*) larvae from eggs classified as either 'control' (uncontaminated) or as 'high' with ΣPCB(21 congeners) concentrations >1mg kg^{-1} dw.

PCB concentration	n	Median	Average rank	Z
Control	100	11.36	118.0	1.60
High	120	11.14	104.2	-1.60
Overall	220		110.5	

H = 2.55 df = 1 $p = 0.110$
H = 2.55 df = 1 $p = 0.110$ (adjusted for ties)

Table 4.13 Kruskal-Wallis analysis comparing length (mm) at hatch (11-dpf) of plaice (*Pleuronectes platessa*) larvae from female broodstock either undosed or dosed with Aroclor 1254 at doses of 0.1-0.175 mg month^{-1} (low dose).

Treatment	n	Median	Average rank	Z
Control	80	6.560	74.6	-3.49
Low dose	98	6.680	101.7	3.49
Overall	178		89.5	

H = 12.17 df = 1 $p < 0.001$
H = 12.17 df = 1 $p < 0.001$ (adjusted for ties)

Table 4.14 ANOVA table comparing length (mm) at metamorphosis (42-dph) of plaice (*Pleuronectes platessa*) larvae from female broodstock either undosed or dosed with Aroclor 1254 at doses of 0.1-0.175 mg month^{-1} (low dose).

Factor	df	Sums of squares	Mean squares	F	*p*
Treatment	1	0.9866	0.9866	3.30	0.071
Spawn(treatment)	6	29.9036	4.9839	16.66	<0.001
Error	152	45.4850	0.2992		
Total	159				

longer significant (Table 4.12). Comparisons were not made to the medium dosed larvae due to a lack of replicates (n = 2).

In 2005, the larvae from the Aroclor 1254-dosed females were significantly larger at hatch compared to those from the control fish (Table 4.13). The control fish gained a greater length within the first week following hatch and maintained this size difference until metamorphosis (Fig. 4.7 B) although at this point, the differences in larval length between treatments were no longer significant yet there was significant variation in larval length at metamorphosis between different female spawns within treatments (Table 4.14). Data from the 2005 experiment were not re-grouped according to egg PCB concentration because this would have resulted in spawns from only two females accounting for each of the egg concentration groups, and the minimum number of replicates within each treatment should be n = 3 for sound statistical analyses.

Data for hatch length were combined across the experiments from both years and investigated with respect to maternal condition, egg PCB concentration and egg size. No correlations existed between hatch length and maternal weight ($r = -0.097$, $p = 0.666$, $n = 25$) or Fulton's condition factor ($r = 0.144$, $p = 0.493$, $n = 25$). There was a significant negative correlation between hatch length and the total egg ΣPCB(21 congeners) concentrations ($r = -0.449$, $p = 0.031$, $n = 23$) with eggs containing a higher PCB dose producing smaller larvae at hatch (Fig. 4.9) (n = 23 because two spawns from treated fish were not analysed for PCB content; see Table 3.3). The relationship between hatch length and the estimated mean egg dry weight was also investigated (Fig. 4.10). There appeared to be a positive relationship between the two factors yet one peculiar outlying point from the high dose group (heaviest egg but one of the shortest mean hatch sizes) prevented this trend from being significant ($r = 0.374$, $p = 0.066$, $n = 25$). Although there was no good reason to do so, removal of this point highlighted the apparent, positive relationship between egg size and larval hatch size ($r = 0.531$, $p = 0.008$, $n = 24$).

Median water temperatures in the 8l larval rearing tanks in 2004 were not significantly different from those in 2005 [Mann-Whitney, $W = 16033.5$, $p = 0.092$, n = 125 (2004), n = 116 (2005)] and the same dietary and light regimes were used.

Consequently, the data for larval growth were combined and re-grouped in order to account for egg ΣPCB(21 congeners) concentration. The groupings used were control, <0.1mg kg^{-1} eggs (low), 0.1-1.0mg kg^{-1} eggs (medium) and >1.0mg kg^{-1} eggs (high). It was intended to apply ANCOVA tests to the raw length data using 'age' as a covariate in order to investigate differences in growth rates between the treatments. However, the data did not meet the requirements of a normal distribution (Anderson-Darling, $p < 0.05$ in all cases) or having equal variances (Bartlett's homogeneity of variance, $p < 0.05$ in all cases) even following $\log_{10}$, $\log_e$ or square root transformations. Therefore, the growth rate from hatch to metamorphosis was calculated by linear regression for individual spawns (Table 4.15). It can be seen that there was variation within each treatment group though observation of the growth curves 2004 and 2005 [Fig. 4.7 and 4.8 (re-grouped)] appeared to show little differences in mean growth rate. The growth rates grouped according to egg PCB concentration met the requirements of ANOVA (Anderson-Darling, A = 0.440, p = 0.264; Bartlett's, T = 3.235, p = 0.357). No significant differences were detected between the growth rates across the treatments (Table 4.16) though the low p-value suggests a possible trend toward lower growth rates at low and medium PCB concentrations.

4.4.4 Routine swimming behaviour

A total of 2250 larvae from spawns over both years were observed for routine swimming behaviour in terms of the number of grid lines crossed on the arena floor in a 30-second period (Figs. 4.11-4.13). Of the three development stages measured, swimming activity was greatest 7-dph in both years (Fig. 4.12). At metamorphosis, the larvae swam around the arena very little, preferring to settle on the bottom and moving only occasionally (Fig. 4.13). The data for routine swimming behaviour were not combined between years as was the growth data because the conditions in the room were considered to be slightly different between years. A different air-conditioning unit was installed in the second year and may have caused an increase in ambient noise levels that may have influenced larval behaviour. Observation of the box plots (Figs. 4.11 - 4.13) highlights an apparent difference between the control larval groups, with those in 2005 appearing to swim around less than those from 2004.

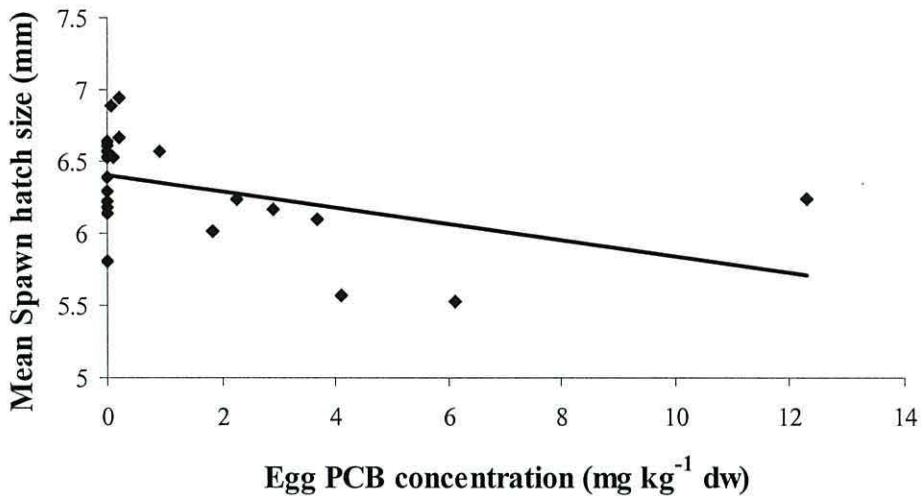


Figure 4.9 The relationship between egg ΣPCB(21 congeners) concentration (mg kg^{-1} dry wt.) and mean spawn hatch length (mm) of plaice (*Pleuronectes platessa*) larvae from females dosed with Aroclor 1254. The regression equation is given by $y = -0.0573x + 6.4081$ ($r^2 = 0.202$, n = 23, $p = 0.031$).

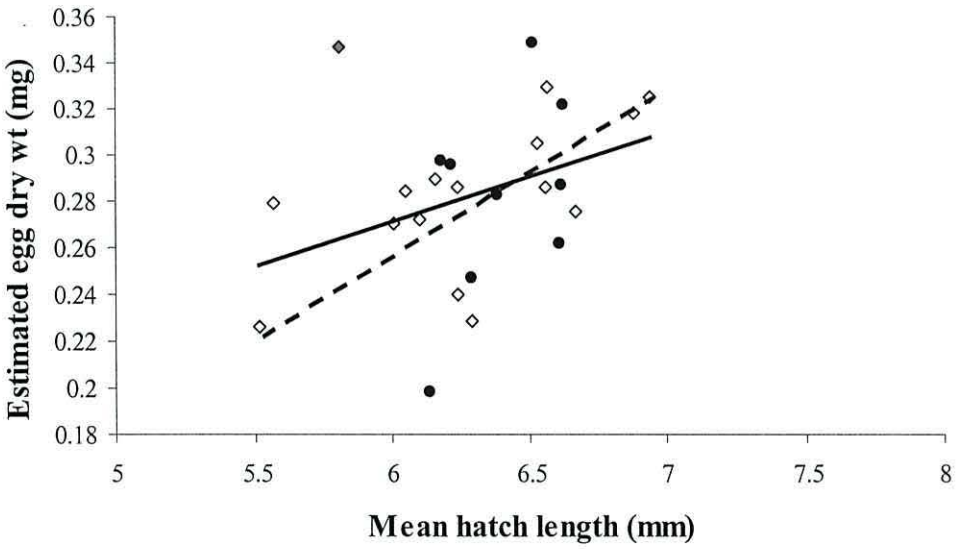
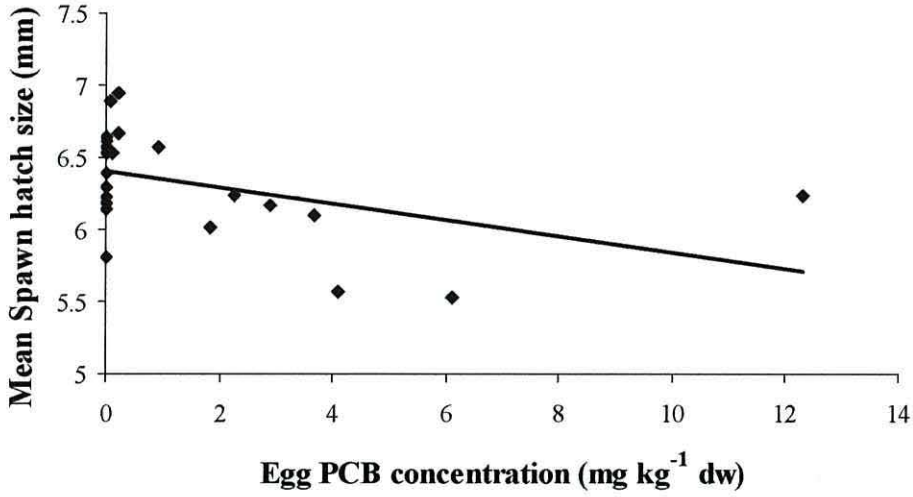


Figure 4.10 The relationship between egg dry weight (mg) and mean hatch length (mm) in plaice (*Pleuronectes platessa*) larvae both dosed (open diamonds) and undosed (closed circles) with Aroclor 1254 ($p = 0.094$). The regression equation (solid line) is given by $y = 0.0305x + 0.0926$ ($r^2 = 0.1287$, n = 25, $p = 0.066$). Removal of the outlying point (red) makes the relationship highly significant ($r^2 = 0.3151$, n = 24, $p = 0.007$) and the regression equation (dotted line) is $y = 0.0451x - 0.0034$.



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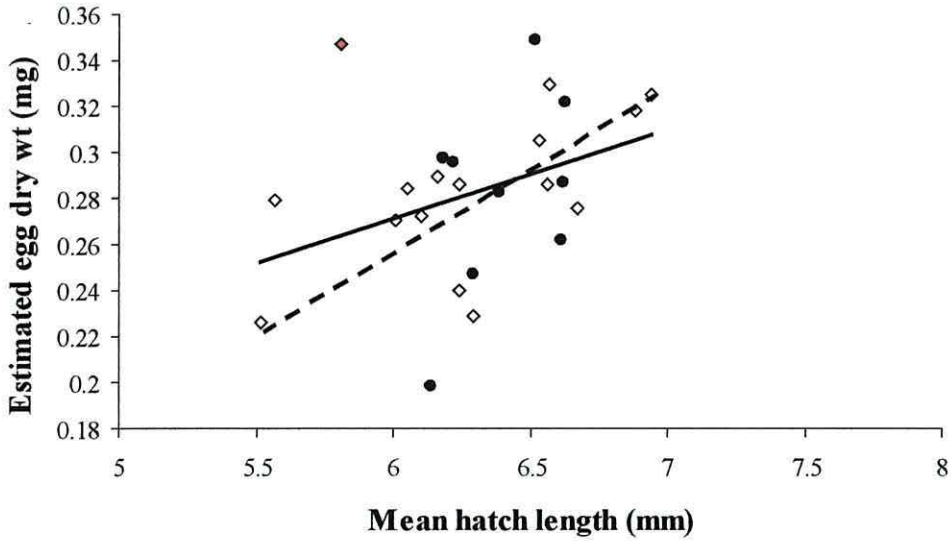

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Table 4.15 Larval growth rates (mm day^{-1} ±SD) calculated by linear regression for spawns collected from plaice (*Pleuronectes platessa*) broodstock dosed with Aroclor 1254. The eggs were either from undosed fish (control) or contained PCBs at concentrations of <0.1mg kg^{-1} dry wt (low), 0.1-1.0mg kg^{-1} dry wt (medium), >1.0mg kg^{-1} dry wt (high).

	Growth rates (mm day^{-1} ±SD)			
	Control	Low	Medium	High
	0.1200	0.1172	0.1122	0.1186
	0.1351	0.1087	0.1207	0.1274
	0.1347	0.0982	0.0958	0.1202
	0.1308		0.0819	0.1121
	0.1540			0.1328
	0.1337			0.1369
	0.0964			
	0.0974			
Mean	0.1253	0.1080	0.1027	0.1247
SD	0.0198	0.0095	0.0173	0.0094

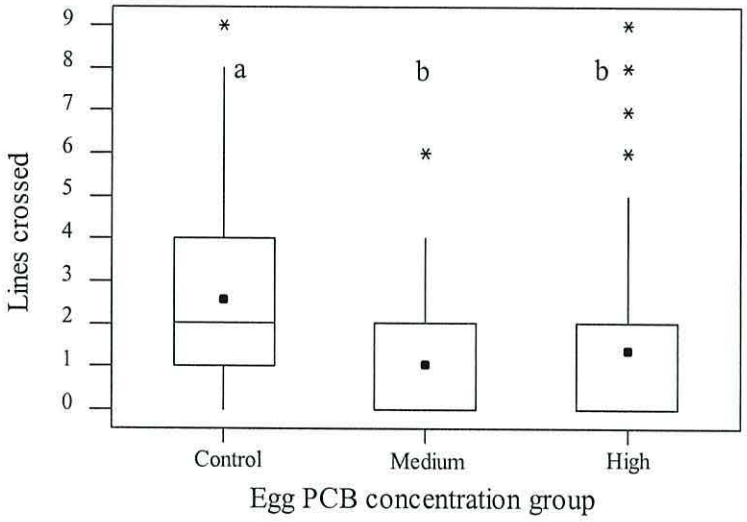
Table 4.16 ANOVA of the growth rates of larval plaice (*Pleuronectes platessa*) either from undosed fish (control) or from females dosed with Aroclor 1254 and eggs containing PCBs at concentrations of <0.1mg kg^{-1} dry wt (low), 0.1-1.0mg kg^{-1} dry wt (medium), >1.0mg kg^{-1} dry wt (high).

Source	df	Adjusted sum of squares	Adjusted mean squares	F	*p*
Treatment	3	0.0019269	0.0006423	2.56	0.089
Error	17	0.0042642	0.0002508		
Total	20				

Table 4.17 Kruskal-Wallis analyses of routine swimming behaviour (number of grid lines crossed in a 30-second period), of plaice (*Pleuronectes platessa*) larvae from broodstock undosed with Aroclor 1254 or from eggs containing either high concentrations (>1.0mg kg^{-1} dry wt), medium concentrations (0.8-1.0mg kg^{-1} dry wt) or low concentrations (<0.2mg kg^{-1} dry wt) of PCBs. Significant differences are marked with *.

Year	Comparison	Development stage	df	n	H	*p*
2004	Undosed vs high dose	Hatch	1	360	47.50	<0.001 *
	Undosed vs medium dose	Hatch	1	270	45.55	<0.001 *
	Medium vs high dose	Hatch	1	270	0.34	0.559
2005	Undosed vs low dose	Hatch	1	270	1.94	0.164
2004	Undosed vs high dose	First feeding	1	360	7.59	0.006 *
	Undosed vs medium dose	First feeding	1	270	1.18	0.277
	Medium vs high dose	First feeding	1	270	1.90	0.168
2005	Undosed vs low dose	First feeding	1	240	7.32	0.007 *
2004	Undosed vs high dose	Metamorphosis	1	330	0.82	0.364
	Undosed vs medium dose	Metamorphosis	1	210	1.59	0.208
	Medium vs high dose	Metamorphosis	1	240	0.48	0.489
2005	Undosed vs low	Metamorphosis	1	240	0.96	0.326

A Hatch 2004



B Hatch 2005

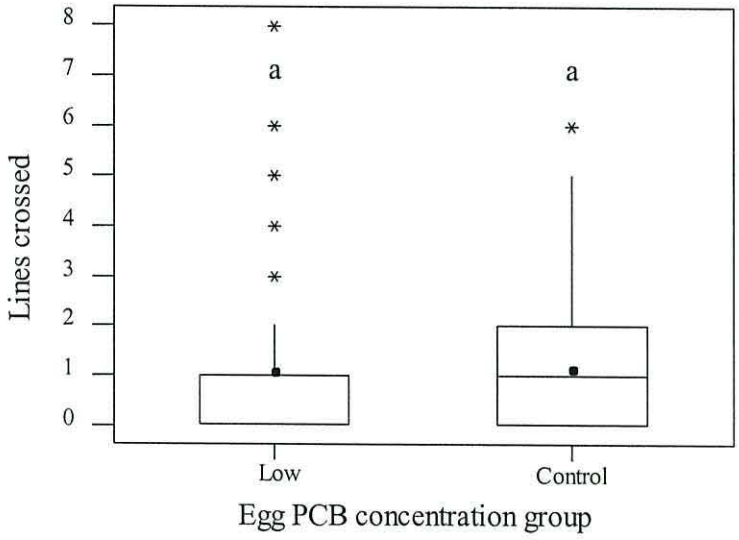
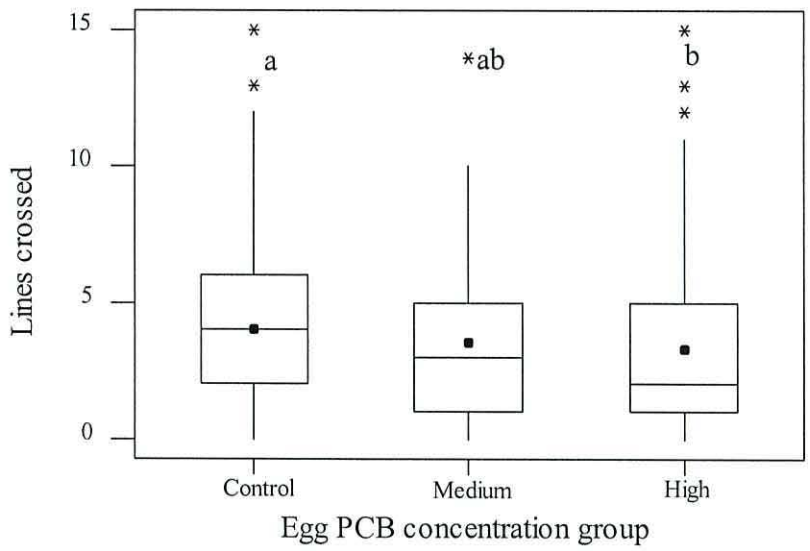


Figure 4.11 Routine swimming activity (number of grid lines crossed in 30-seconds) of newly hatched plaice (*Pleuronectes platessa*) larvae from eggs either undosed or with ΣPCB(21 congener) concentrations of high (>1.0mg kg^{-1} dry wt.), medium (0.2 - 1.0mg kg^{-1} dry wt.) or low (<0.1mg kg^{-1} dry wt.) exposed via maternal transfer in 2004 (**A**) and 2005 (**B**). Boxplots show the median (solid line) and mean (black dot) number of lines crossed, interquartile range and outliers. No median line means median = 0.

A First feeding 2004



B First Feeding 2005

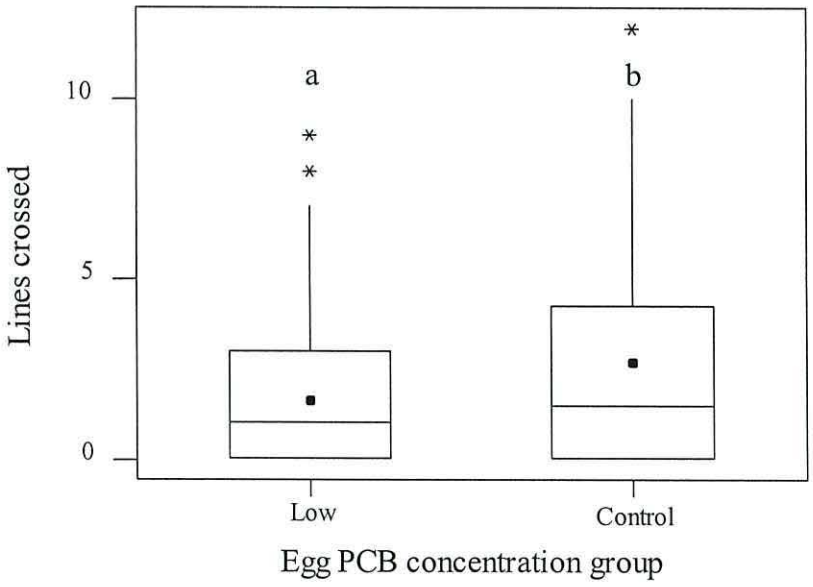
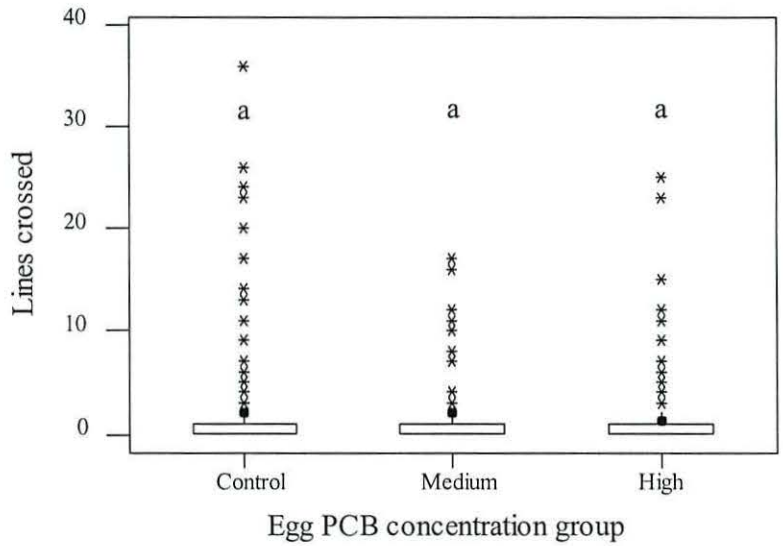


Figure 4.12 Routine swimming activity (number of grid lines crossed in 30-seconds) of plaice (*Pleuronectes platessa*) larvae at 8-days post hatch from eggs either undosed or with ΣPCB(21 congener) concentrations of high (>1.0mg kg^{-1} dry wt.), medium (0.2 - 1.0mg kg^{-1} dry wt.) or low (<0.1mg kg^{-1} dry wt.) exposed via maternal transfer in 2004 (**A**) and 2005 (**B**). Boxplots show the median (solid line) and mean (black dot) number of lines crossed, interquartile range and outliers.

A Metamorphosis 2004



B Metamorphosis 2005

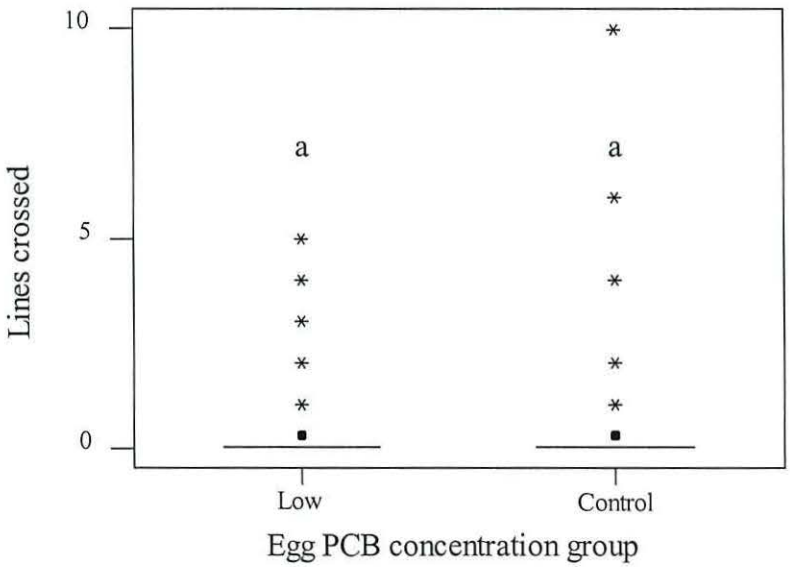


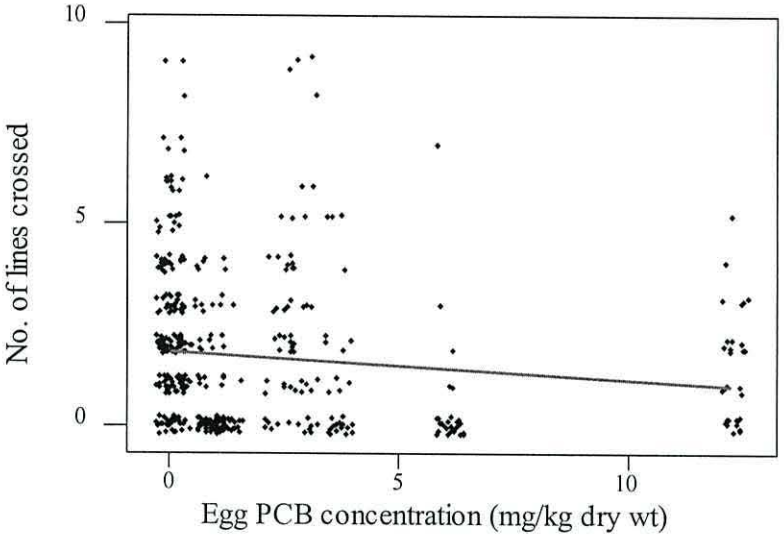
Figure 4.13 Routine swimming activity (number of grid lines crossed) in 30-seconds of newly metamorphosed juvenile plaice (*Pleuronectes platessa*) at 42-days post hatch from eggs either undosed or with ΣPCB(21 congener) concentrations of high (>1.0mg kg^{-1} dry wt.), medium (0.2 - 1.0mg kg^{-1} dry wt.) or low (<0.1mg kg^{-1} dry wt.) exposed via maternal transfer in 2004 (**A**) and 2005 (**B**). Boxplots show the median (solid line) and mean (black dot) number of lines crossed, interquartile range and outliers. No median line means median = 0.

Data did not meet the requirements of ANOVA under any transformations and therefore the non-parametric Kruskal-Wallis test was used. There were significant differences in the number of grid lines crossed in 30-seconds by larvae at hatch only in 2004 (Table 4.17 and Figs. 4.11) between larvae from eggs with both medium and high PCB concentrations compared to the control larvae. These differences were still apparent at first-feeding in the high-dosed larvae in 2004 but also in the low-dosed larvae from 2005. No differences were observed at metamorphosis and the newly settled plaice from all treatments swam very little around the behavioural arenas.

Given the significant differences that were found between treatments, correlations of egg PCB content and routine swimming activity were investigated (Figs. 4.14 and 4.15). Observation of the scatter plots confirmed that there was much variation in swimming activity across all egg PCB concentrations. Nevertheless, the raw data for routine swimming activity in 2004 of high- and medium-dosed larvae showed significant negative correlations with egg ΣPCB(21 congeners) concentrations at hatch ($r = -0.159$, $p = 0.001$, $n = 540$) and first feeding ($r = -0.101$, $p = 0.033$, $n = 540$) (Fig. 4.14). In 2005 there was significant correlation at hatch ($r = -0.216$, $p = 0.001$, $n = 270$) but not at first feeding ($r = -0.123$, $p = 0.076$, $n = 270$), though a similar trend existed (Fig. 4.15). The plaice swam around the arena very little at metamorphosis resulting in the collection of frequency data consisting mainly of 0s (no lines crossed) and 1s (1 line crossed) and consequently correlations with egg PCB concentrations could not be investigated.

Because the data could only be subjected to the Kruskal-Wallis test to investigate differences based on egg PCB content, the data from each PCB content group in 2004 and 2005 were analysed separately to test for variation at the female and individual replicate test (behavioural arena) level. In this case, after $\log_{10}$ transformation most data sets could be analysed using a nested ANOVA, nesting the individual tests within each spawn. Elsewhere, Kruskal-Wallis tests were used to test for differences between spawns within the separate treatment groups. No significant differences were detected between any of the replicate behavioural arena tests within each female spawn but occasional significant differences were detected between female spawns within each treatment group (Table 4.18). The occurrence of these differences did not

A – 2004, 24-hours post hatch



B – 2004, 8-days post hatch

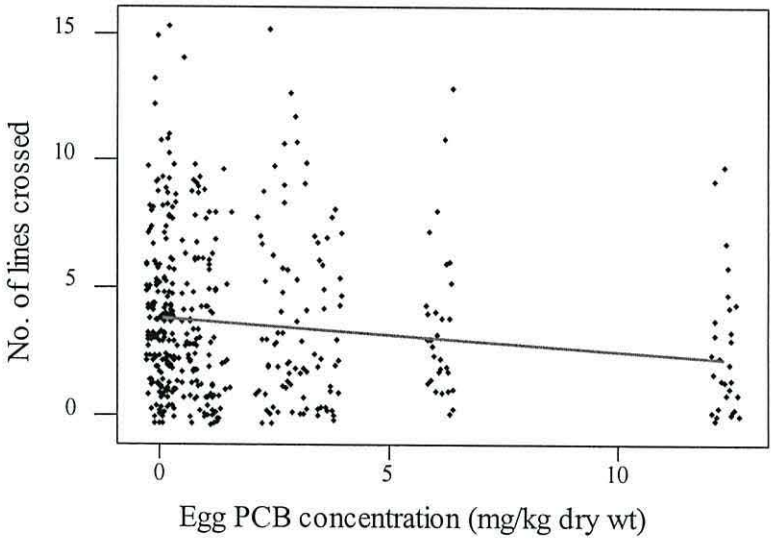
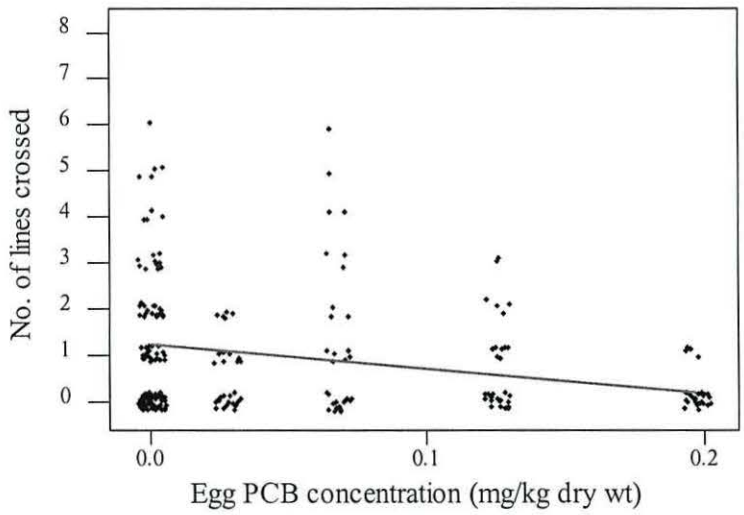


Figure 4.14 Number of 2cm gridlines crossed in 30-seconds by (**A**) 24-hour and (**B**) 8-day old plaice (*Pleuronectes platessa*) larvae in 2004 from spawns with different egg PCB concentrations (mg kg^{-1} dry wt). Significant negative correlations existed at both stages giving the regression lines (**A**) $y = -0.0965x + 1.9745$, $p = 0.001$, n = 540 and (**B**) $y = -0.0978x + 3.8304$, $p = 0.033$, n = 540. Jitter has been added to the data points in order to show the density/spread of data points.

A – 2005, 24-hours post hatch



B – 2005, 8-days post hatch

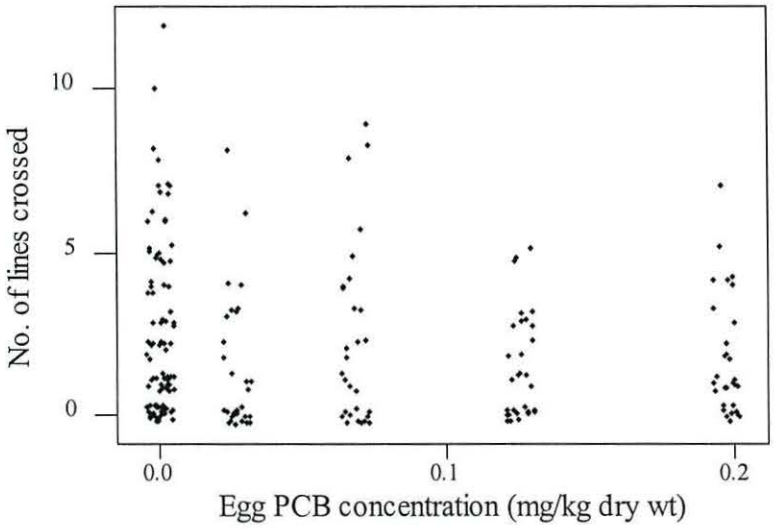


Figure 4.15 Number of 2cm gridlines crossed in 30-seconds by (**A**) 24-hour and (**B**) 8-day old plaice (*Pleuronectes platessa*) larvae in 2005 from spawns with different egg PCB concentrations (mg kg^{-1} dry wt). Significant correlation existed at hatch (**A**) $y = -4.0489x + 1.1119$, $p = 0.001$, n = 270 and a non-significant trend existed at first feeding (**B**) r = -0.123, $p = 0.076$, n = 270. Jitter has been added to the data points in order to show the density/spread of data points.

follow a clear pattern as they were detected in all life stages across experiments from 2004 and 2005.

4.4.5 Vibration response

A total of 1920 larvae were observed for their response to the vibratory stimulus over the experiments in both years. On observation of the data, the mean and median percentage response to the vibration stimulus was greater in 2004 compared to 2005 at all stages of development though there were no obvious differences between any of the treatments (Figs. 4.16 - 4.18). The percentage response of larvae to the vibratory stimulus in each test was arcsine transformed before analysis with nested ANOVA. There were no significant differences between treatments but differences in the percentage response were detected between spawns from different females within treatments at metamorphosis in 2004 and first-feeding in 2005 (Table 4.19).

As there were no differences in the percentages of larvae responding to the vibratory stimulus, differences between treatments in the latency of response were investigated for those larvae that did respond to the vibrational stimulus. The latency of response was similar for larvae at hatch and at first-feeding with mean and median response times at around four and three frames in 2004 and 2005 respectively (Figs. 4.19 - 4.21). At metamorphosis the response time had reduced to two to three frames in both years (Fig. 4.21). The data did not meet the requirements of ANOVA under any transformations and the non-parametric Kruskal-Wallis test was. No significant differences in the latency of response to the vibrational stimulus were detected between any treatment groups in 2004 and 2005 (Table 4.20).

Table 4.18 ANOVA and Kruskal-Wallis tests for differences in routine swimming activity of plaice (*Pleuronectes platessa*) larvae between replicate tests and/or between female spawns in each PCB treatment group at hatch, first-feeding and metamorphosis in 2004 and 2005. Significant differences are marked with *.

Source	df	Adjusted sum of squares	Adjusted mean squares	F	*p*
Control larvae at hatch, 2004					
Female	5	0.28071	0.05614	0.87	0.503
Test(female)	12	1.10453	0.09204	1.43	0.161
Error	135	8.71278	0.06454		
Total	152				
Medium egg PCB concentration larvae at hatch, 2004					
Female	2	0.16325	0.08162	1.51	0.235
Test(female)	4	0.39954	0.09988	1.84	0.141
Error	37	2.00594	0.05421		
Total	43				
High egg PCB concentration larvae at hatch, 2004					
Female	5	0.31194	0.06239	0.72	0.609
Test(female)	10	0.39975	0.03998	0.46	0.908
Error	70	6.04291	0.08663		
Total	85				
Control larvae at first feeding, 2004					
Female	5	2.37637	0.47527	5.19	<0.001 *
Test(female)	12	1.27792	0.10649	1.16	0.316
Error	146	13.38145	0.09165		
Total	163				
Medium egg PCB concentration larvae at first feeding, 2004					
Female	2	0.2457	0.1229	1.19	0.309
Test(female)	6	0.4430	0.0738	0.72	0.637
Error	72	7.4088	0.1029		
Total	80				
High egg PCB concentration larvae at first feeding, 2004					
Kruskal-Wallis Test for differences between females					
H = 4.74	df = 5	n = 180	*p* = 0.449		
Control larvae at metamorphosis, 2004					
Kruskal-Wallis Test for differences between females					
H = 16.27	df = 4	n = 150	*p* = 0.003 *		
Medium egg PCB concentration larvae at metamorphosis, 2004					
Kruskal-Wallis Test for differences between females					
H = 1.49	df = 1	n = 60	*p* = 0.222		
High egg PCB concentration larvae at metamorphosis, 2004					
Kruskal-Wallis Test for differences between females					
H = 16.53	df = 5	n = 180	*p* = 0.005 *		
Control larvae at hatch, 2005					
Kruskal-Wallis Test for differences between females					
H = 3.47	df = 3	n = 120	*p* = 0.324		

Contd. overleaf

Table 4.18 *Contd.*

Low egg PCB concentration larvae at hatch, 2005

Female	4	0.78121	0.19530	2.95	0.028 *
Test(female)	8	0.13439	0.01680	0.25	0.978
Error	52	3.43993	0.06615		
Total	64				

Control larvae at ff, 2005

Kruskal-Wallis Test for differences between females

H = 7.40 df = 2 n = 90 $p = 0.025$

Low egg PCB concentration larvae at hatch, 2005

Female	4	0.54774	0.13694	1.77	0.144
Test(female)	10	1.48873	0.14881	1.93	0.056
Error	70	5.40565	0.07722		
Total	84				

Control larvae at metamorphosis, 2005

Kruskal-Wallis Test for differences between females

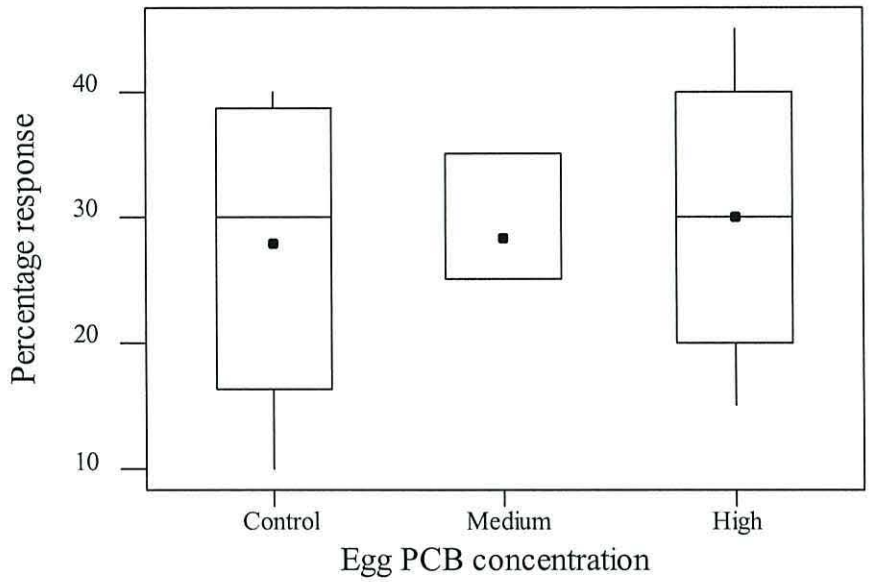
H = 0.79 df = 2 n = 90 $p = 0.672$

Medium egg PCB concentration larvae at metamorphosis, 2004

Kruskal-Wallis Test for differences between females

H = 2.98 df = 4 n = 150 $p = 0.561$

A Hatch, 2004



B Hatch 2005

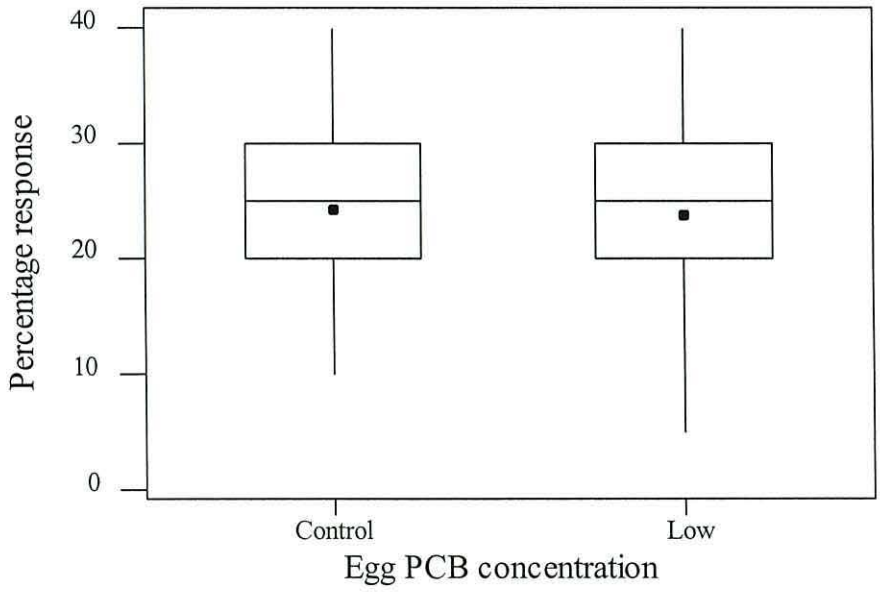
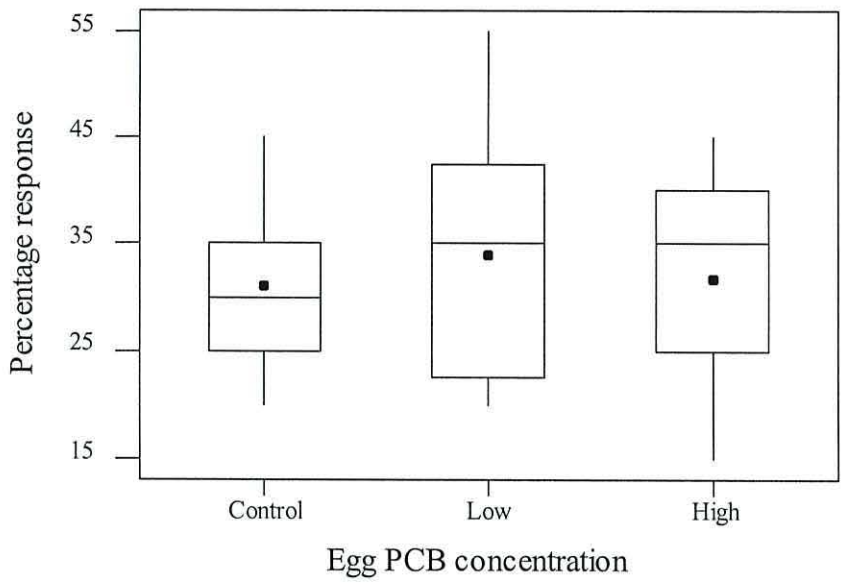


Figure 4.16 Percentage response to a vibratory stimulus of newly hatched plaice (*Pleuronectes platessa*) larvae from eggs either undosed or with ΣPCB(21 congener) concentrations of high (>1.0mg kg^{-1} dry wt.), medium (0.2 - 1.0mg kg^{-1} dry wt.) or low (<0.1mg kg^{-1} dry wt.) exposed via maternal transfer in 2004 (**A**) and 2005 (**B**). Boxplots show the median (solid line; =25 for medium in 2004) and mean (black dot) percent response, interquartile range and outliers.

A First-feeding, 2004



B First-feeding, 2005

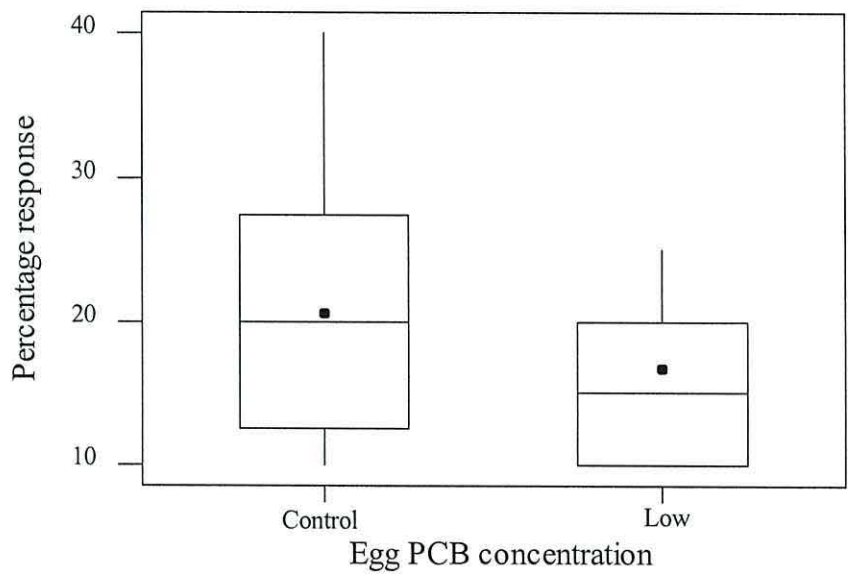
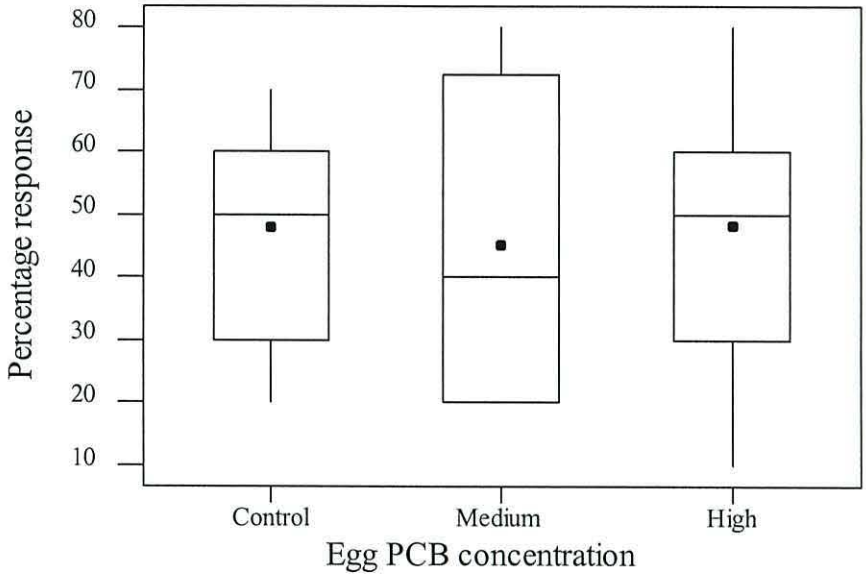


Figure 4.17 Percentage larval response to a vibratory stimulus of plaice (*Pleuronectes platessa*) larvae at first-feeding from eggs either undosed or with ΣPCB(21 congener) concentrations of high (>1.0mg kg^{-1} dry wt.), medium (0.2 - 1.0mg kg^{-1} dry wt.) or low (<0.1mg kg^{-1} dry wt.) exposed via maternal transfer in 2004 (A) and 2005 (B). Boxplots show the median (solid line) and mean (black dot) percent response, interquartile range and outliers.

A Metamorphosis, 2004



B Metamorphosis, 2005

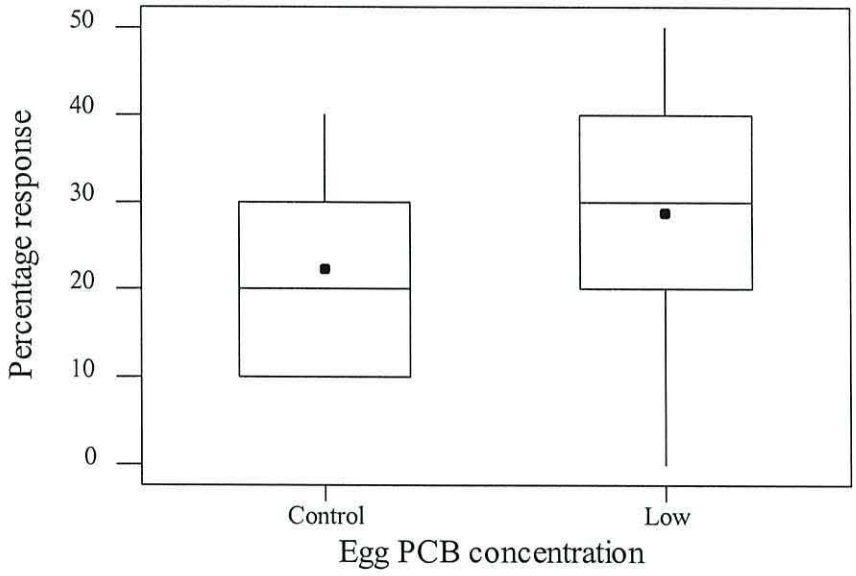
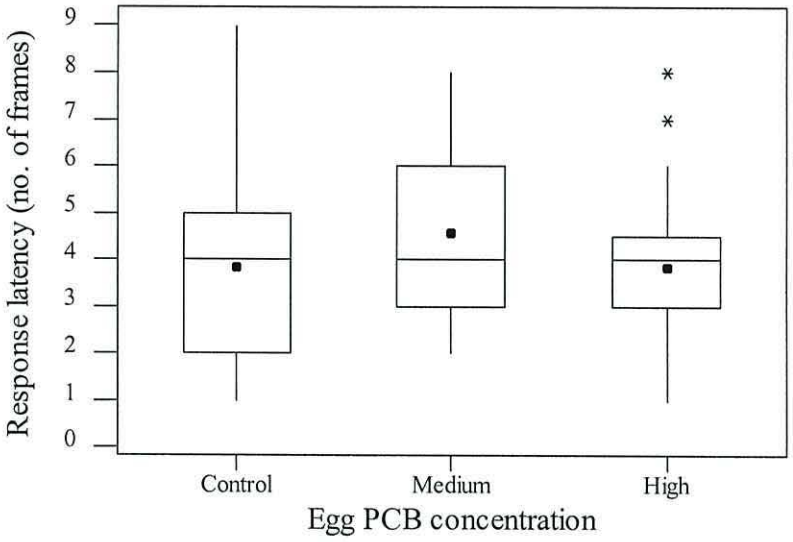


Figure 4.18 Percentage response to a vibratory stimulus of plaice (*Pleuronectes platessa*) larvae at metamorphosis, from eggs either undosed or with ΣPCB(21 congener) concentrations of high (>1.0mg kg^{-1} dry wt.), medium (0.2 - 1.0mg kg^{-1} dry wt.) or low (<0.1mg kg^{-1} dry wt.) exposed via maternal transfer in 2004 (**A**) and 2005 (**B**). Boxplots show the median (solid line) and mean (black dot) percent response, interquartile range and outliers.

Table 4.19 ANOVA comparisons of percentage response to a vibratory stimulus of plaice (*Pleuronectes platessa*) larvae from broodstock either undosed or given high (2-3.5mg), medium (1-1.75mg) or low (0.1-0.175) monthly doses of Aroclor 1254.

Year/PCB dose	Development stage	Comparison	df	n	Adjusted sum of squares	Adjusted mean squares	F	*p*
2004	Hatch	Treatments	2	33	0.00368	0.00184	0.17	0.847
Undosed, high, medium		Females(treatments)	8	33	0.08560	0.01070	0.97	0.482
		Error	22		0.24217	0.01101		
		Total	32					
	First feeding	Treatments	2	42	0.013447	0.006724	0.53	0.595
		Females(treatments)	10	42	0.095520	0.009552	1.20	0.333
		Error	29		0.266984	0.009206		
		Total	41					
	Metamorphosis	Treatments	2	39	0.00943	0.00472	0.13	0.880
		Females(treatments)	8	39	0.73076	0.09134	2.48	0.036
		Error	28		1.02965	0.03677		
		Total	38					
2005	Hatch	Treatments	1	27	0.000175	0.000175	0.03	0.865
Undosed, low		Females(treatments)	7	27	0.072487	0.010355	1.77	0.155
		Error	18		0.105084	0.005838		
		Total	26					
	First feeding	Treatments	1	24	0.009186	0.009186	2.99	0.103
		Females(treatments)	6	24	0.078253	0.013042	4.24	0.010
		Error	16		0.049226	0.003077		
		Total	23					
	Metamorphosis	Treatments	1	24	0.02597	0.02597	1.63	0.221
		Females(treatments)	6	24	0.10910	0.01818	1.24	0.385
		Error	16		0.25557	0.01597		
		Total	23					

A Hatch, 2004



B Hatch, 2005

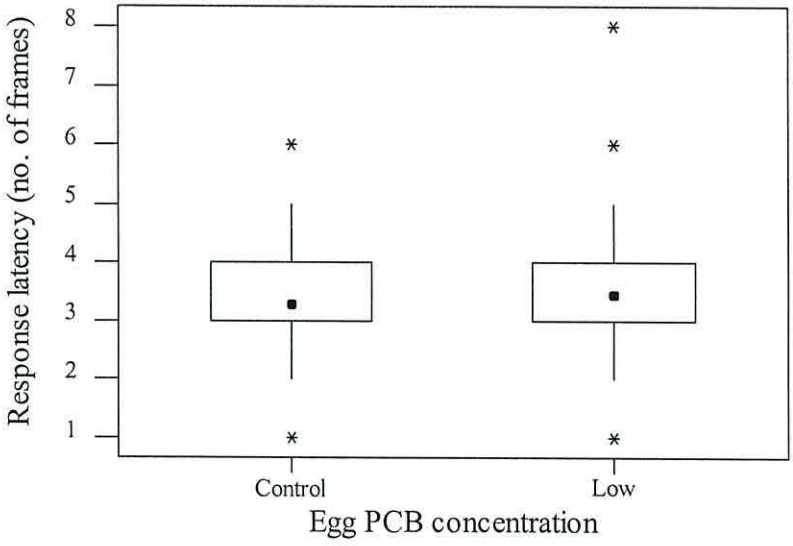
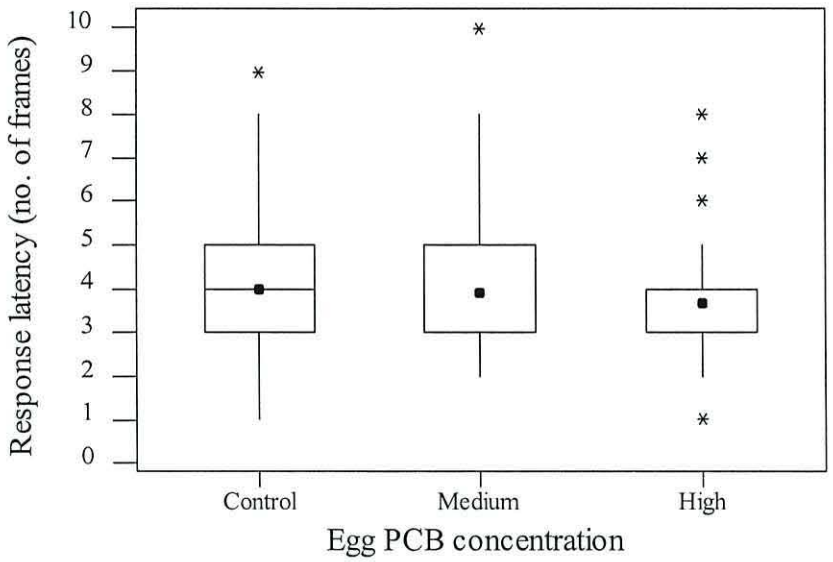


Figure 4.19 Response latency (no. of frames) to a vibratory stimulus of newly hatched plaice (*Pleuronectes platessa*) larvae from eggs either undosed or with ΣPCB(21 congener) concentrations of high (>1.0mg kg^{-1} dry wt.), medium (0.2 - 1.0mg kg^{-1} dry wt.) or low (<0.1mg kg^{-1} dry wt.) exposed via maternal transfer in 2004 (**A**) and 2005 (**B**). Boxplots show the median (=3 in both treatments) and mean (black dot) percent response, interquartile range and outliers.

A First-feeding, 2004



B First-feeding, 2005

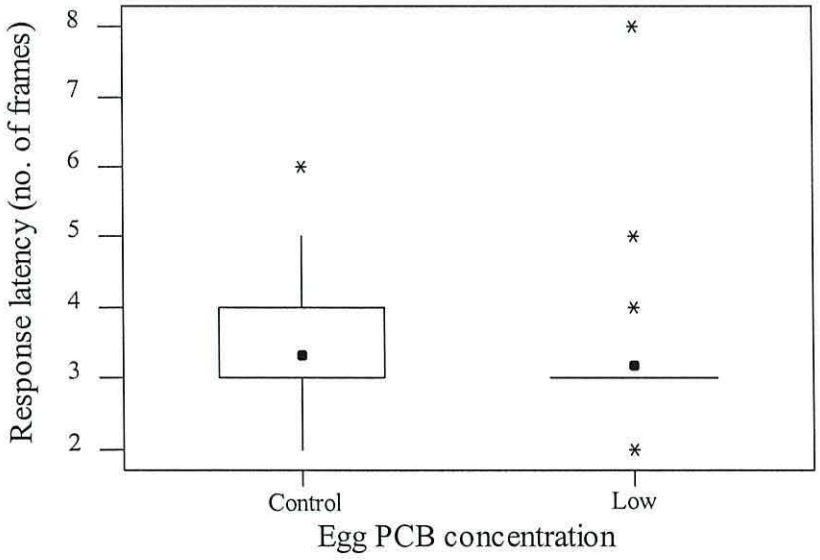
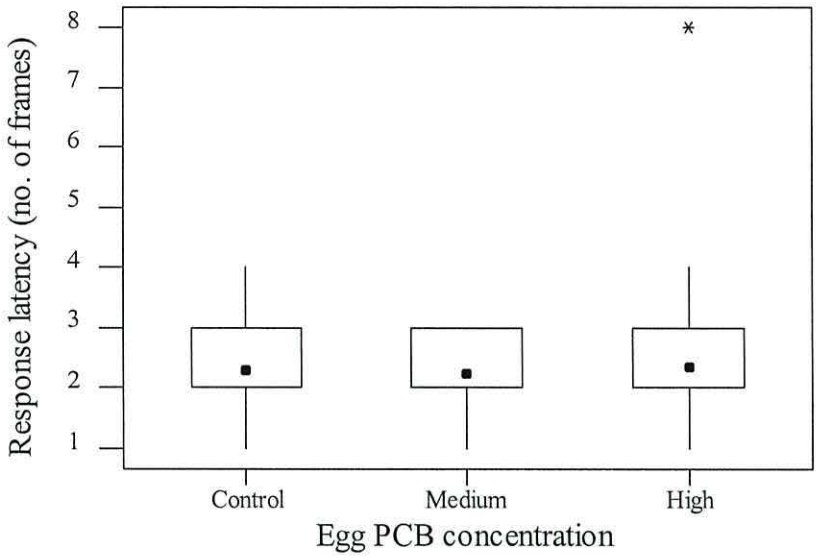


Figure 4.20 Total percentage response to a vibratory stimulus of plaice (*Pleuronectes platessa*) larvae at 8-days post hatch from eggs either undosed or with ΣPCB(21 congener) concentrations of high (>1.0mg kg^{-1} dry wt.), medium (0.2 - 1.0mg kg^{-1} dry wt.) or low (<0.1mg kg^{-1} dry wt.) exposed via maternal transfer in 2004 (**A**) and 2005 (**B**). Boxplots show the median (solid line, =3 where not visible) and mean (black dot) percent response, interquartile range and outliers.

A Metamorphosis, 2004



B Metamorphosis, 2005

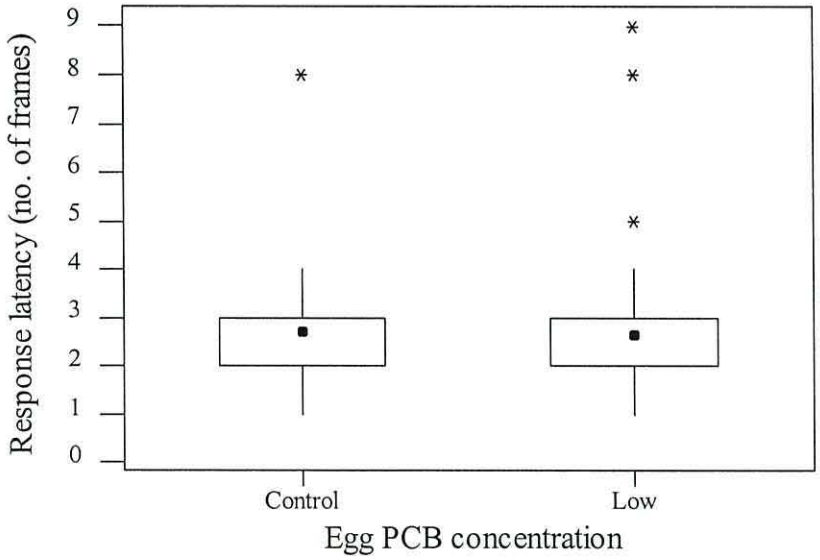


Figure 4.21 Total percentage response to a vibratory stimulus of newly metamorphosed juvenile plaice (*Pleuronectes platessa*) from eggs either undosed or with ΣPCB(21 congener) concentrations of high (>1.0mg kg^{-1} dry wt.), medium (0.2 - 1.0mg kg^{-1} dry wt.) or low (<0.1mg kg^{-1} dry wt.) exposed via maternal transfer in 2004 (**A**) and 2005 (**B**). Boxplots show the median (=2 in all cases) and mean (black dot) percent response, interquartile range and outliers.

Table 4.20 Kruskal-Wallis analysis of latency of response to a vibratory stimulus of plaice (*Pleuronectes platessa*) larvae from eggs either undosed or with ΣPCB(21 congeners) concentrations of high (>1.0mg kg^{-1} dry wt.), medium (0.2 - 1.0mg kg^{-1} dry wt.) or low (<0.1mg kg^{-1} dry wt.) exposed via maternal transfer of Aroclor 1254 in 2004 and 2005.

Year/PCB dose	Comparison	Development stage	df	n	H	*p*
2004	Undosed vs high	Hatch	1	176	0.24	0.621
	Undosed vs medium		1	85	2.51	0.113
	Medium vs high		1	127	2.61	0.106
	Undosed vs high	First feeding	1	208	1.96	0.162
	Undosed vs medium		1	155	0.16	0.691
	Medium vs high		1	175	0.64	0.424
	Undosed vs high	Metamorphosis	1	159	0.01	0.942
	Undosed vs medium		1	99	0.21	0.648
	Medium vs high		1	114	0.18	0.668
2005	Undosed vs low	Hatch	1	134	0.49	0.485
	Undosed vs low	First feeding	1	87	1.35	0.246
	Undosed vs low	Metamorphosis	1	63	0.90	0.344

4.5 Discussion

As reported in chapter 3, the concentrations of PCBs in both the plaice broodstock and eggs used in these experiments (Table 3.3) are representative of those found under field conditions for plaice as well as other marine and freshwater fish species (Table 3.14). Although the plaice may spend much of its life offshore, those stages of the lifecycle inhabiting inshore areas or areas with localised, elevated contamination, remain at risk from accumulating high contaminant body burdens including PCBs.

4.5.1 Aquarium conditions

The conditions in both broodstock tanks and larval rearing tanks were kept as similar as possible between experiments conducted in 2004 and 2005. Water temperatures were lower in 2005 due to the installation of a superior aquarium system and colder ambient seawater temperatures in the Menai Strait. In 2004, fish in all treatment groups began to spawn at approximately the same time and continued to do so until sufficient spawns had been collected for all treatment groups. The apparent peak in high dose fish spawning in 2004 (Fig. 4.2 A) cannot be linked easily to the treatments administered to the fish and as all tank conditions were as uniform as possible, the peak is most probably due to random differences in fish spawning between the tanks.

Larval growth is influenced by many endogenous and exogenous factors including genetics, maternal contribution (i.e. yolk size), food, temperature, oxygen and salinity (Jones, 2002). Conditions in the rearing tanks were monitored twice daily throughout the larval growth period. The similarity in larval tank temperatures between both years (section 4.4.3) together with the use of the same water system (i.e. supply, filtration and water baths), feeding regimes and lack of accumulation of ammonia, nitrates or nitrites meant that fluctuations in exogenous influences on larval growth were minimized. Therefore, it was feasible to combine the data from both years in order for most analyses to be completed by egg ΣPCB(21 congeners) concentration and also to account for individual differences in PCB uptake. The error bars for the growth data (Figs. 4.7 and 4.8) are smallest at hatch and although they increase with

age, a natural process known as "growth depensation" and caused by cumulative endogenous and exogenous effects, they remain small. This supports the evidence that tank conditions were carefully and uniformly controlled and allowed significant differences to be observed between the treatments. Growth depensation is commonly seen in laboratory measurements of larval length (Ehrlich et al., 1976; Ryer & Olla, 1996; Smith & Fuiman, 2003) and is also thought to be a natural phenomenon (Fuiman et al., 2005).

4.5.2 Survival

Although significant differences were detected in the egg dry weights between fish from different treatment regimes (Chapter 3, section 3.4.6), in the majority of cases, no significant differences in proportional survival at 3, 11 and 18-dpf were detected between the Aroclor 1254-dosed groups, except in 2005 (Tables 4.3 to 4.5). There were no significant correlations detected between proportional survival and either egg PCB concentrations (Table 3.3) or egg size (Fig. 4.5). The significant differences detected in the numbers surviving at 11- and 18-dpf between the control and low dosed groups in 2005 in which the low dosed larvae had better survival (Fig. 4.4 B) could be due to the PCBs in the eggs. However, given the lack of correlation between survival and egg PCB concentration, the effect of the small sample size for the control group fish (n = 3) compared to the low dose fish (n = 5) is more likely. Also as the fish were hand-stripped of eggs when judged ready to spawn it is possible that some fish were stripped too early or too late which might have had an impact on egg viability (Fonds et al., 1995). In both years, there was significant variation in survival of larvae from different females within each treatment group which might be attributable to the timing of spawning, natural variation or individual PCB body burdens (Tables 4.4 and 4.5). Fuiman et al. (2005) reported occurrence of the "batch effect" in red drum (*Sciaenops ocellatus*) larvae whereby intrinsic variability in larval viability exists between spawns and may be due to maternal investment in egg quality. Therefore, if the batch effect were occurring here, larger sample sizes of female spawns would be likely to yield more accurate results. This is discussed again, later in this section.

There was no difference in the initial rate of egg mortality in the first 3-dpf between any of the treatments in both 2004 and 2005 (Tables 4.4 and 4.6). This, combined with the results above suggesting a lack of significant negative impacts of PCBs on the hatch success of plaice eggs fits well with other studies. Fonds et al. (1995) found no significant effects of Clophen A40 on the hatch success of dab (*Limanda limanda*) eggs exposed in the same manner as this study whilst Hansen et al. (1985) reported only minor effects on hatch success of Baltic herring (*Clupea harengus*) at PCB concentrations over 120μg kg^{-1} wet wt.

The toxic effects of contaminants may manifest themselves only at certain stages of development and this seemed to become more apparent for Aroclor 1254 in these experiments in terms of survival post-hatch. When the percentage of hatched larvae surviving to the first-feeding stage (18-dpf) was analysed, significant increases in mortality were only detected at the highest egg PCB concentrations (Table 4.7). This might indicate that PCB toxicity occurs during the early larval stage in plaice as opposed to the embryonic stages as differences in embryo survival to hatch (11-dpf) were not significantly different from one another and would coincide with the mobilisation of PCBs from the yolk as it is metabolised. Mauck et al. (1978) exposed brook trout (*Salvelinus fontinalis*) embryos and fry to Aroclor 1254 and found no differences in mortality of the embryos or sac fry but survival was affected at 48-dph following yolk absorption.

Effects of PCBs on the survival of fish larvae are often variable and depend on the species and type of PCBs employed in experiments. Matta et al. (1998) investigated the survival of rainbow trout (*Oncorhynchus mykiss*) fry exposed to Aroclor 1260 from median hatch (25-dpf) to yolk resorption at 31-dpf and found no differences in survival with whole body PCB concentrations of up to 2.5mg kg^{-1} compared to control larvae. No mention was made as to whether these concentrations were on a wet or dry weight basis, making comparison to this study difficult. A hydroxylated PCB (a PCB metabolite) was shown to significantly reduce survival of westslope cutthroat trout (*Oncorhynchus clarki lewisi*) fry and rainbow trout fry at concentrations 1-2 orders of magnitude higher than those determined in this study (Matta et al., 1997). However, as the metabolite was not measured in routine environmental samples from the fish' habitats it could not be stated whether the

concentrations at which the effects were observed were realistic. In a later study, Matta et al. (2001) exposed adult mummichogs (*Fundulus heteroclitus*) to Aroclor 1268 and found no effect on larval survival even at concentrations similar to those found at a hazardous waste site inhabited by the species.

In contrast, studies with other PCB mixtures or individual PCB congeners have shown larval survival to be reduced significantly. McCarthy et al. (2003) reported unpublished data indicating reduced survival of Atlantic croaker (*Micropogonias undulatus*) larvae following maternal exposure to Aroclor 1254. Embryos from female zebrafish (*Danio rerio*) exposed to a mixture of eight PCB congeners suffered significantly increased mortality compared to fish from undosed females even though there was no observed impact on fecundity (Westerlund et al., 2000).

Whilst the variability in the results reported above might be due to inter-specific differences in susceptibility, the use of different PCB mixtures will inevitably expose the experimental subjects to different concentrations of specific congeners. Studies evaluating the toxicities of specific congeners can be useful in determining exactly which congeners have the most toxic effects. Olsson et al. (1999) exposed female zebrafish to CB-60, -104 and -190. CBs 60 and 104 had the greatest effect on embryo and larval mortality whilst CB-190 was implicated in causing morphological abnormalities of the cranium. None of the congeners had an effect on maternal fecundity.

The differences in PCB toxicities reported from studies of this kind, could be due to the different commercial PCB mixtures and their respective congener profiles but also due to interspecific variation in susceptibility to the different mixtures or specific congeners. Weis & Weis (1982) list several studies in which this has been reported: Aroclor 1254 being more toxic than Aroclor 1242 to the fathead minnow (*Pimephales promelas*) (Nebeker et al., 1974) but Aroclor 1242 being more toxic than Aroclor 1254 to cutthroat trout (Mayer et al., 1977). Results such as these suggest that experiments using several different PCB mixtures and/or individual congeners should be tested on a species before conclusions are reached regarding susceptibility.

Although results from laboratory studies such as those mentioned above can be variable, in general they correlate well with results found during field studies whereby the impacts of contaminants on fecundity may be low or insignificant but embryo or larval survival is reduced. For example, perch (*Perca fluviatilis*) downstream from a Baltic paper mill exhibited no differences in spawning frequencies compared to fish from other areas. Adult and larval abundance was reduced significantly at the contaminated site, indicating higher mortality in the late embryonic or early larval developmental periods (Karås et al., 1991). The main difficulty with field-based studies of this kind is determining the exact causes of any observed effects due to the complex mixture of contaminants found in wild fish. Embryo and larval survival have been reduced in both flounder (von Westernhagen et al., 1981) and Arctic charr (Monod, 1985) though synergistic chemical effects in both studies could not be ruled out.

4.5.3 Growth

Although no differences were observed in the growth rates of the larvae with respect to egg PCB concentrations, the significant reduction in hatch size of the larvae at the highest egg PCB concentrations was clear (Tables 4.9 – 4.10, Fig. 4.7 & 4.8). The mean size of larvae from all groups exposed to PCBs remained lower than that of the control larvae throughout the growth period with the exception of the first two weeks data for medium egg PCB concentration larvae. However, as stated previously, the lack of sufficient replicates in the medium group means those data should be treated tentatively. Maternal influence seems to play a role in determining egg dry weight (Fig. 3.7) which in turn is correlated positively with size at hatch (Fig. 4.10). Despite this, the influence of PCB concentrations >1.0mg kg^{-1} in the eggs appears to override the maternal influence and reduce the overall size at hatch (Tables 4.9 and 4.11, Fig. 4.9) whereas there was no correlation with maternal condition factor (p153); larvae remain a smaller size throughout development at least until metamorphosis. Therefore, whilst PCBs might have little effect over maternal egg production (section 3.4.5) they may be exerting more influence over the embryos and larvae that must invest energy in metabolising or depurating the contaminants as ontogeny progresses.

As with the survival data, growth rates from other studies on fish larvae using PCBs and other organochlorine chemicals are variable and appear to depend on the PCB mixture and species used. In the study by DeFoe et al. (1978), fathead minnow larvae exposed separately to Aroclor 1248 and 1260 were significantly shorter than control larvae after 30-days acute exposure via the water at concentrations of 1.3 – 4.4μg l^{-1} with the exception of the lowest concentration of Aroclor 1248 (1.1μg l^{-1}). Westin et al. (1983) found no effect of dietary PCB exposure on the growth rates of striped bass larvae. Atlantic croaker larvae did not exhibit a change in growth rates following maternal exposure to *o,p'*-DDT or *p,p'*-DDE (Faulk et al., 1999), whilst in the same species the larval growth rates were impaired by maternal transfer of Aroclor 1254 at realistic environmental concentrations (McCarthy et al., 2003). In contrast, exposure to environmentally realistic concentrations of Aroclor 1268 actually increased the growth rates of *Fundulus heteroclitus* larvae following maternal exposure (Matta et al., 2001). Most recently, Aroclor 1254 was reported to have no significant effect on the body length of young carp when exposed at water concentrations of 10μg l^{-1} for 50 days though a significant reduction did occur when fish were exposed to a mixture of Aroclor 1254 (5μg l^{-1}) and TBT (2μg l^{-1}) (Schmidt et al., 2005). Total body concentrations were not reported.

The two to four day delay observed for the highest dosed larvae in this study to reach the same size as control larvae could have important ecological implications for survival under natural conditions. In this study, larval metamorphosis always far exceeded 50% at the 42-dph endpoint for larval measurements and behaviour in all larval rearing tanks although no total counts were made of this. By remaining a smaller size for a longer time, the duration of the larval stage in the PCB-exposed plaice was not necessarily extended. The onset of metamorphosis in plaice is thought to be under greater influence of temperature and time as opposed to body length (Riley, 1966). Therefore, although development and growth rates were not retarded, if the same patterns in the data were observed in the wild, smaller larvae might remain at greater risk of predation from a greater range of predators for longer than larger counterparts. Predation mortality has been demonstrated to decline rapidly with an increase in larval size (Peterson & Wrobleski, 1984). Increased mortality during the larval stage, even by a relatively small amount, can influence the abundance of subsequent year-classes within a population (Houde, 2002; Jones, 2002; Rose et al.,

2003; Alvarez et al., 2006). Since mortality rates in fish larvae can be in excess of 20% per day (Houde, 2002), even a two to four day delay in attaining the same size as other individuals within a cohort might expose smaller larvae to predators for longer, especially if the duration of the pelagic/planktonic stage is increased. However, mesocosm experiments have indicated that larger fish larvae are sometimes more susceptible to predators than smaller larvae if predators prefer to capture larger prey instead of small prey (Fuiman, 1989; Litvak & Leggett, 1992). Therefore care should be taken when predicting field outcomes using larval size as a predictor of mortality.

Predation might not be the only obstacle to the survival of smaller larvae. Around the time of yolk absorption, small mouth gape restricts the number and diversity of available prey items on which a larva can feed (Jones, 2002). Larvae that are smaller at hatch are disadvantaged in terms of size and growth if they cannot feed on larger, more nutritious prey that would increase their rate of growth. Whether or not this is a problem for plaice larvae is another matter. Plaice larvae are large at hatch, averaging 6.5mm with a large gape and start out life in the wild feeding on diatoms and larval molluscs (Wimpenny, 1953). In the laboratory, diatoms were not used and the first food consisted of *Artemia* nauplii in all tanks, implying that in the wild, smaller larvae would be unlikely to be affected by prey size at the first-feeding stage. Larger larvae or newly metamorphosed juveniles in the field however might be able to prey on larger food items such as copepods, polychaetes and bivalve siphons (Macer, 1967) sooner than smaller larvae, thus accelerating their development and reducing predation mortality risk more rapidly. Investigations of prey choice and prey capture success in the laboratory could help determine if smaller PCB contaminated larvae really are at a disadvantage to their larger counterparts in this respect.

4.5.4 Behaviour

Many behavioural studies of pollutant effects on fish behaviour examine parameters such as routine swimming activity, foraging success or predator evasion (Smith & Weis, 1997; Zhou & Weis, 1998; Gray et al., 1999; Weis et al., 1999; Weis et al., 2001; McCarthy et al., 2003; Alvarez et al., 2006; Alvarez & Fuiman, 2006). In this study, only routine swimming behaviour and response to a simulated predatory threat

were tested. Routine swimming activity can be as good an indicator of behavioural impairment as feeding behaviour and it reflects the likely rates of encounter between the larvae and their predators or prey (Faulk et al., 1999; Rose et al., 2003). Predator evasion is certainly ecologically relevant as individual survival at the very least, depends on it. There were significant reductions in the routine swimming performance of the high- and medium- dosed larvae within 24-hours of hatching in this study. These differences were not significant following yolk absorption at 8-dph for the medium-dosed larvae but were for the high-dosed larvae (Fig. 4.12 A). Routine swimming activity at 8-dph was greater than that observed within 24-hours of hatch by approximately 2-fold (Figs. 4.11-4.12). This increase in swimming behaviour is normal for larval plaice. At hatch, the larvae make spasmodic wriggling movements followed by periods of inactivity, which can last up to several minutes (Wyatt, 1972) and explains why often only one or two grid lines were crossed in any 30 second period in this study. Over the next 1-2 days of development, the flexing movements move toward the caudal fin resulting in the characteristic larval swimming behaviour with periods of inactivity becoming shorter (Wyatt, 1972).

Larval response to the simulated predator attack was considered an ecologically relevant parameter to assess since predation is regarded as the principal cause of mortality on larval stages (Houde, 2002) and increased mortality might have repercussions for community structure and/or future population sizes (Weis et al., 1999; Rose et al., 2003; Alvarez et al., 2006). Sensory and locomotory ability improve as ontogeny progresses and results in improved predator detection and evasion with age (Fuiman, 2002). In this study there was little change in the percentage or latency of response to the startle stimulus between hatch and first-feeding across any of the treatments. By metamorphosis, larvae in all treatments exhibited a reduced latency of response to the vibration and the percentage response increased in all but the control fish in 2005. Reasons for the lack of increased percentage response in those larvae are unclear. No differences were detected in the percentage or latency of response to the startle stimulus between the treatments.

The method used in this study for the measurement of routine swimming activity was effective and has been used successfully in other studies (Ide et al., 2003; Wisenden et al., 2004) but no differences were observed in the responses to the startle stimulus.

This is not to say that there were definitely no differences in latency of response between larvae from different treatments because the larvae were filmed using only a conventional video recorder (section 4.3.4) that recorded at a rate of 25 frames per second (fps) producing a time lapse between each frame lasting 40ms. McCarthy et al. (2003) used a conventional video recorder able to record at 60fps, with each frame representing only 16.7ms, and were able to detect changes in response latency to a similar startle stimulus. Both herring and plaice larvae will exhibit a C-start escape response (body flexes into a c-shaped curve before propelling the larva forward) to a tactile stimulus within 17-33ms of being touched (Blaxter & Batty, 1985; Batty & Blaxter, 1992). Use of a high-speed video recorder would have increased the precision with which latency of response was measured in this study. This was attempted but proved impossible given the poor resolution obtained from the NAC400 video equipment loaned from the BBSRC. Lighting the larval arena from below might have helped improve resolution (Batty, 1983) but the nature of the experimental equipment precluded this as the post upon which the fish were supported and the hammer struck, produced a large shadow across the centre of the arena. Despite this, the measures of the percentage response to the vibration within 10 frames/400ms of it occurring are likely to be correct since the positions of the larvae's eyes could be marked precisely on the screen allowing deviation from the original position at the point of hammer strike to be observed. In summation, although larvae showed similar capabilities to respond to the startle stimulus regardless of treatment, differences in the quality of the response in terms of latency remain unclear.

The results from the routine swimming analysis suggest that the effects of maternal PCB exposure on larval routine swimming are manifested during yolk absorption in the early larval stages and do not appear to persist into later development (i.e. metamorphosis). This lack of observed effect later in development might be because any PCBs present in the yolk sac had been metabolised following yolk absorption and either depurated or deposited in a body tissue where their impacts might be benign or minimised (Alvarez et al., 2006) although further investigations would be required to investigate this. As the larvae grow and lay down more uncontaminated body tissue, the total PCB body burden would decrease unless further PCB exposure occurred, potentially placing the body burden below any threshold concentration for observed effects. As no further exposures to Aroclor 1254 were performed following hatch, it

cannot be said with certainty whether or not plaice larvae remain susceptible to further PCB exposure following absorption of the yolk sac in terms of swimming activity or otherwise. Alternatively, the increased larval mortality observed between hatch and first-feeding at the highest PCB concentrations might leave only those larvae most tolerant to PCBs, upon which the behavioural impacts are minimised. Only chemical analysis and physiological assays designed to determine levels of enzymes capable of metabolising xenobiotics could clarify this.

Table 4.21 lists a number of previous studies that have investigated the impacts of various contaminants from both the field and laboratory on adult and larval fish behaviour. Many of the studies listed in Table 4.21 report significant changes in some or all of the behavioural parameters measured indicating the validity of using behavioural assessments of contaminant exposure. As with the findings of this study, results from behavioural assays can be variable and may depend on the parameters investigated. In the case of prey capture ability shown by mummichog larvae (Weis et al., 2003) the results were highly variable as seen with routine swimming in this study but significant results were still detected. The negative trends regarding routine swimming activity in this study are highly variable (Figs. 4.11 – 4.15) and could be viewed with some scepticism. Tests on more larvae from eggs at the high end of the PCB concentration range would assist in the confirmation of these findings. Nevertheless, larvae from the medium and high Aroclor 1254 dosing regimes in the 2004 experiment exhibited a significant reduction in routine swimming activity compared to control larvae. PCBs have the potential to adversely affect thyroid activity in humans, which partly controls metabolic rate (Cheek et al., 1999) and therefore this might be an underlying cause of the reduction in routine swimming activity observed in the present study, following exposure to PCBs.

4.5.5 Behavioural biomarkers

The use of behavioural traits in toxicological studies has been investigated in many species (see above) and the advantages and disadvantages of its use as a biomarker have been discussed at length (Peakall, 1996; Weis et al., 1999; Rose et al., 2003; Clotfelter et al., 2004; Zala & Penn, 2004). The number of behavioural studies used

Table 4.21 Impacts of pollutants on fish behaviour from field collected and laboratory dosed fish arranged by contaminant type.

Author(s)	Species [a]	Age	Pollutant and concentration	Impact	Environmentally realistic dose?
Buckler et al. (1995)	Atlantic salmon	Alevin	pH<6.5	Larval feeding and swimming behaviour were impaired.	pH was representative of acid-sensitive surface waters with low acid-neutralizing capacity.
Smith et al. (1995)	Mummichog	Adult	Mercury – might be caused by co-contaminants.	Reduced prey-capture ability of fish from polluted site. Fish were less likely to attack prey or be successful when they did so.	Fish collected from field sites. Aquarium conditions not mentioned.
Smith & Weis (1997)	Mummichog	Adult	Mercury – might be caused by co-contaminants.	Reduced prey-capture ability and predator avoidance. Wild caught fish found to have more detritus in diet, reduced growth and lifespan. Fish from the polluted site suffered greater mortality in the presence of a predator, the blue crab, *Callinectes sapidus*. Correlation existed between fish predatory behaviour and brain Hg concentrations.	Yes, the fish were collected from field sites.
Zhou & Weis (1998)	Mummichog	Larvae and/or embryos	Methylmercury solution (5, 10 or 20 $\mu g\ l^{-1}$)	Depressed swimming performance, some increased vulnerability to predation due to hyperactivity.	More than the water column but less than sediments at the sites of parental populations
Zhou et al. (2001)	Mummichog	Larvae	Methylmercury solution (5, 10$\mu g\ l^{-1}$)	Reduced prey capture rates that improved with growth. Some reduced prey capture efficiency and some reduced growth.	
Alvarez et al. (2006)	Atlantic croaker	Larvae	Methylmercury in food (0.04 – 4.6ng g^{-1} eggs)	No significant effect on larval growth. Reduced swimming activity at various developmental stages. No changes observed in response distance or speed to a visual startle stimulus. Response speed to a vibratory stimulus decreased with increasing MeHg concentrations but response distance and responsiveness were unaffected.	Yes
Wibe et al. (2001)	3-spine stickleback	Adult males	Bis(tributyltin)oxide (TBTO) – 2.7 - 27ppb	Reduced escape response of treated fish to a simulated predator attack and a longer time to initiate this response. High mortality of fish at the highest dose.	Even the lowest concentrations were high by environmental standards, but not unknown.

Contd. overleaf

Table 4.21 *Contd.*

Little et al. (1993)	Bluegill	Juveniles	Pyrethroid insecticide	Gross body tremors acted as highly sensitive indicators among pulse exposed fish.	"Behavioural responses were observed at concentrations an order of magnitude less than concentrations impacting on growth or survival in simulated field studies." (Little et al., 1993)
Brewer et al. (2000)	Rainbow trout	Juveniles	Malathion (insecticide) (20, 40 $\mu g\ l^{-1}$)	Decreases in distance and speed swam and a more linear path than control fish after 24h in both exposures.	Concentrations of both insecticides were based on preliminary tests of lethality for each chemical.
			Diazinon (insecticide) (250, 500, 1000 $\mu g\ l^{-1}$)	Decreases in distance and speed swam and turning rate compared to control fish after 96h in the 250 and 500 $\mu g\ l^{-1}$ exposures.	
(Alvarez & Fuiman, 2006)	Red drum	Larvae	Malathion (insecticide) (1 and 10$\mu g\ l^{-1}$)	Aqueous exposure to malathion did not result in significant differences in growth rates, swimming activity, response to a visual stimulus or respiration rate.	Yes
(Alvarez & Fuiman, 2005)	Red drum	Larvae	Atrazine (herbicide) (40 and 80$\mu g\ l^{-1}$)	Reduced growth rates, increased routine swimming, hyperactivity were significantly affected by atrazine but response to simulated and actual predators and respiration rates were not.	Yes
Zhou & Weis (1999)	Mummichog	Larvae	PCBs and metals (Hg, Cu, Pb, Zn, Cd) in sediment at various field sites.	Larvae from unpolluted sites had greater spontaneous activity, swimming performance and stamina. 1-month old larvae from polluted sites were more vulnerable to predation.	Adults were field-collected and stripped for eggs and milt. Eggs and larvae were reared in clean water.
Faulk et al. (1999)	Atlantic croaker	Larvae	DDT – 0.07-0.20 $\mu g\ g^{-1}$ egg tissue	Routine swimming speed and activity and response to visual and vibratory stimuli were negatively affected by parental DDT exposure.	Yes
McCarthy et al. (2003)	Atlantic croaker	Larvae	Aroclor 1254 0.1 - 6.3 $\mu g\ g^{-1}$ egg	Larvae exposed through parental transfer exhibited impaired responses to startle stimuli. Routine swimming speed and activity were unaffected.	Yes

Contd overleaf

Table 4.21 *Contd.*

Weis et al. (2003)	Mummichog	Larvae	PCBs 0 - 0.69 μg g^{-1} sediment from various field sites. Metal contamination was also present – Hg, Cu, Pb, Zn, Cd.	Prey capture ability was related to sediment contaminant concentrations. Significant negative correlations of prey capture were seen with Hg, Pb, Zn, Cd and PCBs but prey capture was highly variable at all but the most polluted site.	Yes
Weis et al. (2001)	Mummichog	Adult	PCBs 0 - 8.2 μg g^{-1} sediment from various field sites. Metal contamination was also present – Hg, Cu, Pb, Zn, Cd.	Highest prey capture rates were at the cleanest sites. Differences related to overall contaminant concentrations rather than specific toxicants.	Yes

[a] SPECIES – Atlantic salmon, *Salmo salar*; mummichog, *Fundulus heteroclitus*; 3-spine stickleback, *Gasterosteus aculeatus*; bluegill sunfish, *Lepomis macrochirus*; rainbow trout, *Oncorhynchus mykiss*; Atlantic croaker, *Micropogonias undulatus*; red drum, *Sciaenops ocellatus*.

to evaluate the effects of endocrine disrupting chemicals is increasing. Advantages include the ease of making particular measurements compared to physiological assays and the apparent increased sensitivity to other endpoints such as mortality. The procedures are non-invasive and do not require animals to be killed. The equipment and procedures required to monitor behaviour are often inexpensive in comparison to biochemical or physiological assays (Peakall, 1996). Most behaviour exhibited by fish is due to their sensory systems responding to stimuli in the environment. Fish larvae may use olfactory, auditory, mechanosensory, chemosensory or visual systems to detect predators and prey in their environment (Blaxter & Ten Hallers-Tjabbes, 1992). Chemical contaminants may affect the functioning of fish sensory organs (e.g. chemoreceptors or the lateral line) changing or reducing information reaching the brain or they may affect fundamental metabolic processes, both of which should be detected by changes in behaviour such as decreased search efficiencies, strike frequencies, prey capture rates, reaction distances and increased prey handling times (Blaxter & Ten Hallers-Tjabbes, 1992; Peakall, 1996). These in turn may then limit growth rates of larvae or juvenile fish and lower survival rates, as they remain susceptible to factors such as predation for longer periods of time in the wild (Faulk et al., 1999; Zhou et al., 2001).

Clotfelter et al. (2004) described behaviour as "the physical manifestation of an animal's integrated physiological response to its environment." For this reason, it has been suggested that behavioural studies should provide a more complete measure of chemical effects than a few biochemical or physiological measures (Zala & Penn, 2004). Criticisms of behavioural studies are that behaviour can sometimes be difficult to measure, highly variable (e.g. Figs. 4.14 – 4.15) or lacking ecological realism (Zala & Penn, 2004) making selection of the appropriate parameters for measurement important. Behaviour can sometimes be a very subjective parameter to measure as it might involve a human decision as to whether or not an organism really is displaying a type of behaviour and thus it becomes open to potential unconscious bias. Peakall (1996) argued that behavioural effects had not been shown conclusively to be more sensitive than other biological, biochemical or physiological parameters and that the results are often more time consuming to collate as well as being less reproducible. Despite this Zala & Penn (2004) argue that it was observed changes in behaviour of

wildlife such as western gulls (*Larus occidentalis*) (Hunt, 1977) that first alerted people to the threats xenobiotics posed to wildlife.

4.5.6 Predictions from behavioural observations

In a conceptual model, Elliott (2002) demonstrated how sub-lethal contaminant concentrations can have various effects on the physiology, behaviour, morphology, and immunology of fish, resulting in changes to population and community ecology. By integrating the results of studies on predator-prey relationships, foraging and routine activity it is possible to expand on the behavioural side of this model to demonstrate how population and community changes could be affected by alterations in daily behaviour patterns caused by chronic, low-level contaminant concentrations (Fig. 4.22).

Taking these predictions of behavioural changes on populations a step further, Rose et al. (2003) used laboratory data and nested models to simulate the effect of PCB contamination on Atlantic croaker populations. The model aimed to demonstrate how fish populations could be affected by contaminants in addition to the widely acknowledged impacts of over-fishing by quantifying the link between behavioural and physiological effects of the contaminants. The effects of PCBs on egg mortality, fecundity and larval responses to predators were included. The model was run on three scenarios involving baseline conditions, PCB effects on first time spawning and PCB effects on lifetime spawning. The results suggested that PCBs were likely to have little effect on croaker populations relative to natural variation on first time spawning. Over a lifetime of spawning, average total abundance was about 10% lower than under baseline conditions though coefficients of variation for juvenile survival were unaffected by PCBs. The authors noted however that they had ignored PCB effects on the predators and prey of the croaker, on the ability of the croaker to catch its prey and stressed the fact that this was only a preliminary result. The addition of more parameters would improve the model allowing it to be used convincingly alongside laboratory data to demonstrate the impacts of contaminants on fish populations (Rose et al., 2003). Alvarez et al. (2006) combined empirical results of larval exposure to MeHg with an individual-based model to calculate impacts on larval survival and stage duration. Low doses (0.05 mg kg^{-1} day^{-1}) of MeHg were

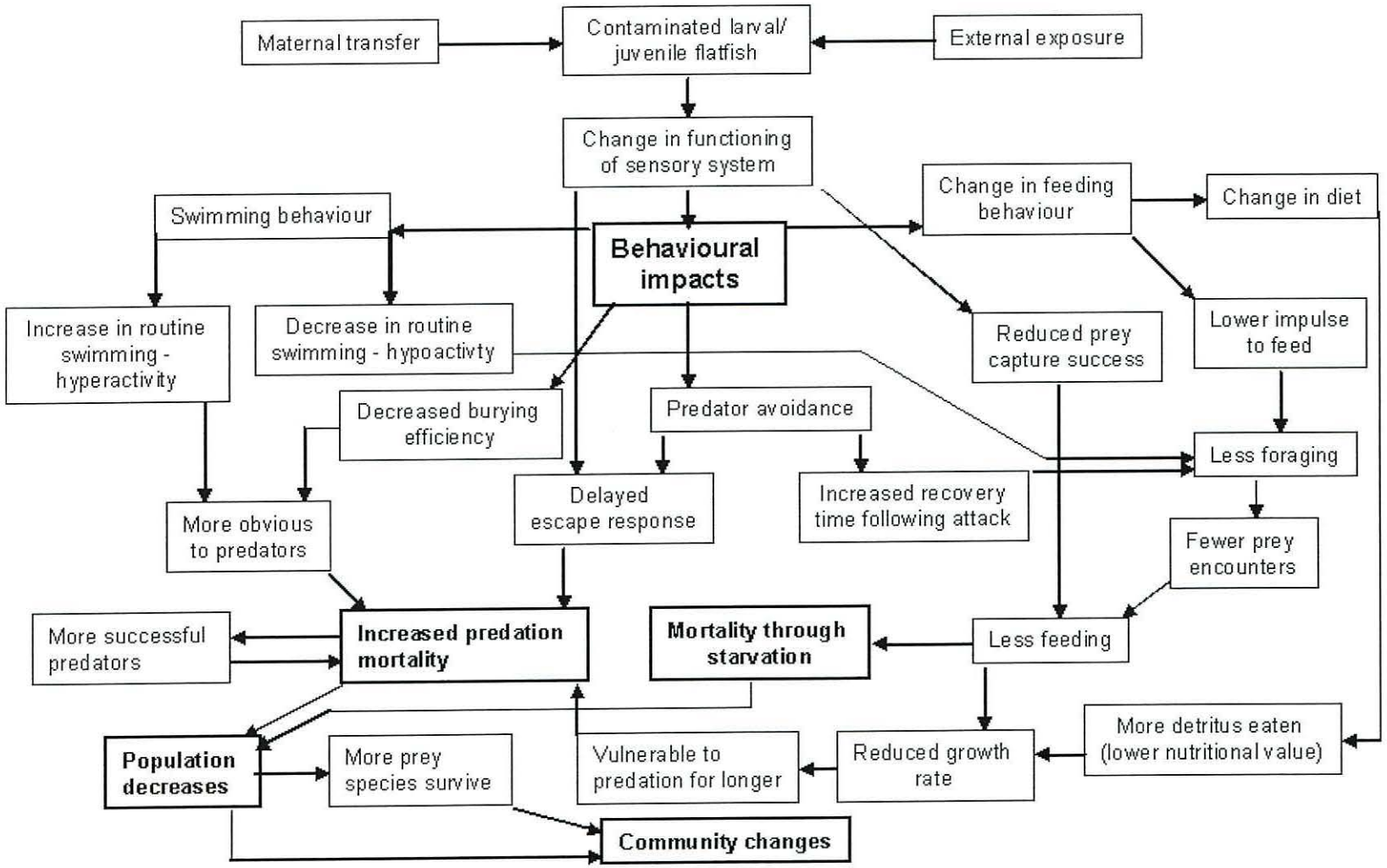


Figure 4.22 Conceptual model of contaminant impacts on flatfish behaviour adapted from Elliott (2002) based on results from previous studies (Weis & Weis, 1989; Zhou & Weis, 1998; Zhou et al., 1998; Faulk et al., 1999; Weis et al., 1999; Zhou et al., 1999; Zhou & Weis, 1999; Weis et al., 2001; Zhou et al., 2001; McCarthy et al., 2003; Weis et al., 2003; Alvarez & Fuiman, 2005; Alvarez et al., 2006). Bold boxes indicate major impacts of behavioural changes e.g. mortality or population/community changes.

calculated to cause an 86% reduction in survival but did not affect stage duration whilst high doses (0.1 mg kg^{-1} day^{-1}) resulted in a 93% reduction in survival and increased stage duration by 20% (Alvarez et al., 2006). It has been suggested that the development of such models, tested thoroughly against laboratory data, offer an alternative approach to extensive field and laboratory testing and could eventually help clarify cause and effects observed in field studies (Rose et al., 2003).

4.5.7 The batch effect

Three major aspects of larval biology have been investigated in this chapter: survival, growth and behaviour. Even in cases when no significant effect of treatment has been observed, significant differences have occurred between spawns from different females within each treatment group e.g. survival (Tables 4.4 and 4.5), growth (Table 4.11) and behaviour (Table 4.18). This phenomenon of intrinsic variability among spawns has been described as the "batch effect" and might be due to variation in maternal investment in egg quality (Fuiman et al., 2005) or genetic influences. Fuiman et al. (2005) suggested that the phenomenon might be related to timing of spawning throughout the reproductive season with poorer quality eggs produced in the early and late batches. The spawns used in this study for analysis of survival, growth and behaviour were most often the first or second spawns obtained from each female, which should have helped avoid this batch effect. However, they were obtained throughout the spawning season from January through to April. Examination of survival, hatch length or routine swimming activity data over this time revealed no changes as the spawning season progressed. Therefore, in this study, timing of spawning appeared to play little role in influencing the size and performance of the plaice larvae. The differences observed between spawns within treatments could therefore be due to different female investments in egg quality or genetic influences. Although this variation was present, it did not preclude detection of significant differences between treatment groups with regard to survival, larval size or routine swimming behaviour.

4.5.8 Conclusions

The results of this study indicate that PCB contamination by maternal transfer at environmentally realistic concentrations can have some adverse effects on development and performance of plaice larvae. At the highest PCB concentrations, larval survival following hatch is significantly reduced and those larvae that do survive exhibit a two to four day delay in attaining the same mean size as larvae from uncontaminated female broodstock. Routine swimming activity of larvae was significantly reduced in high- and medium-dosed larvae at hatch and in low- and high-dosed larvae at first-feeding. By metamorphosis no differences were detected in swimming activity between any treatment groups. The disruption of growth and behaviour in the wild might have impacts on recruitment and community structures if survival is reduced. Behavioural studies have been used with mixed success to assess sub-lethal impacts of xenobiotics, though the parameters measured must be chosen carefully. Behaviour is considered by some to be a more sensitive measure of sublethal xenobiotic impacts on organisms than biochemical or physiological parameters. In the following section of this thesis, physiological parameters of exposed larvae and juveniles are assessed to investigate further impacts of PCB exposure in larval and juvenile plaice.

Chapter 5

The effect of Aroclor 1254 on the cellular energy allocation of the early life stages of the plaice, *Pleuronectes platessa*

5.1 Abstract

The cellular energy allocation (CEA) assay has been designed to test the effects of abiotic stress, on the energy metabolism of organisms. Differences in total carbohydrate, lipid and protein reserves (Ea) are assessed and energy consumption (Ec) is measured by assessment of the electron transport system (ETS) activity. These four measurements are then integrated to a single CEA value (CEA = Ea/Ec) that gives a measure of the net energy budget of an organism or a population. This study aimed to assess the CEA in larval (n = 3 spawns per treatment) and juvenile 0+ plaice (n = 3 replicate tanks per treatment) exposed to the commercial PCB mixture, Aroclor 1254, following maternal transfer of, or dietary exposure to the contaminants and to consider the suitability of the application of the CEA assay.

All energy reserves and energy consumption increased with age and size in the larval exposure experiment. PCB concentrations in eggs were 1.0-126.0μg kg^{-1} dry wt. (low dose) and 847.8-4166.9μg kg^{-1} dry wt. (high dose). At hatch, significant differences existed in lipid energy reserves, ETS activity and CEA values between control and high-dosed larvae ($p = 0.012$, $p = 0.049$ and $p = 0.020$ respectively) following exposure by maternal transfer. There were few significant differences at other stages of larval development; sugar energy in fertilised eggs was significantly lower in high-dosed larvae ($p = 0.036$) and ETS activity was significantly different between low- and high-dosed larvae at metamorphosis ($p = 0.015$). In the juvenile dietary exposure experiment whole body PCB concentrations were 55.4-835.7μg kg^{-1} dry wt. (low dose) and 182.9-1032.2μg kg^{-1} dry wt. (high dose). ETS activity was significantly reduced in both the low- and high-dosed treatments compared to control fish ($p = 0.014$ and $p < 0.001$ respectively). CEA was significantly increased in high-dosed fish only in comparison to control fish ($p = 0.006$). In both experiments, the effects observed, occurred when the metabolism of PCBs was most likely to occur i.e. during yolk-sac absorption and dietary uptake, and the PCB concentrations were environmentally realistic. Use of the CEA technique for evaluating PCB-induced toxic stress in fish larvae is promising but increased replicate larval spawns, a greater range of low PCB concentrations and/or use of liver tissues would further evaluate its sensitivity.

5.2 Introduction

Xenobiotic contamination can disrupt many aspects of an organism's physiology that are detectable even at sub-lethal concentrations. Disruption of an organism's physiology might result in reduced performance in other aspects of its biology such as growth or behaviour that could put its survival at risk (Faulk et al., 1999; McCarthy et al., 2003; Alvarez et al., 2006). Various assays have been used to investigate the physiological changes that occur following xenobiotic exposure and can be used in monitoring programs to detect effects of pollutants even after episodic contamination events have occurred (Einsporn et al., 2005; Orrego et al., 2005; Vaccaro et al., 2005). These techniques often use the induction of enzymes involved in detoxification as a biomarker of contaminant effects (Alberts et al., 1994; Matthiessen et al., 1998; Orrego et al., 2005).

Detoxification reactions are catalysed by the cytochrome P450 family of enzymes, part of a group called mixed function oxygenases, which catalyse a series of reactions to make toxic compounds sufficiently water-soluble in order that they may leave the cell and be excreted (Alberts et al., 1994; Matthiessen et al., 1998). One of these mono-oxygenases is known to use 7-ethoxyresorufin as an artificial substrate, the activity of which is named 7-ethoxyresorufin-O-deethylase (EROD) activity and is often measured as an indicator of exposure to certain pollutants (Holm et al., 1994; Arukwe et al., 2001; Ruus et al., 2006). Measurements of EROD induction have been used successfully to detect contaminant-induced responses in plaice exposed to harbour-dredged spoil in a mesocosm study (Eggens et al., 1996). PCBs are also known to increase EROD activity, inducing the cytochrome P4501A enzyme system in tilapia (*Tilapia guinasana*), with the highly chlorinated PCBs having the greatest effect (Yeung *et al.*, 2003). Although EROD activity can be affected by sex, season and reproductive stage, it can also be used as a sensitive biomarker of contamination and give indications of metabolic rate (Matthiessen *et al.*, 1998; Marchand *et al.*, 2002).

Detoxification of xenobiotics by enzyme action is energetically costly for an organism and can result in reduced ecological performance. For example, Calow and Sibly (1990) reviewed the "metabolic cost" hypothesis which suggests that metabolic changes in an organism that occur as a result of toxic stress, might result in a

depletion of energy reserves or changes in energy consumption at the expense of growth and/or reproduction (De Coen & Janssen, 1997). The scope for growth (SfG) assay was developed as a means of measuring the energy absorbed by an organism by measuring feeding rates or assimilation efficiencies as well as measuring the energy consumed (respiration rate) and excreted (ammonia and amino acids) and providing a general index of stress. SfG has been used in mussels (*Mytilus edulis*) to examine the effects of a polluted environment (Widdows, 1978; Widdows & Johnson, 1988). SfG has also been used successfully to evaluate the impacts of the pesticide chlorpyrifos on the mysid shrimp, *Neomysis integer* (Roast et al., 1999) and of zinc on the freshwater shrimp, *Gammarus pulex* (Maltby & Naylor, 1990). Despite its success the SfG assay has been regarded as labour intensive and difficult to employ in routine environmental impact assessments (De Coen & Janssen, 1997).

The cellular energy allocation (CEA) assay was developed at the Laboratory of Environmental Toxicology and Aquatic Ecology at Ghent University (De Coen & Janssen, 1997). The CEA assay was designed as a biomarker technique to assess short-term changes in energy metabolism as a result of abiotic stress, with the intention of using the results to assist in making longer-term predictions of effects at the population level (De Coen & Janssen, 1997). Abiotic stress may include changes in environmental parameters such as temperature or salinity but also includes contamination with xenobiotic compounds.

The CEA assay takes an integrative approach to assess the status of an organism's or a population's energy reserves because it measures the total energy reserves (lipid, protein and carbohydrate content) and the rate of energy consumption (via assessment of the respiratory, electron transport system (ETS) activity) and integrates them into one value reflecting the net energy budget (De Coen & Janssen, 1997). The ETS is situated in the mitochondrial membrane within a cell and consists of an elaborate chain of flavoproteins, cytochromes (proteins) and metallic ions that transport electrons to oxygen molecules to create water and ATP as two of the by-products of respiration (Packard, 1971; Alberts et al., 1994).

The CEA methodology has been used successfully as a biomarker for toxicant-induced stress in *Daphnia magna* following lindane and mercury exposure and also in

N. integer following exposure to both tributyltin and chlorpyrifos (De Coen & Janssen, 1997; Verslycke et al., 2003; Verslycke et al., 2004b). The aim of the research in this chapter was to investigate the potential of the CEA methodology as an indicator of PCB-induced stress in the eggs, larvae and juveniles of the European plaice following exposure to Aroclor 1254 by maternal transfer and in the case of individual, juvenile fish, by dietary exposure. It was hypothesised that the "metabolic cost" of detoxifying or excreting contaminants would be detected in the net energy budget measured using the CEA technique.

5.3 Methods

Following preliminary investigations to establish a method of homogenising whole fish before sub-sampling homogenate for CEA analysis, two separate experiments were performed to establish (i) the suitability of the CEA assay for detecting effects of PCB exposure on juvenile fish and (ii) the effects of PCB exposure on the CEA of larval and juvenile plaice. Experiment 1 examined the impacts of dietary PCB exposure on juvenile 0+ plaice whilst Experiment 2 investigated the impacts of maternal PCB transfer on eggs and larval plaice following oral dosing of female broodstock. The methods for each experiment are described below before detailed explanation of the CEA methodology.

5.3.1 Preliminary investigations

Before using the CEA methodology (De Coen & Janssen, 1997; Verslycke et al., 2003) on experimental samples, it was necessary to test whether the technique could be used effectively with homogenate obtained from whole fish. The advantage of using homogenate taken from whole fish to assess available energy and energy consumption would be that CEA values could be calculated for individual juvenile fish rather than being compiled from pooled data as is the case when using individual larvae or small, individual invertebrates (Verslycke et al., 2004a).

Initial investigations were carried out with the Aquatic Ecology and Toxicology Group at Ghent University in Belgium, on zebrafish (*Danio rerio*) in May 2004. Livers (<20mg each) were removed from fish killed by an overdose of anaesthetic (2-phenoxyethanol) and used individually for each step of the CEA methodology (n = 3 at each step). Sub-samples of whole fish homogenate (homogenised by pestle and mortar with or without liquid nitrogen) were also used for analysis of sugars, lipids and proteins (n = 3 per assay). Insufficient time was available to attempt the ETS assay on these samples. In the UK, whole common gobies (*Pomatoschistus minutus*) collected from Dulas Bay and Foryd Bay, Anglesey, were used to test the CEA technique in the laboratory in conjunction with an MSc project student (Godsell, 2005) and to further determine the viability of using whole fish (n = 10 from each

site) in the assays. In the latter case the whole fish were homogenised using a Teflon-tipped homogeniser whilst keeping the sample in a tube cooled with ice and water.

The CEA data for the Dulas and Foryd Bay gobies were subjected to repeatability tests (Lessells & Boag, 1987) (as described in section 4.3.3) in order to determine whether or not it was necessary to take three samples from each fish for each separate step of the CEA assay. The results of the repeatability analyses are shown in Table 5.1a. Whilst the data for lipid and ETS analyses gave high r-values (0.8 - 0.9), the repeatability coefficients for the sugar and protein analyses did not. This indicated that the assay results were very variable, possibly due to incomplete homogenisation, and one measurement alone of either protein or sugar energy content was unlikely to give results that were representative of an individual fish. Therefore it was decided to run triplicate samples for each assay.

5.3.2 Experiment 1 - Juvenile fish collection, husbandry and PCB exposure

Juvenile 0+ plaice (25 – 40mm total body length, n = ca. 250) were collected at low water using a push net with 5mm mesh from Llanfairfechan beach, North Wales in June 2004 and transported back to the laboratory. Fish were maintained in 8-litre plastic aquaria at a density of 30 fish per tank and fed *ad libitum* on ground pellet feed (Dana Feed). Tanks were siphoned clean and new food added each day. Sea water from the Menai Strait that had passed through settlement tanks and a sand filter was then passed through a 5μm mesh before flowing through the tanks at a rate of 4 l hr^{-1} at 12 ±1°C with salinity at 32-33 ppt. A small air stone was placed in each tank to provide aeration. Fish were maintained in these conditions for approximately six weeks after which fish were selected and placed into nine identical 8-litre tanks at random at a density of 15 fish per tank.

After a further 7-days acclimation, fish in six tanks were exposed to the commercial PCB mixture Aroclor 1254 through their diet. The desired Aroclor 1254 doses for the fish were 0.2 and 1.0mg Aroclor 1254 kg^{-1} fish d^{-1}. The juvenile fish used in the experiment weighed approximately 1g each. It was assumed that the juvenile plaice

Table 5.1a Repeatability analyses (Lessells & Boag, 1987) on the individual components of the cellular energy allocation assay conducted on common gobies, *Pomatoschistus minutus*, collected from Foryd and Dulas Bays, Anglesey. For more details on repeatability analysis, see section 4.3.3.

Assay step	F ratio (df)	n_o	Repeatability (r)
Protein analysis	1.14 (18,38)[a]	3	0.045
Sugar analysis	2.96 (18,37)*	3	0.396
Lipid analysis	26.77 (18,38)***	3	0.896
ETS	13.70 (19,40)***	3	0.809

[a] $p = 0.354$; * $p < 0.05$; *** $p = 0.001$

Table 5.1b Repeatability analyses (Lessells & Boag, 1987) on the individual components of the cellular energy allocation assay conducted on juvenile plaice, *Pleuronectes platessa*, either unexposed or exposed to Aroclor 1254 through their diet. For more details on repeatability analysis, see section 4.3.3.

Assay step	F ratio (df)	n_o	Repeatability (r)
Protein analysis	7.64 (44,79)***	2.762	0.706
Sugar analysis	10.18 (44,78)***	2.732	0.771
Lipid analysis	39.26 (44,79)***	2.762	0.933
ETS	16.72 (44,79)***	2.762	0.851

*** $p = 0.001$

may eat up to 3 – 5% of their body weight in any day during the experimental procedure (30-50mg each) and it was decided to add 1.0g of the ground pellet feed to each tank every day. Aroclor 1254 was dissolved in hexane at a low concentration ($8\mu g\ ml^{-1}$) and a high concentration ($40\mu g\ ml^{-1}$). Measures of 0.5ml of these Aroclor solutions were added a drop at a time with a Pasteur pipette to separate 1g batches of crushed pellet feed before being mixed and dried under a flow of nitrogen to evaporate the hexane. This produced multiple 1g batches of food containing Aroclor 1254 at low ($4\mu g\ g^{-1}$) and high ($20\mu g\ g^{-1}$) concentrations. Subsequent GC/MS analysis of the food samples showed the actual concentrations in the food to be $2.1\mu g\ g^{-1}$ for the low dose food and $15.8\mu g\ g^{-1}$ for the high dose food (see later in Table 5.9). Therefore, if a hypothetical individual fish (n = 15 per tank) weighing 1g ingested exactly $1/15^{th}$ of the food each day it would consume $0.14\mu g$ Aroclor 1254 day^{-1} in a low dose tank or $1.03\mu g$ Aroclor 1254 day^{-1} in a high dose tank; doses similar to those desired. It was expected that the amount of food and subsequent Aroclor exposure would vary depending on the feeding habits and sizes of individual fish.

For the control diet, pure hexane was added to the food and evaporated before being fed to the fish in the remaining three tanks. Food was added to the tanks every day for seven days. Before adding new food, uneaten food and faeces from the previous day were siphoned out from the tanks. After seven days, food was withheld for 48-hours to allow evacuation of gut content to occur and thus prevent undigested food contributing to the energy reserve fractions measured. Five fish were sampled from each tank and shock-frozen in liquid nitrogen before being stored at –80°C. Remaining fish were shock-frozen and stored at –20°C before being analysed by GC/MS for PCB content.

Five fish from each tank were homogenised separately using a Teflon-tipped homogeniser and cooled on ice to limit enzyme activity and prevent the breakdown of proteins. Given the results of the repeatability tests on the data for the gobies (Table 5.1a), extra care was taken to ensure thorough homogenisation of each fish before subdivision into 12 x 1.5ml pre-weighed eppendorfs with an estimated 10-20mg of material per tube. The tubes were then re-weighed before freezing at –80°C. Where possible, three samples from each fish were selected at random for each stage of the CEA analysis and homogenised very thoroughly at the start of each assay. For 11

fish, only two samples could be taken for lipid, protein and ETS analysis and for 12 fish, only two samples were taken for sugar analysis because these fish were too small to provide enough material for 12 sub-samples. The remaining fish in each tank were freeze-dried and analysed for PCB content (see section 2.3.3) for the field-caught juveniles.

Repeatability tests were conducted on the data obtained for each of the four CEA assays (Table 5.1b). Because in some cases only two samples were analysed per fish, this needed to be taken into account when calculating n_0 for calculation of the repeatability r-value by using the formula below (Lessells & Boag, 1987):

$$n_0 = [1/(a-1)] \cdot \left[\sum_{i=1}^{a} n_1 - \left(\sum_{i=1}^{a} n_1^{\,2} / \sum_{i=1}^{a} n_1 \right) \right]$$

where a is the number of groups and n_1 is the sample size in the ith group.

Ensuring that thorough homogenisation had taken place appeared to improve the results greatly for each of the CEA assays with no r-values lower than 0.7 being obtained. All four steps of the assay were significantly repeatable as the ANOVAs were significant. Although the r-values had improved it was still deemed appropriate to take three samples per spawn for each set of analyses in the following experiment to ensure that the final results were as accurate as possible.

5.3.3 Experiment 2 - Larval exposure

Three individually tagged females in the low-dosed broodstock tank in 2005 were given the same doses as high-dosed fish from 2004 (see chapters 3 and 4) to provide three high-dosed replicates of larvae for the CEA work. Larval fish were also reared from three extra spawns obtained from each of the control and the low-dosed females during the broodstock experiment carried out in 2005 (described in section 3.3). All larvae for the CEA study were reared in the same way as the larvae in the main larval

experiments (Chapter 4). Eggs were also sub-sampled in the same way for PCB analysis in the same way (Chapters 3 and 4).

Egg samples were collected from the 8l rearing tanks post-fertilisation when they were floating on the surface; an indication of viability. Larvae were sampled within 24-hours of hatch, at 8-dph and at 42-dph (metamorphosis). Twelve replicates of approximately 20 eggs or larvae from each spawn were shock-frozen in liquid nitrogen at fertilisation, hatch (11-dpf) and first-feeding (18-dpf). Metamorphosed larvae were frozen individually. This provided three replicate samples of approximately 20 to 30mg wet wt. for each of the measurements of sugar, lipid and protein energy and energy consumption at each life stage and from each spawn. On 10 occasions across all assays completed, only two samples were taken for analysis due to the low numbers of eggs or larvae available and on four occasions for hatched and first-feeding high-dosed larvae just one sample was taken due to the low numbers of individuals available. However, these samples still contained approximately 20 individuals from the same spawn. Frozen fish were placed in pre-weighed 1.5ml eppendorfs, weighed and stored at –80 °C until all samples were analysed using the CEA methodology. Sub-samples of eggs from each spawn (ca. 20g wet wt.) and remaining post-metamorphosed fish (following the CEA sampling) were freeze-dried and analysed by GC/MS (see section 2.3.3).

5.3.4 General sample preparation

Although whole juvenile fish from experiment 1 had already been homogenised before division into eppendorfs, all samples in both the juvenile and larval fish experiments were homogenised at the start of each analysis with a Teflon-tipped homogeniser to ensure proper mixing with the appropriate reagents used in each stage. All procedures, unless otherwise stated, were completed on ice to reduce enzyme activity and retain sample viability. A Dynex Technologies MRX II microplate photometer linked to a PC was used to measure absorbance and enzyme activity for all four analyses.

5.3.5 Lipid analysis

Sub-samples were homogenised in 100µl of deionised water in 1.5ml eppendorfs for 1-minute and a further 100µl of deionised water was added. 500µl of chloroform (SIGMA-Aldrich, 36691-9), 500µl of methanol (SIGMA, M-3641) and a further 250µl of deionised water were pipetted into all samples, which were then vortex-mixed. Samples were centrifuged (SCIQUIP, 2-16) for 10-minutes (3000rpm at 4°C). The upper fluid phase (water and methanol) was discarded and 3 x 100µl measures of the lower fluid phase (chloroform and lipids) were placed in separate 5ml glass tubes and dried at 60°C for 30-minutes. When dry, 500µl of concentrated sulphuric acid was added to the tubes and the samples were vortex-mixed.

To make the fatty acid standards, 62.5mg of tripalmtin (C16:0, SIGMA T-5888) was dissolved in 10ml of chloroform. A 1:2 serial dilution with chloroform provided concentrations of 6.25, 3.12, 1.56, 0.78, 0.39, 0.19, 0.09 mg ml^{-1}. A blank of 1ml chloroform was also produced. Each tube was vortex-mixed before 100µl was taken from each and added to a new tube. To each of these, 500µl of concentrated sulphuric acid was added and the tubes vortex mixed.

Both standards and samples were heated at 200°C for 15-minutes and allowed to cool. 1.5ml of deionised water was added and each tube was vortex mixed. From each sample 250µl was placed in a 96-well microtitre plate (Nunclon surface) along with one set of standards and three blanks. Plates were shaken for six seconds at 9Hz (540rpm) and absorbance at 340nm was measured.

5.3.6 Protein analysis

Samples were homogenised for 1-minute in 100µl of 0.5M Tris-HCl (Trizma base, SIGMA, T-1503; HCl, Aldrich, 31,8949) at pH 6.8 in 1.5ml eppendorfs. A further 100µl of Tris-HCl was added and samples were centrifuged for 10-minutes (4500rpm at 4°C). A 1:40 dilution was then carried out by taking 25µl of supernatant and adding it to 975µl of deionised water. Samples were vortex-mixed and three

replicates of 25µl taken from each and placed in a 96-well microtitre plate (Nunclon surface) kept on ice.

A 1:2 serial dilution with de-ionised water of a stock protein (SIGMA, P-7656) in the microtitre wells, gave standards with concentrations of 0.4, 0.2, 0.1, 0.05, 0.025 mg ml^{-1}. A set of standards and three blanks (25µl deionised water) were added to each plate.

250µl of Bradford reagent (SIGMA, B-6916) was added rapidly to each well with a multi-tip pipette and the plates incubated in the dark for 15-minutes at room temperature. Plates were shaken for six seconds at 9Hz (540rpm) and absorbance at 590nm was measured.

5.3.7 Sugar analysis

100µl of deionised water was added to each sample in the 1.5ml eppendorfs before homogenising them for one minute and adding a further 100µl of deionised water. To each sample 100µl of 15% trichloroacetic acid (TCA) (SIGMA-Aldrich, T-6399) was added before they were vortex mixed and left on ice for 10-minutes to allow the proteins to precipitate out. The samples were then centrifuged for 10-minutes (3500rpm at 4°C) and the supernatants removed and placed in new eppendorfs. The pellets were re-suspended in 200µl of 5% TCA, vortex-mixed and centrifuged again (3500rpm at 4°C) in case any sugar remained in the first pellet. The supernatant was removed from the second wash, added to the former and the supernatants vortex-mixed. 250µl of supernatant was removed from each eppendorf and put into new 2ml eppendorfs before adding 250µl of 5% phenol (Aldrich, 24,232-2) and 1ml concentrated sulphuric acid (Aldrich, 32,050-1). Again, the samples were vortex-mixed and 3 x 300µl replicates were placed in a 96-well microtitre plate.

For the standards, 1:2 serial dilutions with deionised water of a 0.5% solution of α-D-glucose (Aldrich, 15,896-8) were prepared to give concentrations of 0.5, 0.25, 0.125, 0.062, 0.031 and 0.016 mg ml^{-1}. 250µl of deionised water was used as the blank for the assay. Phenol and sulphuric acid were added to the standards as for the samples

and a set of standards and three blanks were added to each plate. Plates were incubated in the dark for 15-minutes at room temperature before shaking for six seconds at 9Hz (540rpm) and absorbance at 490nm was measured.

5.3.8 Electron Transport System activity

The methods used for the ETS assay were similar to those used by Packard (1971) with modifications developed by the Aquatic Ecology and Toxicology Group at Ghent University in Belgium and described herein. Complex buffer solutions were required for the ETS assay in order to preserve the action of the enzymes involved. A homogenate buffer was prepared that comprised of 476ml 0.1M di-sodium hydrogen phosphate (Na_2HPO_4) (Aldrich, 21,988-6) and 24ml 0.1M di-potassium hydrogen phosphate (K_2HPO_4) (Aldrich, 23,450-8) resulting in a 0.1M PO_4 buffer of pH8.5 at room temperature. 3.0275g of Tris-HCl, 4.51mg of magnesium sulphate ($MgSO_4$) (Aldrich, 20,809-4), 0.75g of polyvinylpyrrolidone (PVP) (SIGMA, PVP-10) and 1ml of Triton X-100 (non-ionic) surfactant (Aldrich, 23,472-9) were added and the buffer was adjusted to pH8.5 at room temperature with 1.0M HCl and 1.0M NaOH.

A substrate buffer was made that consisted of a 0.1M PO_4 buffer as described above with 3.0275g of Tris-HCl and 1ml of Triton X-100. The solution was adjusted to pH8.5 at room temperature by adding 1.0M NaOH solution. A buffered substrate solution was prepared by adding 48.236mg of 1.7nM NADH (nicotinamide adenine dinucleotide) disodium salt (SIGMA, 93205) and 8.334mg of 0.25nM NADPH (nicotinamide adenine dinucleotide phosphate) tetrasodium salt (SIGMA, N-7505) to 40ml of the substrate buffer.

P-iodonitrotetrazolium violet (INT) (SIGMA, I-8377) was prepared by adding 40mg to 20ml of deionised water and sonicating the mixture for one hour to dissolve the INT. Triton X-100 keeps the formazon in solution to maintain enzyme activity that is measured using a spectrometer (Owens & King, 1975).

Samples were homogenised for one minute in 200µl of homogenate buffer before adding a further 200µl of homogenate buffer. Samples were then centrifuged for 10-

minutes (3500rpm at 4°C). The supernatants were retained and vortex mixed. In microtitre plates kept at room temperature (unmeasured, ca. 20°C, see later discussion) 180µl of buffered substrate solution was pipetted into each well followed by 3 x 60µl replicates of supernatant. Bubbles formed during the pipetting as a result of the surfactant were burst using a needle in order to prevent variation in absorbance measurements. 60µl of INT (p-iodonitrotetrazolium violet at 2mg ml^{-1}) was added quickly to each well. Control wells contained 60µl of homogenate buffer, 180µl buffered substrate solution and 60µl INT solution. As soon as the INT was added the plates were shaken at 9Hz (540rpm) for six seconds and the kinetic reaction was measured every eight seconds at 490nm and *Vmax* (the maximum rate of reaction) was calculated automatically by the Dynex Technologies Revelation software. Within each experiment, all ETS samples were prepared together and each plate was run on the same day within 5-10 minutes of each other. During the reaction in the ETS assay, p-iodonitrotetrazolium violet (INT) oxidises the co-enzyme Q-cytochrome B complex within the electron transport chain, producing formazon (reduced INT) (Packard, 1971). Formazon has a strong, red colour and it is the rate of this colour formation that allows calculation of the ETS activity.

The following formula was used to calculate ETS activity (mols oxygen used per minute per sample) after Owens & King (1975).

$$\text{Mol } O_2\text{/min/sample} = Vmax \times 300\mu l \times (400\mu l/60\mu l)/((15900\times10^6\ \mu mol\ \text{formazon.cm}) \times 0.7795cm \times (2\ \text{mol formazon/mol } O_2))$$

Where:

Vmax	= maximum rate of reaction
0.7795cm	= focal length of microplate well

5.3.9 Energy reserve calibration charts

Each microtitre plate analysed for energy reserve fractions contained one set of standards. For each assay the standards from four plates were averaged and used to calculate the quantities of lipids, sugars or proteins in each sample. The results from

the standard solutions prepared for the sugar, protein and lipid analyses were used to produce standard calibration graphs (see examples in Figs. 5.1 and 5.2). The calibration graphs were then used to convert the sample absorbances into the concentrations in solution (mg ml^{-1}) before calculating the quantity of each reserve fraction in each sample (mJ mg^{-1} ww).

5.3.10 CEA calculations

The concentrations of each energy reserve (sugar, proteins and lipids; mg mg^{-1} ww) were converted into energetic equivalents using their energies of combustion (17 500, 24 000 and 39 500 mJ mg^{-1} respectively) given by Gnaiger (1983). Oxygen consumption derived from the ETS data was transformed into energy consumption by dividing by the sample wet weight, multiplying by 60 and then multiplying by the oxyenthalpic equivalents for an average protein, lipid and sugar mixture (484 kJ mol^{-1} O_2) also given by Gnaiger (1983). This produced the ETS data for each egg or larval sample from each spawn or each juvenile fish, with units of mJ mg^{-1} wet wt hr^{-1}.

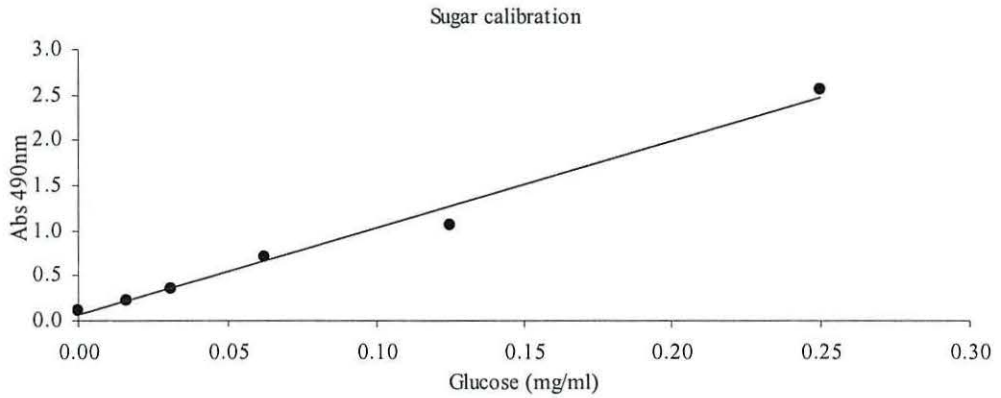
The CEA was calculated as described below:

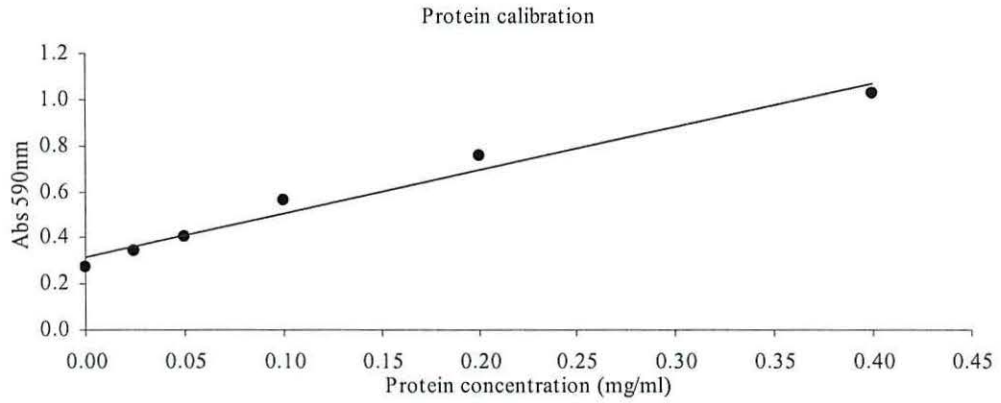
$$CEA = Ea/Ec$$

Where:

CEA = cellular energy allocation (hr)
Ea = energy available (sum of lipid, protein and sugar reserves) (mJ mg^{-1} wet wt.)
Ec = rate of energy consumption (mJ mg^{-1} wet wt. h^{-1})

The CEA score is basically a ratio used for inter-group comparisons and its units are only of theoretical importance. A literal explanation of the units would be the theoretical amount of time remining until all the energy reserves were used, though this is an unrealistic estimate as death would occur earlier given that the summed energy reserves include all body tissues in this case.





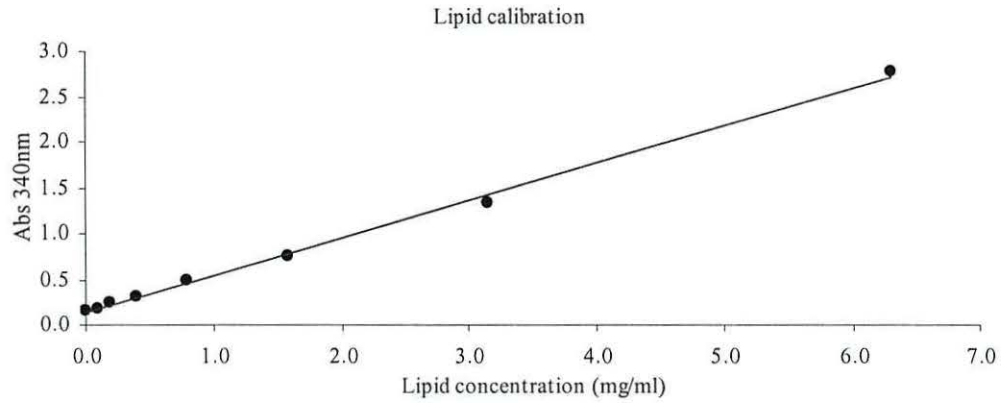


Figure 5.1 Energy reserve calibration graphs, each based on the average readings of four sets of standards analysed alongside the experimental samples from the juvenile dietary exposure experiment. Sugar, α-D-glucose ($y = 9.652x + 0.0564$, $R^2 = 0.9873$); protein, bovine albumen ($y = 1.8827x + 0.3152$, $R^2 = 0.9732$); lipid, tripalmtin ($y = 0.4096x + 0.1405$, $R^2 = 0.997$).

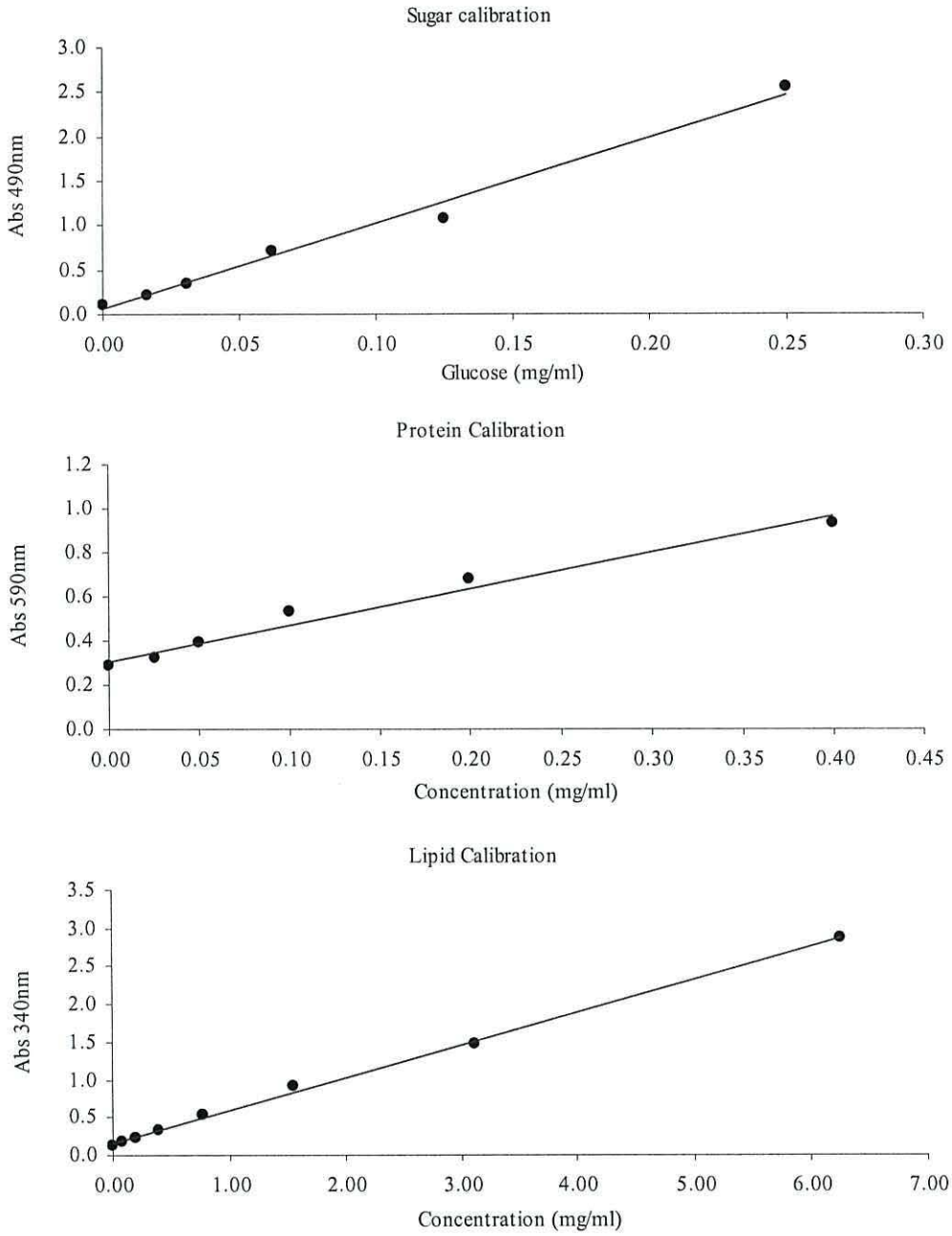


Figure 5.2 Energy reserve calibration graphs, each based on the average readings of four sets of standards analysed alongside the experimental samples from the maternal PCB transfer experiment. Sugar, α-D-glucose ($y = 9.652x + 0.0564$, $R^2 = 0.9873$); protein, bovine albumen ($y = 1.6615x + 0.3044$, $R^2 = 0.9800$); lipid, tripalmtin ($y = 0.434x + 0.1585$, $R^2 = 0.9987$). Values for sugar calibration were taken from the juvenile experiment due to problems with the standards prepared for the larval experiment.

5.3.11 Statistical analysis

All analyses were carried out with Minitab™ v13.2. Data were checked for normality and homogeneity of variance using Anderson-Darling and Bartlett's tests respectively. Data were log-transformed when $p > 0.05$ and if the criteria for ANOVA were still not met, the non-parametric Kruskal-Wallis test was used to check for significant differences in the median results. Nested ANOVAs were used where possible to account for variation caused by potential maternal influences. When significant results were detected, Tukey's honestly significant difference (HSD) pairwise comparisons were applied to detect exactly where significant differences occurred.

5.4 Results

5.4.1 Preliminary experiments

The preliminary work was designed to investigate the viability of different methods of sample preparation. Work with zebra fish was restricted by the time available during the visit to Ghent University and therefore not every stage of the CEA analysis was completed. However, considerable variation existed between the replicate measures of sugar and protein energy reserves in the liver in comparison to the lipid reserves (Table 5.2a). The coefficients of variation (CV) in lipid energy was greater in the whole fish homogenate compared to the liver samples yet still low in comparision to the CVs obtained by Verslycke et al. (2004a) for lipid energy of *N.integer* that ranged from 11 to 62 (mean = 37) depending upon the location of the sampling sites. The CVs for proteins and sugars in the study by Verslycke et al. (2004a) were 9 – 37 (mean = 21) and 16 – 66 (mean = 36) respectively. The high variation in the protein and sugar results suggested that complete homogenisation of the samples had not occurred and a different method of preparation should be investigated.

Data for the common goby (samples prepared using a teflon-tipped homogeniser) resulted in improved CVs for proteins although CVs increased for both sugars and lipids. CVs for lipids and proteins were not too dissimilar to those obtained by Verslycke et al. (2004a) and it was considered that with more thorough homogenisation, repeatability would improve (Tables 5.1a and b). This method of sample preparation was therefore used for the juvenile fish in Experiment 1.

5.4.2 Water temperatures

The mean water temperature of the rearing tanks in the juvenile Aroclor 1254 exposure experiment was 13.2°C ±0.7 (SD) over the nine-day experimental period. For the rearing tanks in the larval exposure experiment, the mean water temperature was 9.2°C ±0.4 (SD) for the period between hatch and metamorphosis. No significant differences were found between treatments in the mean water temperatures of the treatment tanks used in the juvenile dietary exposure experiment in 2004 (ANOVA,

Table 5.2a Mean (±SD) energy fractions (mJ mg^{-1} ww) and coefficients of variation (CV) determined from zebra fish (*Danio rerio*) samples (n = 3 for each analysis) analysed using different methods of preparation. Whole fish were homogenised using a pestle and mortar either at room temperature or in liquid nitrogen. Entries left blank (-) denote no samples analysed.

Sample type	**E sugar** (mJ mg^{-1} ww)	**E proteins** (mJ mg^{-1} ww)	**E lipids** (mJ mg^{-1} ww)
Liver only	265.89	492.80	3646.11
SD	178.06	1482.44	111.69
CV	67	301	3
Whole fish (room temperature)	143.31	1044.90	-
SD	69.60	538.30	-
CV	49	52	-
Whole fish (liquid N_2)	-	512.39	1387.57
SD	-	79.54	176.05
CV	-	16	13

Table 5.2b Mean (±SD) energy fractions (mJ mg^{-1} ww) and coefficients of variation (CV) determined from common goby (*Pomatoschistus minutus*) samples (n = 10 from each field site). Whole fish were homogenised using a Teflon-tipped homogeniser whilst keeping the sample in a tube in ice and water (data collected in preparation for this study and as work for an M.Sc. thesis (Godsell, 2005).

Field site		Fish (mg ww)	Homogenate (mg ww)	E sugar (mJ mg^{-1} ww)	E protein (mJ mg^{-1} ww)	E lipid (mJ mg^{-1} ww)	Ec (mJ mg^{-1} ww hr^{-1})
Dulas Bay	Mean	712.3	18.91	21.98	1499.0	2935.0	19.80
	SD	167.2	5.234	16.77	423.2	781.9	5.54
	CV	23	28	76	28	27	28
Foryd Bay	Mean	685.0	15.17	19.49	1444.0	4646.0	9.02
	SD	131.0	5.001	20.22	453.1	1570.0	3.34
	CV	19	33	104	31	34	37

$F_{2,90} = 0.15$, $p = 0.859$) or between the median temperatures in the larval rearing tanks in the 2005 maternal exposure experiment (Kruskal-Wallis, H = 0.06, df = 2, n = 500, $p = 0.971$).

5.4.3 Variation in CEA results

All data from the four steps of the CEA assay are based on weight-specific values. This is because in the case of the maternal exposure experiment, the samples used consisted either of multiple eggs and larvae or individuals used for each step. In the juvenile exposure experiment that used sub-samples from each juvenile, each fish sampled had a different weight, so comparison of the separate energy fractions had to be kept on a weight-specific basis. The CEA values in the juvenile experiment were calculated for individual fish but used the weight-specific values to avoid over-complicating the calculations. Coefficients of variation in the juvenile experiment were 35-63% for sugar energy, 17-20% for protein energy, 36-42% for lipid energy and 23-31% for ETS activity and showed a general improvement compared to the goby data (Table 5.2b). In the larval experiment, all CVs ranged between 3 and 40% with the exception of sugar energy at first-feeding which ranged between 25 and 65% and protein energy which ranged between 26 and 50% at metamorphosis.

5.4.4 Larval experiment results

Each energy reserve (lipids, proteins and carbohydrates) increased on a mJ mg^{-1} wet wt basis during the period of development from hatch until metamorphosis but very few significant differences were found between treatments at any stage of development (Tables 5.3 to 5.6). Consequently the data for all three treatments were pooled in order to demonstrate graphically the changes that occurred in the whole body proximate concentrations, whole body total energetic values and the percentage contribution of lipids, proteins and carbohydrates to whole body total energy content as ontogeny progressed (Figs. 5.3 and 5.4). Whilst the total energy for each type of energy reserve measured (lipids, proteins and carbohydrates) increased in magnitude throughout development, the relative contributions of protein and glucose to the

Tables 5.3 – 5.7 Effects of maternal exposure to Aroclor 1254, on the cellular energy allocation (CEA) in fertilized eggs (5.3), larvae <24hrs post-hatch (5.4), larvae at 8-dph (5.5) and metamorphosed larvae (5.6) of the European plaice *P.platessa*. Table 5.7 shows the effects of dietary exposure to Aroclor 1254 on the CEA of field-caught 0+ plaice exposed for 8-days. Data (mean ±SD) were subjected to nested ANOVAs. Energy reserves are expressed as mJ mg ww^{-1} and units of energy consumption (ETS activity) are mJ mg ww hr^{-1}. Data in red text has different letters to highlight significant differences either at the 5% level following Tukey's HSD pairwise comparisons. * = p <0.05.

5.3 Fertilised eggs

Maternal dose (mg kg^{-1} fish $month^{-1}$)	Energy allocation					
	Sugar reserve (mJ mg^{-1} ww)	Protein reserve (mJ mg^{-1} ww)	Lipid reserve (mJ mg^{-1} ww)	E_a (mJ mg^{-1} ww)	E_c (mJ mg^{-1} ww hr^{-1})	CEA
Undosed	4.0^{a} *	123.8	1496.9	1624.6	3.5	465.8
	±0.6	±30.3	±427.2	±357.2	±0.9	±74.3
0.14-0.24	3.8^{ab}	110.2	1381.5	1495.4	3.1	484.7
	±1.5	±14.3	±299.7	±132.8	±0.5	±31.1
3.32-4.33	3.3^{b} *	129.7	1356.3	1489.3	3.4	447.4
	±0.7	±28.9	±327.9	±148.7	±0.7	±111.8

5.4 Larvae <24 hours post-hatch

Maternal dose (mg kg^{-1} fish $month^{-1}$)	Energy allocation					
	Sugar reserve (mJ mg^{-1} ww)	Protein reserve (mJ mg^{-1} ww)	Lipid reserve (mJ mg^{-1} ww)	E_a (mJ mg^{-1} ww)	E_c (mJ mg^{-1} ww hr^{-1})	CEA
Undosed	14.9	360.6	1651.7^{a} *	1993.5	18.7^{a} *	103.7^{a} *
	±2.5	±134.8	±250.4	±305.6	±4.8	±33.3
0.14-0.24	15.6	367.3	1990.3^{ab}	2359.4	15.9^{ab}	149.5^{ab}
	±2.1	±80.4	±302.1	±81.0	±4.3	±16.1
3.32-4.33	13.0	346.1	2160.4^{b} *	2673.1	12.1^{b} *	216.5^{b}*
	±1.8	±129.4	±314.7	±492.5	±1.7	±50.4

5.5 Larvae at 8-days post-hatch

Maternal dose (mg kg^{-1} fish $month^{-1}$)	Energy allocation					
	Sugar reserve (mJ mg^{-1} ww)	Protein reserve (mJ mg^{-1} ww)	Lipid reserve (mJ mg^{-1} ww)	E_a (mJ mg^{-1} ww)	E_c (mJ mg^{-1} ww hr^{-1})	CEA
Undosed	30.8	489.5	2100.2	2575.3	29.2	90.2
	±7.7	±88.2	±492.6	±292.4	±7.2	±16.6
0.14-0.24	28.9	491.7	2206.2	2623.7	31.9	81.5
	±18.6	±28.3	±318.9	±320.2	±8.2	±13.5
3.32-4.33	25.9	512.0	1919.7	2489.4	27.7	82.3
	±10.31	±54.6	±525.1	±743.1	±7.8	±22.5

5.6 Metamorphosed larvae (42-days post-hatch)

Maternal dose (mg kg^{-1} fish $month^{-1}$)	Energy allocation					
	Sugar reserve (mJ mg^{-1} ww)	Protein reserve (mJ mg^{-1} ww)	Lipid reserve (mJ mg^{-1} ww)	E_a (mJ mg^{-1} ww)	E_c (mJ mg^{-1} ww hr^{-1})	CEA
Undosed	112.9	729.9	2462.9	3307.8	45.4^{ab}	72.9
	±26.5	±364.2	±888.7	±872.8	±7.5	±18.2
0.14-0.24	116.5	581.8	2919.6	3617.9	54.5^{a} *	67.6
	±23.5	±196.0	±667.0	±270.1	±14.1	±9.5
3.32-4.33	91.1	789.2	2424.5	3307.8	38.7^{b} *	86.9
	±29.1	±209.1	±799.2	±475.7	±11.1	±15.7

Contd. overleaf

Contd.

5.7 0+ plaice

Dietary dose (mg kg^{-1} fish day^{-1})	Energy allocation					
	Sugar reserve (mJ mg^{-1} ww)	Protein reserve (mJ mg^{-1} ww)	Lipid reserve (mJ mg^{-1} ww)	E_a (mJ mg^{-1} ww)	E_c (mJ mg^{-1} ww hr^{-1})	CEA
Undosed	45.4	1057.3	2783.9	3886.6	**23.4^{a}**	**175.4^{a}**
	±25.0	±179.8	±1162.1	±1313.1	**±7.3**	**±69.9**
0.2	45.3	1017.2	2610.6	3673.1	**19.0^{b}**	**203.2^{ab}**
	±28.1	±168.5	±1080.4	±1232.0	**±4.3**	**±88.2**
1.0	39.4	982.0	2450.6	3472.0	**13.5^{c}**	**271.0^{b}**
	±14.0	±198.8	±894.6	±1028.4	**±4.2**	**±84.7**

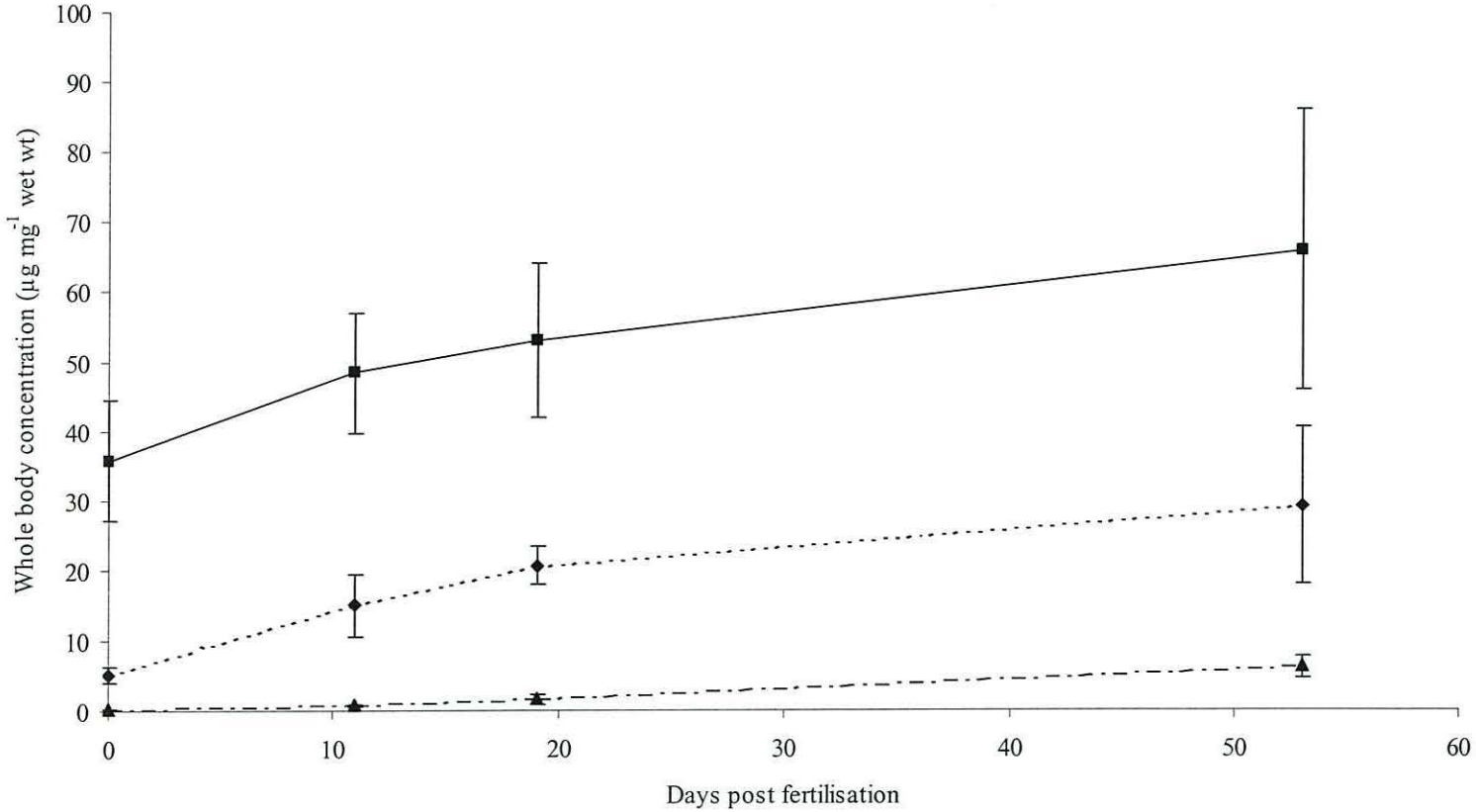
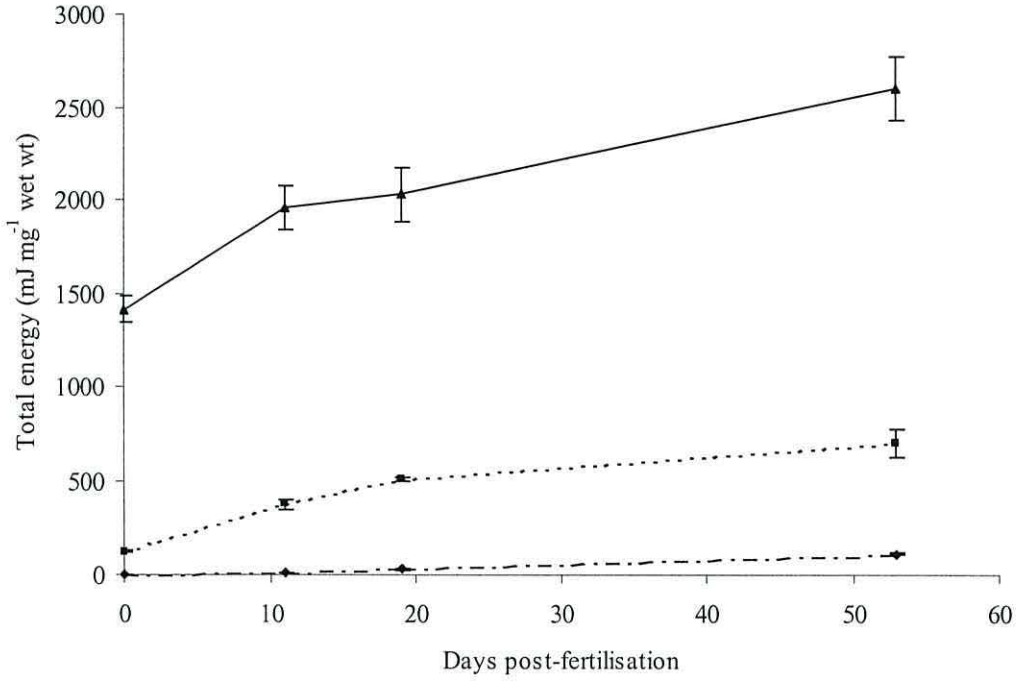


Figure 5.3 Changes in the mean ±SE whole body concentrations of lipids (solid line), proteins (short dashes) and carbohydrates (long and short dashes) following fertilisation of plaice (*Pleuronectes platessa*) eggs until complete metamorphosis of the larvae at 53-days post-fertilisation (no. of spawns = 9). Concentrations are plotted against age because larval length or weight were not measured during the experiment.

A



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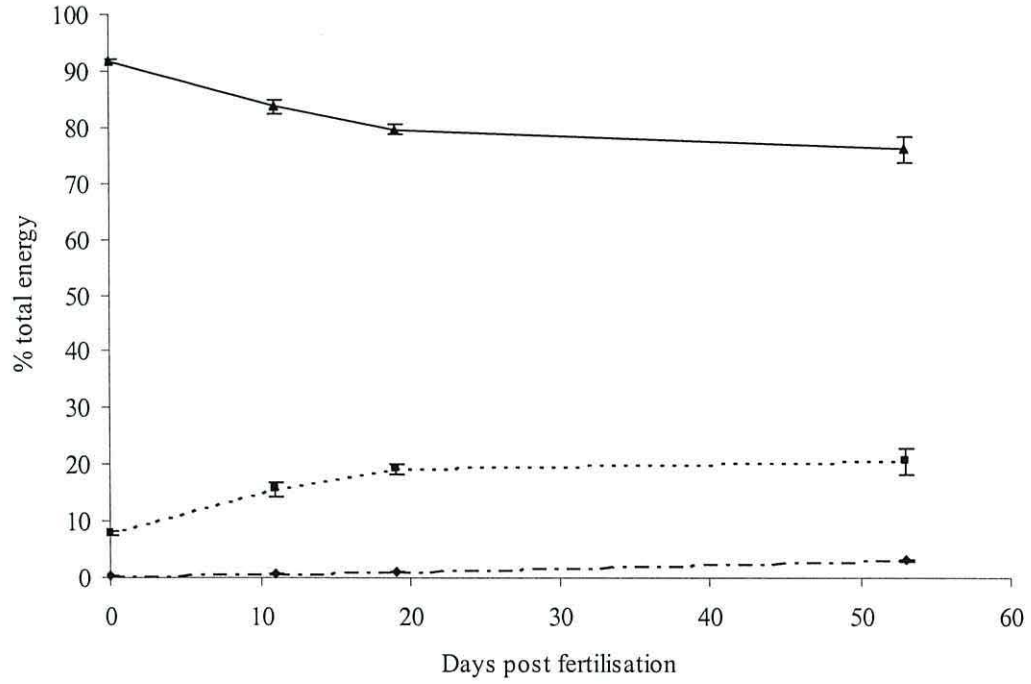


Figure 5.4 Plots showing the changes in (**A**) mean (±SE) energy reserves (mJ mg^{-1} wet wt.) and (**B**) mean (±SE) percentage composition of energy reserves of pooled samples of plaice (*Pleuronectes platessa*) eggs and larvae following fertilisation of eggs from separate female spawns (n = 9). Lipids (solid line), proteins (short dashes) and carbohydrates (long and short dashes).

overall energy pool increased with increasing age, whilst that of lipids decreased (Fig. 5.4). The average protein concentration across all larval samples at hatch was 15 ±4.5μg mg^{-1} wet wt and this increased by 95% to 29.2 ±11.3μg mg^{-1} wet wt at metamorphosis, whereas lipids increased from 48.4 ±8.7μg mg^{-1} wet wt by 36.2% to 65.9 ±20.1μg mg^{-1} wet wt over the same time period (Tables 5.3 to 5.7). In comparison, sugar concentrations at hatch were on average only 0.8 ±0.1μg mg^{-1} wet wt increasing to 6.1 ±0.2μg mg^{-1} wet wt at metamorphosis; an increase of 762.5% (Tables 5.3 to 5.7).

Larval exposure to PCBs via maternal transfer gave a series of significantly different (p <0.05) results with respect to lipid energy, ETS activity and CEA values between treatments in newly hatched larvae (Table 5.4). These differences were not observed in later developmental stages with the exception of ETS activity at metamorphosis (Table 5.6). For the fertilised eggs there were significant differences between control and high-dosed eggs with regard to sugar energy ($p = 0.036$) (Table 5.5). At hatch, there were significant differences in lipid reserves, ETS activity and CEA values between control and high-dosed larvae ($p = 0.0123$, $p = 0.0485$ and $p = 0.020$ respectively). These significant differences between control and high-dosed larvae at hatch did not occur at first-feeding (8-dph). The significant difference in ETS activity at metamorphosis between low- and high-dosed fish (Table 5.6) ($p = 0.0145$) may possibly be a Type II error given the lack of any differences observed at first-feeding. Additionally, the pattern of the changes in ETS activity with PCB dose, did not follow those of ETS activity at hatch (Table 5.4) or the changes observed in the juvenile CEA assay (Table 5.7).

Although some differences were detected in sugar reserves of eggs (ANOVA, $F_{2,9} = 3.65$, $p = 0.047$) (Table 5.3) and lipid reserves of newly hatched larvae (ANOVA, $F_{2,9} = 6.81$, $p = 0.014$) (Table 5.4), these were not enough to cause significant differences between treatments in terms of total energy reserves (Ea). The patterns in energy composition (Figs. 5.3 – 5.4) demonstrate the relative increase in protein and sugar reserves, which occurred as the larvae developed into juvenile fish. In quantitative terms, protein and lipid were the most abundant of the three energy reserves (7.37 – 23.86% and 73.29 – 92.38% respectively) with sugar making up only 0.22 – 3.42% of the reserve, though the quantity increased with age.

The nested ANOVAs indicated that there were significant differences in egg protein energy reserves (ANOVA, $F_{6, 27} = 7.39, p < 0.001$), metamorphosis protein energy reserves (ANOVA, $F_{6, 27} = 5.61, p = 0.002$) and sugar energy reserves in eggs (ANOVA, $F_{6, 27} = 9.08, p < 0.001$) and larvae at hatch (ANOVA, $F_{6, 27} = 3.80, p = 0.024$) and first-feeding (ANOVA, $F_{6, 27} = 5.57, p = 0.006$) between female spawns within treatments. For all other energy fractions at each life stage analysed, no significant differences were detected between female spawns within treatments.

5.4.5 Juvenile experiment results

Juveniles dosed with PCBs in their diet exhibited significant reductions in the rate of energy consumption at low- and high-doses compared to control fish ($p = 0.0135$ and $p < 0.001$ respectively) (Table 5.7). However, this only resulted in significant differences between control and high-dosed fish with respect to CEA ($p = 0.006$). No significant differences were observed between any of the energy reserve fractions (Table 5.7) when subjected to Tukey's HSD pairwise comparisons ($p > 0.05$ in all cases). The nested ANOVA tests detected significant differences in ETS activity between tanks within treatments (ANOVA, $F_{6, 9} = 6.55, p < 0.001$) but this was not the case for individual or summed energy reserve fractions ($p > 0.05$ in all cases). Pairwise comparisons showed most, but not all, of the differences to be due to higher variation between tanks in the control group and not the PCB treated fish tanks. This did not preclude the ANOVA from detecting significantly different ETS activities between treatments.

5.4.6 CEA values

The CEA index (Ea/Ec) provides an estimate of the overall energy budget at each developmental stage. CEA is affected by extreme values and as a result, significant differences are often detected between treatments when differences also exist between Ec values (Tables 5.4 and 5.7). Log CEA values declined with age in all treatments in the larval experiment (Fig. 5.5). Table 5.8 gives the regression equations and R^2

values for all regression lines in Fig. 5.5. As the obvious effect of PCB contamination on ETS activity appears to disappear at post yolk absorption, so too does the impact on CEA. Dietary exposure of juvenile fish to PCBs later in ontogeny, again results in a decrease in ETS activity and a subsequent increase in CEA (Table 5.7).

5.4.7 PCB analyses

The LDs for the 21 PCB congeners detected in the GC/MS analyses ranged from 1.4 – 70.4μg l^{-1} sample for both the juvenile plaice exposed to Aroclor 1254 through their diet and the eggs obtained from female plaice dosed orally with Aroclor 1254. The range of ΣPCB(21 congeners) concentrations in the juvenile dietary exposure experiment was 55 – 835.7μg kg^{-1} dry wt. in low-dosed fish and was 182.9 – 1032.2μg kg^{-1} dry wt. in high-dosed fish (Table 5.9). PCBs were not detected in the control fish (Table 5.9). PCBs were detected in the eggs stripped from female broodstock and fertilised to provide the larvae at concentrations of 1.0 – 126.0μg kg^{-1} dry wt. in eggs from low-dosed females and 847.8 – 4166.9μg kg^{-1} dry wt. in eggs from high-dosed females (Table 5.10). PCBs were not detected in fish sampled at metamorphosis (Table 5.10). Table 5.11 shows the concentrations of PCBs detected in the livers of the female broodstock at the end of the spawning period. The liver PCB concentrations were higher than those of the female broodstock given high PCB doses in 2004 (Table 3.3) and might be because these fish spawned only once, giving less opportunity for PCB depuration.

Significant correlations were found between ΣPCB (21 congeners) egg concentrations and lipid energy ($r = 0.835$, $p = 0.010$), total available energy ($r = 0.844$, $p = 0.008$) and CEA ($r = 0.849$, $p = 0.008$) at hatch (Fig. 5.6). Error bars are not given in Figs. 5.9B and D as the Ea and CEA values are calculated for each individual spawn, of which n = 9 in this experiment. As it was not possible to analyse eggs from female number 412 for PCB concentration, the data is omitted from Fig. 5.6 and only eight data points are presented. The heavy influence of one data point at the highest PCB concentration however means that these data should be treated tentatively.

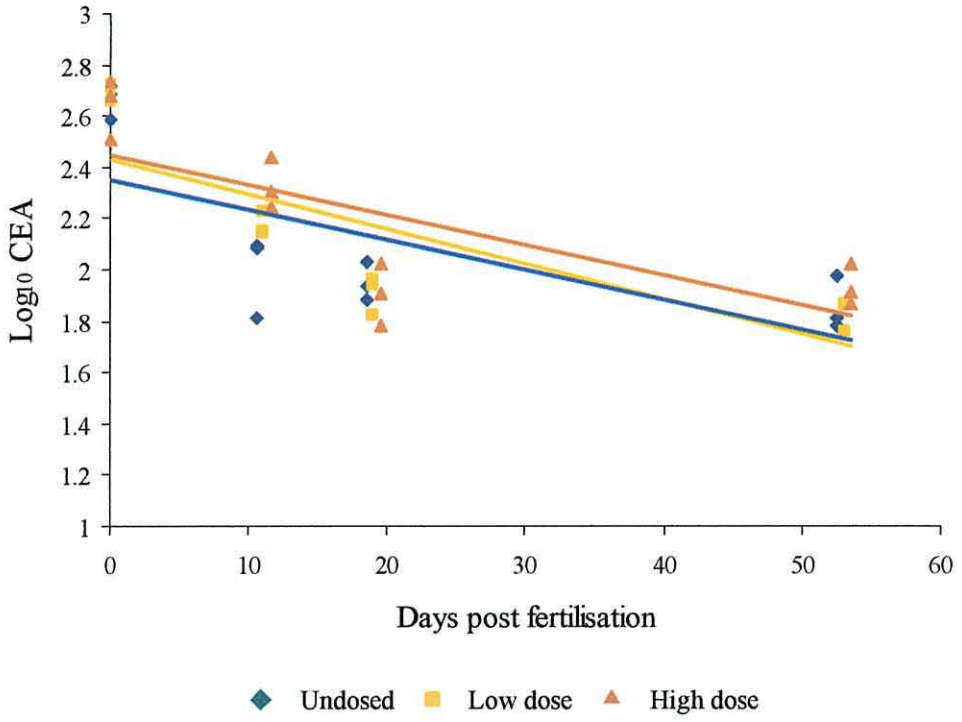


Figure 5.5 Log_{10} CEA values plotted against (A) larval standard length and (B) days post-fertilisation. Larvae were obtained from eggs from either uncontaminated female plaice broodstock (blue) of from females exposed to Aroclor 1254 at a low dose (yellow) of 0.14-0.24 or a high dose (orange) of 3.32-4.33 mg kg^{-1} $month^{-1}$. No significant differences or interactions existed between treatments and larval size (ANCOVA df=2, p>0.05 in all cases, n = 26) or days post fertilisation (ANCOVA df=2, p>0.05, in all cases, n = 36) with respect to Ea and Ec. Regression equations for the lines are given in Table 5.8.

Table 5.8 Equations and R^2 values for the regression lines shown in Fig. 5.5 for log CEA vs days post-fertilisation of plaice (*Pleuronectes platessa*) eggs and larvae. ANCOVA was applied to all the data sets and no significant differences in the rates of change of log CEA were detected between treatments (p >0.05 in all cases).

Treatment	R^2	Regression equation
Control	0.492 *	$y = 2.331^{e-0.0053x}$
Low dose	0.6575 **	$y = 2.434^{e-0.0052x}$
High dose	0.5422 **	$y = 2.4148^{e-0.0062x}$

[a], p >0.1; *, p <0.05; **, p <0.01

Table 5.9 Whole-body polychlorinated biphenyl concentrations (μg kg^{-1} dw) (ΣICES 6 and ΣPCB 21 congeners) in juvenile 0+ plaice (*Pleuronectes platessa*) exposed to Aroclor 1254 through their diet (analysis of diet in first three rows). n.d.=not detected; <LD denotes values falling below the limit of detection but retained for comparison to values from other experimental tanks.

Treatment	No. of fish	Sample wet wt (g)	Sample dry wt (g)	PCB dose (mg kg^{-1} fish day^{-1})	ICES 6 Congeners (μg kg^{-1}dw)						ΣPCB (21 congeners) (μg kg^{-1} dw)
					52	101	118	153	138	180	
Undosed food	-	1.16	1.14	-	nd	nd	nd	nd	nd	nd	nd
Low dosed food	-	1.10	1.08	-	114.1	274.4	227.0	149.2	152.5	28.7	2095.0
High dosed food	-	1.16	1.10	-	760.9	2105.5	2123.9	1129.3	1233.4	117.6	15806.9
Undosed tank 4	9	8.770	1.62	0	nd	nd	nd	nd	nd	nd	nd
Undosed tank 5	8	7.693	1.37	0	nd	nd	nd	nd	nd	nd	nd
Undosed tank 9	9	8.768	1.63	0	nd	nd	nd	nd	nd	nd	nd
Low dose tank 1	9	8.065	1.25	0.2	38.2	109.1	95.5	58	62.6	5.3	835.7
Low dose tank 7	8	7.768	1.47	0.2	41.4	86.3	77.6	57.3	57.4	9.9	731.1
Low dose tank 8	8	6.885	1.23	0.2	7.7	<LD (4.96)	nd	<LD (5.37)	<LD (4.88)	nd	<LD (55.4)
High dose tank 2	8	6.678	1.22	1.0	13.1	<LD (21.6)	<LD (17.6)	<LD (10.2)	<LD (11.7)	nd	<LD (182.9)
High dose tank 3	9	6.913	1.28	1.0	58.1	113.4	111.5	59.6	59.5	7.9	940.4
High dose tank 6	9	6.284	1.11	1.0	54.9	132.9	114.9	75.1	73.5	10.3	1032.2

Table 5.10 Polychlorinated biphenyl concentrations (μg kg^{-1}dw) (ΣICES 6 and ΣPCB 21 congeners) in the eggs and metamorphosed larvae (Juv.) from female plaice (*Pleuronectes platessa*) broodstock in the PCB treated groups. Abbreviations: n.s. = no sample; n.d. = not detected; trace = below practical quantification limit (2xLD); <LD denotes values falling below the quantification limit but retained for comparison to other experimental values.

Fish ID code	Sample	Wet wt (g)	Dry wt (g)	Maternal PCB dose (mg kg^{-1} fish $month^{-1}$)	ICES 6 Congeners (μg kg^{-1}dw)						ΣPCB (21 congeners) (μg kg^{-1} dw)
					52	101	118	153	138	180	
423-2	Eggs	9.48	0.65	0.19	nd	trace (7.7)	21.7	<LD (6.0)	<LD (12.9)	<LD (3.2)	<LD (126.0)
424-2	Eggs	16.39	1.14	0.14	nd	nd	nd	nd	nd	<LD(1.2)	<LD (1.0)
426-1	Eggs	14.59	0.99	0.24	nd	nd	10.2	<LD (6.7)	trace (8.2)	<LD (2.0)	<LD (68.1)
412	Eggs	ns	ns	4.33	ns	ns	ns	ns	ns	ns	ns
413	Eggs	22.05	1.81	3.32	39.1	95.5	94.7	62.2	69.6	11.4	847.8
478	Eggs	9.14	0.69	3.93	167.7	462.9	464.1	273.8	365.1	46.4	4166.9
514, 523, 527	Eggs	11.00 - 18.82	0.70 - 1.04	0	nd	nd	nd	nd	nd	nd	nd
423, 424, 426	Juv.	-	0.15 - 0.55	0.14 - 0.24	nd	nd	nd	nd	nd	nd	nd
412, 413, 478	Juv.	-	0.37 - 0.42	3.32 - 3.93	nd	nd	nd	nd	nd	nd	nd
514, 523, 527	Juv.	-	0.15 - 0.16	0	nd	nd	nd	nd	nd	nd	nd

Table 5.11 Polychlorinated biphenyl concentrations (µg kg^{-1}dw) (ΣICES 6 and ΣPCB 21 congeners) in the livers of female plaice (*Pleuronectes platessa*) broodstock exposed to Aroclor 1254. Abbreviations: n.d. = not detected; trace = below practical quantification limit (2xLD); <LD denotes values falling below the LD but retained for comparison to other experimental values.

Fish ID code	Sample dry wt (g)	Maternal PCB dose (mg kg^{-1} fish $month^{-1}$)	Maternal liver ICES 6 Congeners µg kg^{-1}dw						Maternal liver ΣPCB (21 congeners) µg kg^{-1} dw
			52	101	118	153	138	180	
423	0.49	0.19	nd	<LD (25.9)	<LD (10.3)	<LD (12.9)	trace (20.6)	<LD (4.1)	<LD (142.6)
424	0.60	0.14	<LD (3.7)	<LD (5.1)	30.1	trace (35.8)	26.3	nd	trace (164.4)
426	0.50	0.24	trace (113.5)	258.4	369.0	241.0	238.8	51.8	2652.8
412	0.64	4.33	3564.2	7811.3	9016.4	4632.0	5095.9	997.3	68861.6
413	0.54	3.32	421.7	2657.2	3558.3	2389.6	2622.4	507.8	23140.1
478	0.62	3.93	8402.4	21493.6	25950.5	14659.2	15427.1	2951.1	187467.0
514, 523, 527	0.70 - 1.04	0	nd	nd	nd	nd	nd	nd	nd

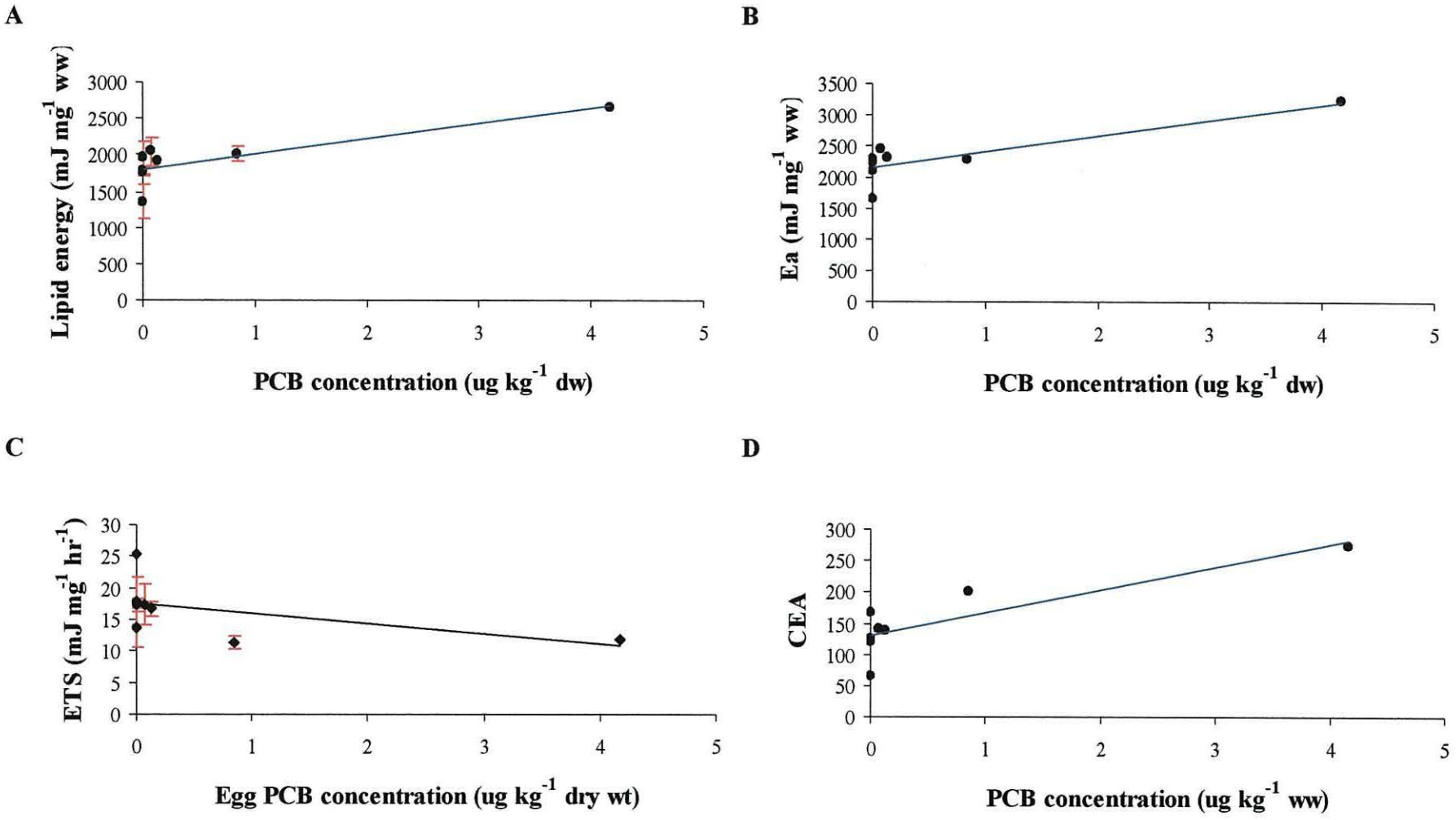
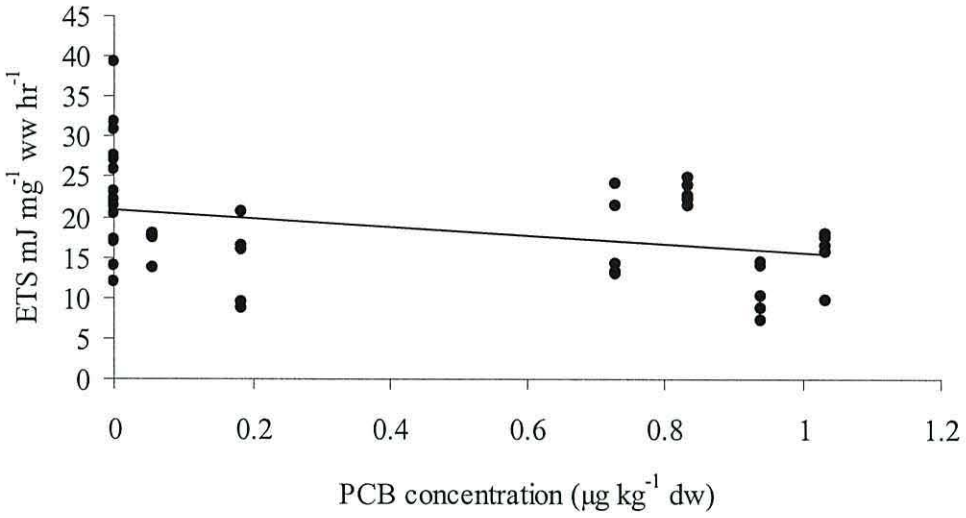


Figure 5.6 Relationship of (**A**) lipid energy reserves ($y = 211.91x + 1801.5$, $R^2 = 0.6979$), (**B**) total energy available (Ea) ($y = 256x + 2153.9$, $R^2 = 0.7118$), (**C**) energy consumption ($y = -1.5975x + 17.478$, $R^2 = 0.2744$) and (**D**) cellular energy allocation (CEA) ($y = 36.188x + 130.46$, $R^2 = 0.7206$) of newly hatched (<24hrs) plaice (*Pleuronectes platessa*) larvae in relation to egg ΣPCB(21 congeners) concentrations (µg kg^{-1} dw). Error bars are not given for charts B and D as the Ea and CEA are indices based on summed totals of energetic parameters for individual spawns.

Correlations of PCB concentrations and ETS activity were not significant in the larvae at hatch ($r = -0.524$, $p = 0.183$). In the juvenile 0+ fish experiment, there was a significant negative correlation between ETS activity and PCB concentration ($r = -0.352$, $p = 0.018$) (Fig. 5.7 A) but not with any other experimental parameters ($p > 0.05$ in all cases) (Figs. 5.7 B and 5.8).

There were no significant correlations of ETS activity with mean water temperatures at hatch or in the juvenile exposure experiment ($p > 0.05$ in both cases) (Fig. 5.9). Therefore, given that there were also no significant differences detected between rearing tank water temperatures (section 5.4.2), this factor is unlikely to have influenced the differences in ETS activity observed between the treatents.

A



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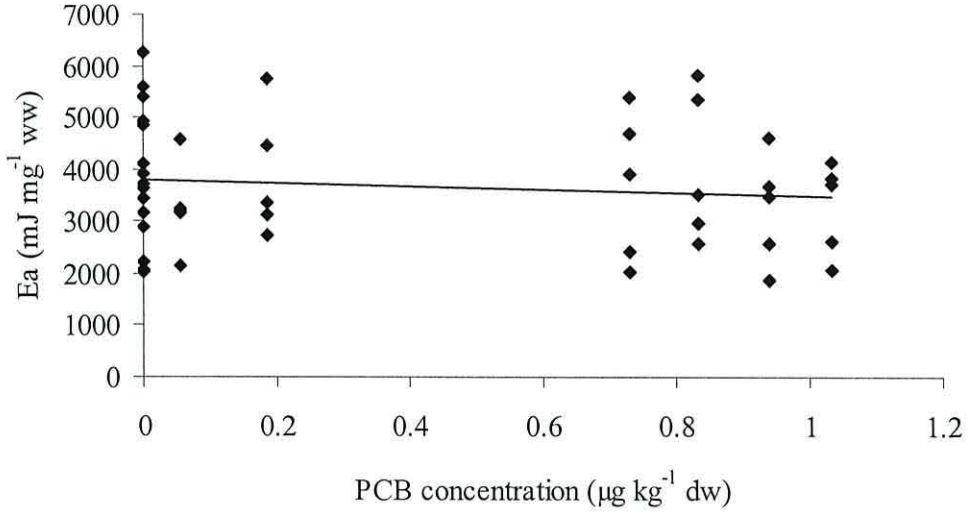


Figure 5.7 Relationship of (**A**) energy consumption rates ($y = -5.4593x + 20.948$, $R^2 = 0.1238$, $p = 0.018$) and (**B**) total energy available (Ea) ($y = -291.84x + 3799.7$, $R^2 = 0.0113$, $p = 0.512$) of juvenile 0+ plaice (*Pleuronectes platessa*) in relation to whole body PCB concentration following dietary exposure to Aroclor 1254.

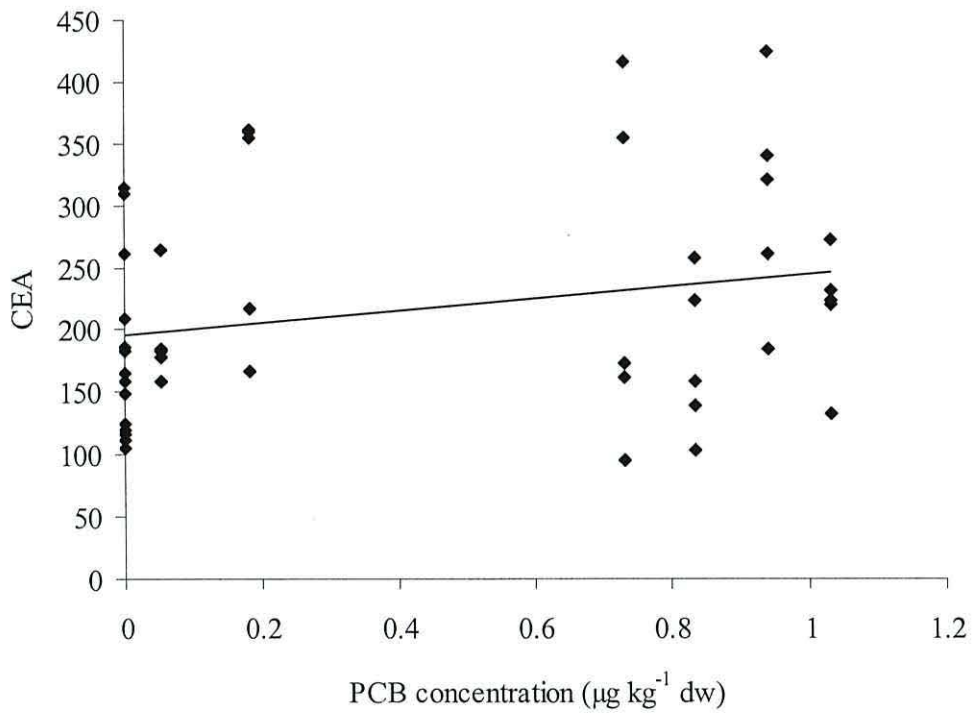
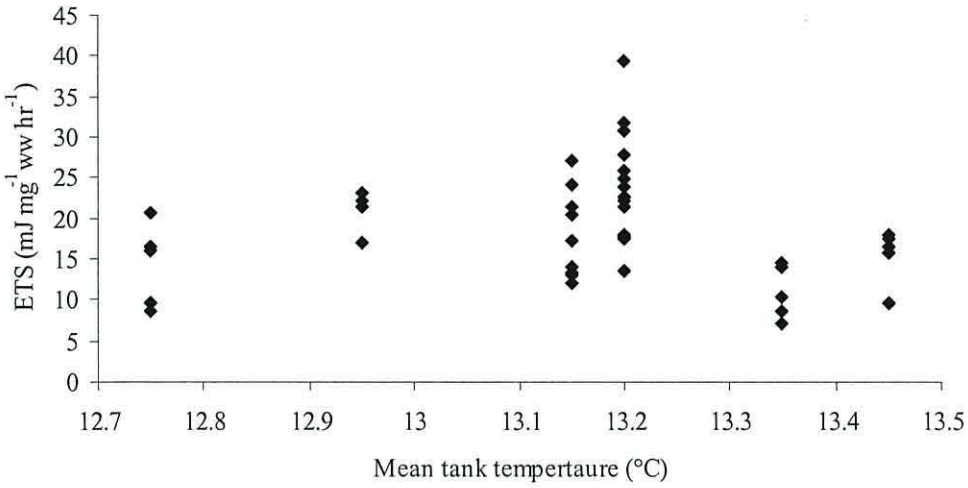


Figure 5.8 Scatter plot of the cellular energy allocation (CEA) of juvenile 0+ plaice (*Pleuronectes platessa*) following dietary exposure to Aroclor 1254 in relation to whole body PCB concentration. No significant relationship was detected ($y = 48.333x + 196.24$, $R^2 = 0.0545$, $p = 0.122$).

A



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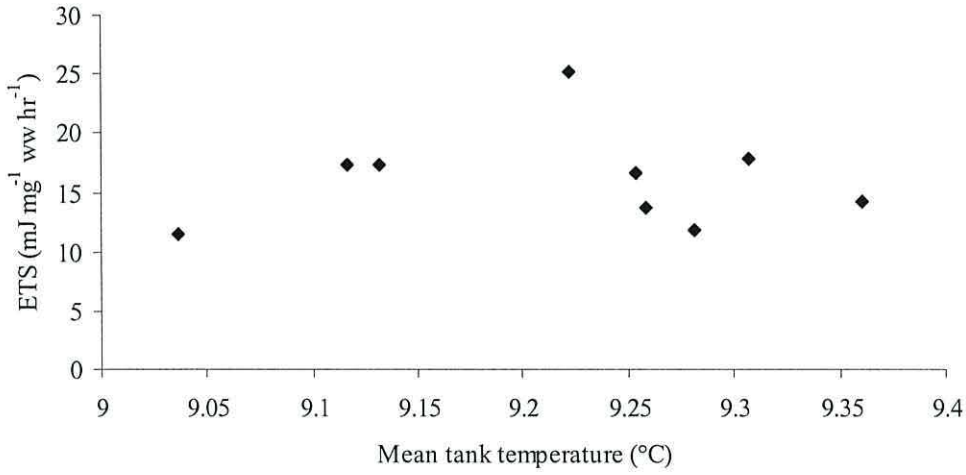

Figure 5.9 Scatter plots of electron transport system (ETS) activity (mJ mg^{-1} ww hr^{-1}) and mean water temperature (°C) of (**A**) juvenile 0+ plaice (*Pleuronectes platessa*) exposed to Aroclor 1254 in their diet (r = -0.039, p = 0.797) and (**B**) plaice larvae exposed to PCBs via maternal transfer (r = -0.053, p = 0.892).

5.5 Discussion

5.5.1 Energy composition

The measure of each type of energy reserve (lipids, proteins and carbohydrates) provides a quantitative measure of the manner in which energy is stored within an organism. The results of this study demonstrate that lipids and proteins are perhaps the most important components of energy reserves given that together they always comprised over 95% of the total reserves (Tables 5.3 – 5.7). Carbohydrates contributed only a small amount of the overall available energy throughout development. The large increase in protein reserves throughout development (Figs. 5.3 and 5.4) is likely to be due to the deposition of muscle mass as the larvae develop (Territo & Smits, 1998) and would provide a valuable energy reserve should a shortage of food occur.

Christensen & Korsgaard (1999) reported that during metamorphosis in plaice larvae, protein catabolism was dominant but this changed to lipid catabolism by the end of metamorphosis, demonstrating the importance of these two energy fractions in the metabolism of plaice larvae. Investigations of the physiological energetics of newly hatched Atlantic cod larvae demonstrated that their catabolic metabolism was predominantly fuelled by amino acids (67%) and lipids (32%), while glycogen only accounted for 1% of the total enthalpy dissipated (Finn et al., 1995a; Finn et al., 1995b). Finn et al. (1995a) suggested that this sequence of catabolic substrate oxidation should be generally applicable to other cold-water fish which spawn eggs that do not contain oil globules such as the plaice (Wimpenny, 1953). In a further study on halibut larvae, Finn et al. (1995c) further demonstrated that proteins primarily, followed by lipids, fuelled metabolism.

Although the proportions of lipid energy in this study outweighs that of proteins, it should be remembered that the results presented here have assessed the total energy reserves and not the particular energy sources metabolised. The results of this study support those of the other studies mentioned above by demonstrating that lipids and proteins are quantitatively the most important energy fractions. Seven days following

first feeding, the larvae were reared on lipid-enriched *Artemia* (ICES oil mix, see section 4.3.1) and this might have contributed to the high concentrations of lipids detected at metamorphosis, as was suggested following CEA studies on *N.integer* (Verslycke & Janssen, 2002). In another study of CEA in adult *N.integer*, Verslycke et al. (2004a) identified protein and lipid as quantitatively the most important energy fractions providing approximately equal percentages of energy, whilst sugar only contributed 1-2% of the total energy content. Although these are results for an invertebrate species they demonstrate consistency in the results obtained between the two laboratories in carrying out the analytical steps.

Costopoulos & Fonds (1989) examined the lipid and protein content of juvenile plaice between 5.7cm and 30cm long (total length). Carbohydrates were not measured but had been reported previously to account only for approximately 1% of the dry weight of plaice white muscle (Johnston & Goldspink, 1973) and this estimate corresponds well with the results of this study (Fig. 5.3). Costopoulos & Fonds (1989) reported that the mean protein content of plaice comprised 70.42 – 76.37% of the dry weight with lipids comprising only 1.86 – 16.46% depending on the condition factor. In this study on larval plaice, although the protein content was lower than that of lipids, the percentage increase in protein content was greater than that of lipid content and further analyses of larger juvenile fish would be necessary to see if this trend continues. Lipid content is highest in plaice with higher condition factors (Costopoulos & Fonds, 1989). Although condition factor was not measured in this study, the larvae and juvenile fish were fed *ad libitum* and likely had good condition factors that may have increased lipid content. The methods of lipid extraction and protein content calculation also differed between the studies and a comparative study would determine whether the analytical methods used influenced the results. Despite these differences in the results, Costopoulos & Fonds (1989) reported the mean ±SD total available energy in the plaice sampled (5.7 – 30cm total length) as 2.392 ±0.176 – 4.546 ±0.163 kJ g^{-1} wet wt., increasing with condition factor. In this study, the mean ±SD value of Ea (across all treatments) for an individual juvenile plaice in Experiment 1 was 3.677 ±1.182 kJ g^{-1} wet wt. In the newly metamorphosed juveniles in Experiment 2 the mean ±SD value of Ea per female spawn was 3.411 ±0.537 kJ g^{-1} wet wt demonstrating that although the lipid and protein contents may differ in

comparison to other studies, the total Ea values reported here lay well within the same range as that reported by Costopoulos & Fonds (1989).

Few significant differences in energy reserve fractions were detected between any of the PCB-exposed groups of larval or juvenile plaice in this study. The only two occasions where significant differences did exist were in the early developmental stages of embryos and newly hatched larvae (carbohydrates in eggs and lipids in newly hatched larvae) (Tables 5.3 and 5.4). This is discussed further in the next section together with the ETS activity data.

5.5.2 Energy consumption and CEA

The ETS activity provides an estimate of the potential whole organism respiration rate (Verslycke & Janssen, 2002). It has been suggested that although measurement of ETS activity gives an overestimate of the actual rate of respiration, the ETS assay reflects the maximum ETS activity possible under saturated substrate (NADH, NADPH) conditions and correlates well to actual respiration rates (Verslycke & Janssen, 2002). Because the assay is run under saturated conditions, any differences observed in ETS activity between treatments are either the result of different quantities of enzymes being produced by the organisms sampled from the treatments, or specific interactions of the PCB congeners with the electron transport chain (Verslycke & Janssen, 2002). Samples from all treatments are run together in multiwell plates, rather than separately so enzyme activity within each treatment group is measured simultaneously, making results very comparable.

In the juvenile exposure experiment, ETS activity was significantly reduced in the high-dosed individuals compared to control and low-dosed individuals (Table 5.7) even though there were few apparent differences in the magnitude of whole body PCB concentrations between individuals sampled for GC/MS analysis (Table 5.9). The significant reduction in ETS activity in newly hatched larvae from eggs with the highest PCB concentrations compared to control larvae was detected in the maternal transfer experiment (Table 5.4) whilst the low-dosed larvae had a lower, but not significantly different, mean ETS activity than control larvae. No significant

differences in ETS activity were detected between treatment in the eggs or in the larvae at first-feeding but significant differences were detected between treatments at metamorphosis (Table 5.6). These differences between treatments in ETS activity at metamorphosis did not follow the same dose-response patterns as those observed at hatch or in the juvenile fish in Experiment 1, the reasons for which are unclear. One possibility is that this result is a Type II error (a truly non-significant result but deemed significant by a test) but as this cannot be proven here, the result should not be disregarded completely. In other studies investigating contaminant effects on the ETS activity, mixed results have been reported. ETS increased in *N.integer* following chlorpyrifos exposure (Verslycke et al., 2004b) but showed a slight, but not significant decrease in the same species following exposure to TBT (Verslycke et al., 2003). The decrease in ETS in this study resulted in elevated CEA values and might be the result of a species-specific response or a toxicant-specific effect. The ETS is based in the mitochondrial membranes within a cell and as such, the effect of the Aroclor 1254 observed in this study could be either endocrine disruption or actual structural damage to the mitochondrial membranes. Both possibilities are addressed below.

Studies completed *in vitro* have shown that PCBs and their metabolites are capable of affect thyroid function and activity in humans by interfering with the thyroid receptor complex (Cheek et al., 1999; Fritsche et al., 2005). Evidence also exists that maternal transfer of organochlorine contaminants via lactation in marine mammals such as the grey seal (*Halichoerus grypus*) may reduce the total levels of thyroid hormones in offspring (Sormo et al., 2005). As stated earlier (see section 1.6.4) thyroid disruption has also been observed in fish living in polluted environments. Zhou et al. (1999) reported impairment of thyroid function in mummichogs (*Fundulus heteroclitus*) from a creek known to be polluted with heavy metals and organic compounds including PCBs, which may have been a factor in the many behavioural differences observed such as reduced prey-capture and predator avoidance ability and impaired routine swimming activity. Brown et al. (2004) exposed lake trout (*Salvelinus namaycush*) to PCB 126 and recorded increased plasma thyroxin levels and increased thyroid epithelial cell height. It should be noted in the latter case that only a single congener was used and that other PCB congeners may have produced the same or different results. Thyroid hormones are required for maintaining metabolic function through

their influence on mitochondrial activity (Sher et al., 1998). Based on this evidence, it is possible that the reduced ETS activity observed in both experiments here might be a subsequent result of reduced thyroid production or competition for thyroid receptors with PCBs.

The second possible explanation is one of structural alteration of the mitochondrial membrane. Tan et al. (2004) exposed thymocytes, cerebral cells and lipid bilayer vesicles from Sprague-Dawley rat pups to PCBs 77 and 52 *in vitro*. PCB 52 was found to increase membrane fluidity of each cell type, which would alter the function of membrane proteins. Mitochondrial membranes within a cell can also be affected by PCB exposure. Nishihara (1984) and Nishihara & Utsumi (1986) observed that ortho-substituted congeners could disrupt mitochondrial integrity, leading to a loss of both membrane potential and ATP generation, increased permeability to large molecules and swelling. Gilroy et al. (1996) also noted that PCB 156 exposure resulted in mitochondria with unorthodox cristae from *in vivo* tests on young Sprague-Dawley rats. Similar evidence of structural changes to mitochondria in fish is not as abundant but exposure of flounder (*Paralichthys olivaceus*) to the insecticide methylparathion, resulted in dilated mitochondria (Li & Zhang, 2002) and demonstrates that fish can respond to contaminants in similar ways to mammals. Indeed, even insects have exhibited mitochondrial morphological abnormalities as a result of heavy metal water pollution (Sorour, 2001). Whether or not the action of PCB exposure in the present study was such that it disrupted thyroid activity or membrane morphology, the possibilities exist that the mitochondrial functions could have been altered, thus affecting the production of ATP and/or resulting in the observed drop in ETS activity. Investigations of both thyroid hormone levels and mitochondrial structure in plaice larvae from exposed females compared to controls would help clarify the effects seen in the present study.

The CEA value integrates all the energetic values to reflect the net energy budget (De Coen & Janssen, 1997). Significant differences in CEA values occurred via dietary exposure in Experiment 1 and at hatch in Experiment 2. In Experiment 2, no differences in CEA values were detected between treatments in eggs, first-feeding or metamorphosed larvae even though in the latter case significant differences were detected in ETS activity. The general trend from both experiments appears to show a

reduction in ETS activity coupled with increased CEA values when PCBs are most likely metabolised i.e. through yolk and food metabolism.

In the maternal exposure experiment, significant effects on CEA values were seen early in development in newly hatched larvae. At this stage only endogenous nutrients in the yolk are metabolised and it is likely that the yolk will contain the highest concentrations of PCBs as they can be transported to the eggs together with the yolk protein vitellogenin (Ungerer & Thomas, 1996). In their investigation of the ontogeny of xenobiotic biotransformation enzyme genes in early larval stages of plaice, Hodgson & George (1998) reported the presence of mRNAs for 'metallothionein' and 'glutathione *S*-transferase' (GST) in eggs which indicated the potential for oxidative defence at a very early stage in development. Genes coding for the enzymes 'non-specific carboxyesterase' and 'phenol UDP-glucuronosyltransferase' peaked at hatch. These enzymes have roles in both the metabolism of endobiotic substances and also of pollutants (Hodgson & George, 1998). CYP1A (part of the cytochrome P450 family) was also expressed at hatch and increased throughout early development and this enzyme is known to be involved in the biotransformation of xenobiotic pollutants (Matthiessen et al., 1998; Yeung et al., 2003). The expression of CYP1A in fish frequently results in increased EROD activity (see section 5.2) following exposure to xenobiotic compounds with a planar structure, which include polyaromatic hydrocarbons (PAHs), PBDEs and PCBs (Eggens et al., 1996; Arukwe et al., 2001; Boon et al., 2002; Yeung et al., 2003) although this does not always occur (Timme-Laragy et al., 2005). The likely presence of these enzymes might explain why significant results were detected at hatch in this study but not thereafter if the PCB congeners responsible had been metabolised. The results suggest that PCB exposure might result in a reduction in ETS activity whilst PCBs are metabolised but following this, normal metabolic processes resume. Whether or not a continuous reduction in ETS activity or increased CEA values would occur in situations of chronic pollutant exposure warrants further investigation, as would studies on the time-lag of potential recovery of ETS activity to normal levels following short-term exposure such as in Experiment 1.

In a recent study on male rats, vitamins C and E were shown to play a protective role against oxidative damage in Leydig cells (involved in testosterone production)

following exposure to Aroclor 1254 (Murugesan et al., 2005). In rats not treated with vitamins C and E, levels of antioxidant enzymes such as GST were significantly diminished compared to controls, whereas this was not the case in Aroclor-treated rats given vitamin C and E supplements. Further evidence exists that *Artemia* enrichment mixtures might also play an ameliorative role against the effects of PCBs in fish larvae exposed through their diet (Papaioannou, 2000). Although *Artemia* were enriched in the experiment in this study, larvae were only fed with enriched food from approximately 14-dph. All data in these experiments and the results in Chapter 4 with the exception of growth, behaviour and CEA at metamorphosis were collected before feeding with enriched *Artemia* began. Given that most observed effects on the CEA had disappeared by first-feeding, the potential impact of enriching the *Artemia* on these results is expected to be minimal.

Larvae were not analysed for PCB content at first-feeding as there were insufficient numbers to allow for this. PCBs were not detected at metamorphosis but they might have been present at concentrations below the limit of detection. In section 3.4.3, the estimate for PCBs in individual eggs at the highest concentrations was 64-4518pg egg^{-1}. Only ca. 20 individual fish were sampled per spawn for PCB analysis. On this basis a maximum of only 0.09µg of PCBs could have been extracted. Assuming 100% extraction efficiency, only 90pg would be injected into the GC/MS apparatus for analysis. Given the high LDs obtained during the PCB analyses in this study, it is unlikely that this small amount could have been detected.

The main aim of this chapter was to investigate whether the CEA assay was sensitive enough to detect changes as a result of PCB exposure. In the results reported for the effects of PCBs on the individual energy reserves, the only significant differences detected between treatments following both the larval and juvenile exposures were in egg sugar energy and larval lipid energy at hatch. Although a significant correlation was found between lipid energy reserves in newly hatched larvae and the egg PCB concentrations (Fig. 5.6 A), this correlation is only based on a small number of data points (no. of spawns = 8) and thus should be treated tentatively. The same patterns of correlation in the data exists for Ea, Ec and CEA (Fig. 5.6 B-D) and these results are likely to be influenced heavily by the extreme value from one particular spawn

from the highest dosed female broodstock. Ideally, a greater number of replicates would provide better correlative evidence.

However, with lower repeatability values for the sugar and protein analyses (Table 5.1), the practice of taking three sub-samples per spawn remains desirable but increasing the number of replicate spawns within each treatment makes the assay more labour intensive, potentially diminishing its suitability as a rapid assessment tool of xenobiotic effects. Retrospective power analysis (Thomas, 1997) was applied to the results of the individual steps of the larval and juvenile CEA assay that gave non-significant results (Tables 5.12 – 5.16). Power values for the tests ranged between 3 and 47%, with a mean value of 14.5% indicating little chance of detecting a significant result. The power analyses were also applied to the significant results from the larval experiment and power values between 51% and 69%. Power values from the juvenile exposure experiment gave values of 78% and 91% for CEA and ETS activity respectively. The results of the power analyses suggest that an increase in replicate spawns per treatment in the larval experiment would have improved the power of the ANOVA tests to detect significant differences between treatments. Some of the suggested minimum sample sizes were very large and often associated with the lowest power values (e.g. 3% and suggested n = 2512 for Ea at 8-dph) making it unlikely that any real significant differences existed between the parameters measured.

During the larval phase ETS activity increased with larval size (Tables 5.3 – 5.6) yet the ETS values obtained for the newly metamorphosed larvae (Table 5.6) were considerably higher than those for the 0+ juveniles (Table 5.7). Reasons for this are not entirely clear. Development of larvae is likely to be an energetically costly process and might require an increase in respiration rate as ontogeny progresses. Christensen & Korsgaard (1999) reported a marked increase in RNA and DNA in metamorphosing plaice larvae, due to the increased cell division taking place, which might require increased respiration rates. Indeed, Nelson & Wilkins (1994) reported respiration rates of rabbitfish (*Siganus randalli*) to increase with larval size but did not report a decrease post-metamorphosis. Larvae were not retained in the laboratory post-metamorphosis because investigations into the changes in ETS with development were not the aim of the study. Also, as the juveniles used in Experiment 1 were field-

Tables 5.12 – 5.16 Power analysis on the results of the cellular energy allocation (CEA) assay on larvae and juveniles of the European plaice *P.platessa* presented in Tables 5.3 – 5.7. ANOVA results were subjected to retrospective power analysis by using the significance levels obtained, the actual differences observed and the number of samples used per treatment. The output from each test indicated (i) the power of the test to detect the differences observed with the sample size used (ii) the minimum difference detectable with this sample size and (iii) a calculation of the minimum sample size required to detect the observed differences. Red text indicates where significant differences were detected with a sample size n = 3.

5.12 Fertilised eggs

	Energy allocation					
	Sugar reserve	Protein reserve	Lipid reserve	E_a	ETS activity	CEA
Significance level obtained	0.031	0.050	0.657	0.742	0.308	0.852
Actual difference observed	0.7	19.5	140.6	135.3	0.4	37.3
Original sample size	3	3	3	3	3	3
Power of test	58%	50%	6%	5%	17%	4%
Minimum detectable difference	0.64	19.50	620.58	805.54	0.77	391.86
Required sample size	2	3	58	106	11	331

5.13 Larvae <24 hours post-hatch

	Energy allocation					
	Sugar reserve	Protein reserve	Lipid reserve	E_a	ETS activity	CEA
Significance level obtained	0.060	0.649	0.014	0.122	0.049	0.024
Actual difference observed	2.6	21.2	508.7	679.6	6.6	112.8
Original sample size	3	3	3	3	3	3
Power of test	47%	7%	69%	34%	50%	62%
Minimum detectable difference	2.71	91.30	405.76	861.35	6.57	97.95
Required sample size	4	56	2	5	3	2

5.14 Larvae at 8-days post-hatch

	Energy allocation					
	Sugar reserve	Protein reserve	Lipid reserve	E_a	ETS activity	CEA
Significance level obtained	0.650	0.614	0.793	0.946	0.684	0.813
Actual difference observed	4.9	22.5	286.5	134.3	4.2	7.9
Original sample size	3	3	3	3	3	3
Power of test	7%	7%	4%	3%	6%	4%
Minimum detectable difference	21.17	87.44	2139.88	3886.49	20.23	65.45
Required sample size	56	45	167	2512	70	206

5.15 Metamorphosed larvae (42-days post-hatch)

	Energy allocation					
	Sugar reserve	Protein reserve	Lipid reserve	E_a	ETS activity	CEA
Significance level obtained	0.174	0.070	0.317	0.774	0.019	0.332
Actual difference observed	25.4	207.4	495.1	310.1	15.8	19.3
Original sample size	3	3	3	3	3	3
Power of test	27%	44%	17%	5%	65%	16%
Minimum detectable difference	36.62	224.35	969.77	2116.66	13.20	38.99
Required sample size	6	4	12	140	2	12

Contd. overleaf

Contd. from overleaf

5.16 0+ plaice

	Energy allocation					
	Sugar reserve	Protein reserve	Lipid reserve	E_a	ETS activity	CEA
Significance level obtained	0.729	0.546	0.710	0.665	0.001	0.006
Actual difference observed	6.0	75.3	333.3	414.6	9.9	95.6
Original sample size	3	3	3	3	3	3
Power of test	5%	9%	6%	6%	91%	78%
Minimum detectable difference	33.94	244.45	1756.80	1876.64	5.89	68.19
Required sample size	96	32	83	61	1	2

caught and subjected to different developmental conditions and selection processes it may not be feasible to compare the ETS activities across the two experiments and further analysis of post-metamorphosis juveniles reared under the same laboratory conditions would confirm if this were the case.

A further possible explanation of the differences between the larval and juvenile ETS activity is that the ETS assay is based on the rate of an enzyme reaction that will in itself be influenced by the ambient temperature. In earlier preliminary work the ETS plates were kept on ice by mistake whilst they were prepared and the reaction rate was inhibited greatly. As these two experiments were completed in two different years, at different times of year, the ambient laboratory temperatures might have played a role in determining the actual rate of reaction. Differences within experiments remain valid as the micro-titre plates were processed one after another but comparisons between experiments or to values from other laboratories should be treated with caution. Despite this, the ETS values in both experiments fall within a similar range to those reported for *N.integer* (Verslycke et al., 2004a).

The significant negative correlation of ETS with whole body PCB concentrations in Experiment 1 (Fig. 5.7) supports the results of the larval analyses although no relationship between ETS and PCB concentration was apparent in newly hatched larvae. The lack of correlation of Ea with PCB concentration should be expected given that no significant results were detected between the treatments (Fig. 5.7 B). Although a significant increase in CEA was detected at the highest dose in the juvenile fish (Table 5.9) there was no correlation with PCB concentration (Fig. 5.8) and the result might be due to the high variation in the CEA values obtained. ETS activity varied significantly between tanks within the control treatment in the juvenile exposure experiment (see section 5.4.3). This experiment calculated values for individual fish rather than pooled samples as in Experiment 2. Therefore, high individual variation in ETS activity is likely to be the cause of this given the lack of correlation of ETS activity with tank water temperatures (Fig. 5.9). Whether or not this variation in ETS activity in fish from control tanks may have been the cause of the significant differences observed between control and dosed juveniles in Experiment 1 is unknown for certain but differences in ETS activity were also detected between low- and high-dose treatments, indicating a dose-response

relationship. Alternative methods for future work to help minimise within-treatment variation are discussed below.

5.5.3 Methods

The repeatability tests on the goby CEA data (Table 5.1a) and the juvenile plaice in experiment 1 (Table 5.1b) demonstrated that the CEA methodology worked well in the laboratory but highlighted the need to ensure complete sample homogenisation. Use of a single body tissue such as liver would be likely to produce less variable results as there would be no issues with ensuring connective tissues (skin and bone) were distributed evenly among sub-samples and might produce more reliable results. Many organic contaminants accumulate in fatty tissues such as the liver (Ungerer & Thomas, 1996) and so the resulting metabolic effects may be observed at a greater level in this tissue as more contaminants are metabolised within the hepatocytes (Alberts et al., 1994). However, when using such small fish, there would be insufficient liver material to calculate the CEA for individual fish and the process of extracting sufficient livers to complete the assay would be extremely time consuming. The use of adult fish livers that could be sub-sampled with speed would certainly warrant investigation. Recently the CEA assay was run using livers of adult Senegalese sole, *Solea senegalensis*, as part of a dietary study, demonstrating the viability of this option (Rueda-Jasso et al., 2004). This would also allow the remaining adult liver material to be analysed for PCBs and provide a more accurate picture of the effects of PCBs on CEA. In Experiment 1 with the juvenile fish, different individual fish were sampled for GC/MS analysis compared to those sampled for the CEA assay and the assumption was made that the fish analysed for PCB content exhibited the same feeding behaviour, and the same PCB body burdens as those sampled for CEA. The random nature of the sampling should ensure that this was more or less the case but it cannot be guaranteed.

The tank temperatures were not significantly different between treatments in both experiments and fluctuated very little (section 5.4.2). This was an important factor to control as low temperatures have been shown to have a negative influence on the energy reserves, most specifically protein and sugars and have the most adverse effect

on CEA in mysid shrimp (Verslycke & Janssen, 2002). In both experiments, the water for each tank came from the same source, through the same filters ensuring that the salinity was the same in each tank. Again, salinity has been shown to have an influence on sugar consumption with estuarine mysids consuming more sugar energy at higher salinities in order to provide a fast energy source to fuel hyperosmoregulation (Verslycke & Janssen, 2002). Although large fluctuations of salinity were unlikely to occur with the experiments reported herein, maintaining the same conditions across all tanks was considered important for a correct understanding of the effects of PCBs on CEA in plaice.

Verslycke & Janssen (2002) and Verslycke et al. (2003) did not feed their experimental subjects (*N.integer*) during their CEA assays in order to avoid problems of interference caused by individual differences in feeding rates or energy assimilation efficiencies. Mysids were fed in a further experiment examining the effects of the pesticide chlorpyrifos and this did not appear to affect the sensitivity of the assay (Verslycke et al., 2004b). The nature of the PCB exposure pathway in Experiment 1 necessitated feeding the juvenile fish and starving them prior to analysis should have reduced the influence of undigested food on the CEA results. Larvae could not be starved prior to analysis as this may have resulted in high mortality rates, preventing analysis at later stages of development. The use of three replicates of ca. 20 larvae per sample for analysis should have ensured that the final results were representative of each spawn and account for differences in feeding rates of individual larvae.

The final whole body concentrations of PCBs detected in the juvenile fish used in Experiment 1 (Table 5.9) were approximately an order of magnitude greater than those detected in field-caught juveniles (Table 2.6). When the concentrations were converted to a wet weight basis (see section 2.5.3) the detected values ranged between 12.3 and 229.2μg kg^{-1} wet wt. Those exceed the values of 5 - 69μg kg^{-1} wet wt detected in plaice muscle tissue in Liverpool Bay during CEFAS surveys (CEFAS, 1987; CEFAS, 1990; CEFAS, 1991) but are within the range of reported values for other fish species in different locations such as mummichogs in the Hudson river (Monosson et al., 2003) or winter flounder in the USA (Johnson et al., 1992) (Table 1.5). The concentrations obtained in the eggs of the plaice larvae in Experiment 2 are in the same range as those used for the experimental work in the previous chapter (see

section 3.5.2) and are comparable to concentrations detected in eggs sampled from field locations (Table 3.14), albeit from different species to that used here. Nonetheless, the results highlight the potential for PCBs to affect the energy metabolism of fish larvae at environmentally relevant concentrations.

5.5.4 Conclusions

The present study suggests that Aroclor 1254 has a negative impact on the ETS activity in newly hatched plaice larvae exposed via maternal transfer and in juvenile fish exposed to Aroclor 1254 via their diet. Generally, results from both experiments are promising and indicate that ETS was the parameter most affected by the PCB treatments resulting in increased CEA indices during the likely periods of PCB metabolism. However, the variation in the data was often high (see standard deviations, Tables 5.3 – 5.7 and CVs in section 5.4.3). Further validation of the methods, with increased replicate spawns of newly hatched and first-feeding larvae over a wider range of lower environmentally-realistic PCB concentrations could add further weight to the evidence of the disruption of ETS activity by PCB exposure. Alternatively, the analyses of just the liver tissue from juvenile or adult fish might yield less variable results due to the homogenous nature of the tissue analysed, thus making the assay more sensitive to contaminant effects. Nevertheless, the results are very encouraging regarding the use of the CEA assay in toxicological studies involving fish larvae and juvenile fish. Further development of the technique using both field and laboratory studies could result in a very suitable, sensitive and reasonably rapid technique for assessing effects of toxic stress on fish.

The way in which these results regarding cellular energy relate to those in previous chapters as well as the integration of behavioural and physiological studies is discussed in the following chapter.

Chapter 6

General Discussion

6 General Discussion

The following discussion aims to integrate the results from the previous four chapters to provide a broad picture of the impacts of maternal PCB transfer on larval plaice at environmentally realistic concentrations. The importance of such an approach to eco-toxicology is discussed and suggestions are made for improving future studies.

6.1 Integrating disciplines

The work presented in the preceding chapters highlights just some of the techniques available to detect the impacts of toxic stress on organisms following exposure to xenobiotic compounds. Often, studies that address toxic effects, either in the laboratory or field use single biomarkers as indicators of a response (Little et al., 1993; Sumpter & Jobling, 1995b; Zhou & Weis, 1998; Roast et al., 1999; Arukwe et al., 2001; Verslycke et al., 2003) and do not always provide information on the final body burdens of their subjects (Schmidt et al., 2005) possibly due to time constraints, limited resources or the perceived efficacy/sensitivity of a particular technique. The advantage of assessing multiple parameters is that a more complete conclusion can be reached regarding the overall effects that toxic exposure can have on a species. The ways in which the impacts of exposure are integrated into the whole-organism response can also be deduced more easily if experimental conditions are as similar as possible throughout the exposure period or across experiments rather than making inter-laboratory comparisons where it might be more difficult to re-create the same conditions as those in another study (Scott & Sloman, 2004). Although such integrated studies can sometimes be time-consuming and laborious, they provide invaluable, comprehensive data sets regarding whole-organism responses to xenobiotics. Recent reviews of the literature have sought to integrate the disciplines of physiology and behaviour in order to demonstrate the interactions between the two and the challenges being faced within the field of ecotoxicology (Clotfelter et al., 2004; Scott & Sloman, 2004; Zala & Penn, 2004).

The work presented in this thesis has brought together the approaches of fieldwork, laboratory studies, organic chemistry, fish biology, animal behaviour and physiological energetics. A broad overview has been presented of the mechanisms of exposure to, and potential impacts of endocrine disrupting chemicals, in this case PCBs, on the early life stages of fish in the wild. The fieldwork (Chapter 2) detected PCBs in the juvenile plaice sampled from nursery areas around the UK, confirming that newly settled 0+ juvenile plaice were exposed to these compounds at certain sites at concentrations ranging from 10.16 to 46.8$\mu g\ kg^{-1}$ dry wt. Although PCBs were not detected in the egg or adult tissue samples it should be remembered that the limits of detection of the GC/MS analyses were relatively high, the overall quantity of eggs sampled was small and the adult plaice were sampled from a relatively uncontaminated location. However, this lack of data regarding PCBs in adult plaice can be supplemented by published literature confirming the presence of PCBs in adult plaice in UK coastal waters (Leah et al., 1997; CEFAS, 1987; CEFAS, 1990; CEFAS, 1991). The evidence from the maternal transfer work (Chapter 3) demonstrates that for plaice, PCBs can be transferred with ease to the next generation and maternal transfer is therefore likely in the field. Indeed maternal transfer has been observed in winter flounder under field conditions in the USA (Johnson et al., 1992) and contaminants have been detected in eggs of various fish species (Table 3.14). Given the evidence for PCB contamination in plaice around the UK coast and the potential for transfer to subsequent generations, it is prudent to consider the possible impacts on the offspring of contaminated females or populations. This was achieved by using further multidisciplinary techniques in order to (i) establish a broad, integrated overview of possible impacts and (ii) to establish the sensitivity of different endpoints with regard to detecting effects at low concentrations.

The analytical techniques employed to investigate differences in congener patterns between tissue-types demonstrated how congener profiles change depending on the mode of exposure i.e. oral or maternal exposure (Figs. 3.7 – 3.10). The results of these analyses suggested that the impacts on the plaice larvae exposed to PCBs via maternal transfer may be reduced given the tendency of the less chlorinated and generally less toxic congeners (Wilson-Yang et al., 1991) to increase in proportion to the more highly toxic congeners. Some studies have researched the impacts of individual congeners on organisms and have shown effects to be both oestrogenic

(Carlson & Williams, 2001; Jung et al., 2005), anti-oestrogenic (Vaccaro et al., 2005) and anti-androgenic (Bonefeld-Jørgensen et al., 2001). Whilst these types of study are invaluable in assessing the potential effects of individual compounds they cannot be fully predictive of field effects given the complex mixtures of compounds that often occur. However, even using the complex mixture of congeners found in Aroclor 1254 in this study does not account for the vast array of other contaminants to which organisms might be exposed and further methods for investigating this are discussed later in this section.

Even though larvae received a lower proportional maternal transfer of highly chlorinated biphenyl congeners, survival of yolk-sac larvae was significantly reduced at the medium and high egg PCB concentrations, whilst survival of the developing embryos did not differ significantly from control larvae (section 4.4.2). The differences observed in size at hatch (section 4.4.3) were correlated with the egg PCB concentrations (Fig. 4.9) and might put survival at risk in the wild if larvae remain smaller for a longer time period and therefore remain susceptible to a wider range of predators, though as mentioned earlier, this theory can be contested (see section 4.5.3). The analysis of routine swimming behaviour provided a similar result with larvae at medium- and high-doses displaying less routine swimming activity compared to control larvae. Whilst no measurements were made of larval swimming speed, this has been shown to increase with increasing larval size in numerous species including plaice (Bailey et al., 2005). It would be interesting to measure routine swimming speeds in this case as well as burst speeds in response to the vibratory stimuli used and investigate correlations with both larval size and PCB concentrations.

Although no correlations existed between ETS activity in newly hatched larvae and egg PCB concentrations (Chapter 5, Fig. 5.9 C), the data were only based on eight replicate spawns, many at the low end of the PCB concentrations and further replicates would provide increased certainty in the presence or absence of a relationship between the two variables. Despite this, ETS activity showed a significant reduction in high-dosed larvae at hatch compared to control larvae (Table 5.4). ETS activity seemed to be suppressed by PCB exposure at the times when PCBs were likely to be being metabolised (see section 5.5.2). The ETS system is involved

in the production of the molecule ATP, which is the principle carrier of chemical energy in cells (Alberts et al., 1994). If ATP production is being inhibited in larvae and juveniles exposed in the experiments in Chapter 5, it might go some way to explaining the reduced swimming activities observed in exposed larvae in Chapter 4. Assays measuring the quantity of ATP being produced in the PCB-exposed and control larvae, the levels of thyroid hormones in those larvae or examination of mitochondrial structure (as discussed in section 5.5.2) would help establish any relationships. It is investigations such as this that might help to further integrate studies of behaviour and physiology, helping to establish strong bodies of "cause and effect" of particular compounds or classes of compounds.

As mentioned in Chapter 1, one of the major issues faced by ecotoxicologists is the unwillingness of endocrine disruptors to "play by the rules" of traditional toxicological studies. The general maxim that "the dose makes the poison" does not often apply and dose-response relationships are often non-linear (Colborn et al., 1996). In this study, the pattern of egg production per gram female (Fig. 3.13) appears to demonstrate the potential for these types of dose-response relationships. The difficulty caused by these U- or inverted U-shaped relationships is that toxicological studies often only make use of a limited range of doses or endpoints and as such, might overlook concentrations at which sub-lethal effects occur. Although this study has established some apparent cause-and-effect relationships of PCBs with the parameters investigated, further work over a wider range of concentrations at the lower end of those used here, would help confirm whether further skewed, U- or inverted U-shaped relationships exist.

In addition to the factors mentioned previously with respect to the duration of such investigations, the numbers of animals/species to use and the numbers of different concentrations requiring investigation, studies examining xenobiotic effects solely in the field or in the laboratory are also fraught with their own suite of problems. Field populations can become resistant to certain pollutants, as was the case with Atlantic tomcod (*Microgadus tomcod*) in the Hudson River (Yuan et al., 2005). In that study, fish from the Hudson river (heavily contaminated with PCBs and PAHs) did not exhibit induction of CYP1A following intraperitoneal injections of CB-77, whereas fish from an unpolluted river did (Yuan et al., 2005). Resistance did not occur to

PAH exposure and was shown to be a heritable trait rather than a non-genetic adaptation. Therefore, when assessing the impacts of xenobiotics on organisms in, or from the field, it is important to either source them from or compare them to individuals from uncontaminated sites as well as different populations. Assessment of more than one species should also be considered when examining the toxic effects of a compound since closely related species might respond in different ways (Clotfelter et al., 2004) as was the case with plaice and flounder in the mesocosm study by Eggens et al. (1996). In that study, the differences between the two species might also have been due to increased natural selection pressures on flounder populations that spend much of their life cycle in inshore and estuarine environments where contamination is likely to be higher and the requirement for resistance all the greater. If just flounder had been used in that study, perhaps different conclusions may have been drawn regarding the toxic status of the particular harbour dredge spoil used whereas use of multiple species reduced the probability of such an outcome.

One of the problems highlighted by Zala & Penn (2004) and Clotfelter et al. (2004) was the use of common laboratory strains or species for ecotoxicological work. Whilst these species/strains might be convenient to rear and utilise in laboratory experiments, they may not be truly representative of their taxonomic groups (Clotfelter et al., 2004) and care should be taken when interpreting results and making predictions regarding effects on field populations. Indeed, care should be taken to find out as much information as possible regarding an organism's response to xenobiotics or its ability to process such compounds. Even the plaice embryos and larvae used in this study are likely to have been able to metabolise the PCBs passed on by maternal transfer given the intrinsic ability to produce the required enzymes at such an early life stage (see section 5.5.2) (Hodgson & George, 1998). Knowledge such as this can be invaluable in elucidating the results of toxicological studies. Nonetheless, this work has tried to meet as many of these requirements as possible by avoiding the use of inbred laboratory strains, not using a typical model species, attaining low PCB concentrations (though even lower concentrations would be more desirable) and investigating the chemical, behavioural, physiological and developmental parameters in relation to exposure. This study has demonstrated the potential effects that a single class of compounds could have on organisms exposed to them in the environment though, as mentioned previously, the latter are exposed to a

range of xenobiotics simultaneously and mesocosm studies such as that by Eggens et al. (1996) are invaluable in this respect. However, with the advancement of chemical technology there remains a need to test individual chemicals in *vivo* as endpoint sensitivity can differ depending on the parameter measured.

In their review of the role of animal behaviour in endocrine disrupting studies, Clotfelter et al. (2004) noted the importance of considering the life stage being tested with respect to endocrine disrupting chemicals. Often, early life stages are particularly sensitive to xenobiotics compared to adults (Colborn et al., 1993) and ecotoxicological investigations should consider all stages of an organism's lifecycle. Even when this barrier has been crossed there exists the issue of deciding on the endpoint for any particular exposure study. Nguyen & Janssen (2002) considered the sensitivity of different endpoints when investigating embryo-larval toxicity tests on the African catfish (*Clarius gariepinus*). In a set of experiments measuring survival, hatch success, morphology and growth following exposure to five pollutants including the pesticide malathion, the sensitivity of the endpoints was summarised as growth > abnormal morphology > larval survival > embryo survival > hatch success.

In order to determine the sensitivity of each parameter measured in this study, the concentrations or ranges of concentrations producing the lowest observed effect (LOEC) have been summarised in Table 6.1. The lowest concentrations at which any significant effect was observed was in the improved survival and hatch size of the embryos at the very lowest egg PCB concentrations (0.1 – 0.175mg kg^{-1} dry wt.) though it is difficult to consider these as adverse effects. In terms of potential adverse effects on the embryos and larvae, the order of sensitivity is: routine swimming activity at hatch/survival from hatch until first feeding > ETS activity/CEA > hatch size > routine swimming at 8-dph. However, it should be noted that many of the PCB concentration ranges overlap one another when presenting these data in such a way. Further replicate studies including lower concentrations would allow the LOECs to be more accurately identified. Nonetheless, it is a measure of behaviour, namely that of routine swimming activity, that has been placed at the top of the list in terms of the LOEC on larvae, supporting the use of behaviour in toxicological studies. Further experiments with lower concentrations would allow concentrations with no observed effects (NOECs) to be derived. However, the importance of selecting multiple

Table 6.1 Summary of the various parameters measured in this study of the response of larval, juvenile and adult plaice (*Pleuronectes platessa*) to either oral or maternal exposure to the commercial PCB mixture, Aroclor 1254.

Parameter measured	Life stage	Exposure route	LOEC mg kg^{-1}	Comments
Reproductive output	Adult	Oral	-	No significant effects observed between 0 and 18mg kg^{-1}
Egg dry weight	Egg	Maternal	<1.0	Eggs in this size class were not significantly different from controls but had a significantly greater dry weight compared to eggs with PCB concentrations >10mg kg^{-1}
Survival	Fertilised eggs until hatch	Maternal	0.1 – 0.175	Improved survival within this concentration range in comparison to larvae from undosed females.
	Hatch until first-feeding	Maternal	0.47 – 0.91	Significant reductions in the percentage surviving from hatch to first feeding. Mean survival was 94.95% for controls but only 90.49% and 89.36% for medium and high dosed larvae respectively.
Hatch size	Larvae <1-dph	Maternal	0.27-1.0	Increased hatch length of larvae from eggs containing PCBs between these concentrations compared to control larvae.
			>1.0	A significant decrease in hatch length was detected compared to control larvae.
Growth rate	0 to 42-dph	Maternal	-	No differences were detected.
Routine swimming	Hatch	Maternal	0.47 – 0.91	Reduced swimming activity.
	8-dph	Maternal	>10.0	Reduced swimming activity.
	Metamorphosis	Maternal	-	No observed differences.
Predator response	0 to 42-dph	Maternal	-	No observed differences.
Energy consumption (ETS)	Hatch	Maternal	0.85 – 4.17	Reduced ETS at $p = 0.05$ but not significant following the Bonferroni correction.
	Juvenile	Oral	0.18 – 1.00	Reduced ETS activity in the highest dosed group.
Cellular energy allocation (CEA)	Hatch	Maternal	0.85 – 4.17	Increased CEA in newly hatched larvae.
	Juvenile	Oral	0.18 – 1.00	Significantly increased CEA scores in the highest dosed fish.

parameters in determining these is paramount given the varying sensitivities of different endpoints. Indeed, simply because some endpoints are unable to detect significant changes, it does not always imply total proof of no change in reality (Meister & van den Brink, 2000).

6.2 What does all this mean for the plaice?

Although not detected in this study, the PCB concentrations expected to occur in plaice eggs around the UK coast are likely to be toward the lower end of the concentration range achieved in this study (see section 3.5.2) and lower still, depending on the sampling locations. Many eggs would need to be sampled from the field with more sensitive analytical procedures (lower LDs) to detect PCBs and other contaminants. Nonetheless, maternal transfer of PCBs does occur in plaice under the laboratory conditions reported here. Field sampling during the spawning season from areas of higher PCB concentrations (e.g. Liverpool Bay, (Camacho-Ibar & McEvoy, 1996) compared to suitable reference areas (Underwood, 2000) would likely confirm this under field conditions.

The results reported here demonstrate that realistic concentrations of PCBs can have an effect on the ecological performance (behaviour, growth, survival, CEA) of plaice larvae. The full extent to which each of these parameters are affected could be investigated in more detail by a team of researchers to provide sufficient data to enable computer models to predict effects at the population level (see section 4.5.6), as has been achieved with PCBs and methylmercury in Atlantic croaker (Rose et al., 2003; Alvarez et al., 2006). Whether or not xenobiotic exposure is a bigger threat to plaice populations than the EU fishing industry is a different topic for discussion. Attempting to separate the impacts of two simultaneous processes such as fishing and endocrine disruption that can affect the reproductive capacities of populations would be a difficult task. Perhaps the most important message is that this study has shown the effects PCBs can have on aquatic organisms in general and the impacts on the ecology of species in contaminated areas should continue to be investigated.

PCBs have a vast body of evidence against them regarding their impacts on wildlife though toxicological studies that use them can still be useful in developing methodologies and demonstrating how sub-lethal impacts can affect the ecology of organisms. Further research should aim to address other emerging chemicals of interest for toxicity (e.g. PBDEs and hexabromocyclododecane (HBCDD), a new fire retardant compound). Investigations using such methods as those both used and suggested in this thesis have a greater degree of relevance than simple in *vitro* or in *vivo* mortality testing.

6.3 Legislation and the future

The new EU Chemicals Policy, REACH (Registration, Evaluation and Authorisation of CHemicals) is due for implementation in 2006. This aims to assess the toxicity of all synthetic chemicals in use in the EU, many of which have undergone little or no testing before being permitted entry into the marketplace. This will be a time-consuming process and it is unlikely that comprehensive measures of effects on many parameters in a range of species will be able to be carried out for every chemical. It is important therefore that researchers and research institutions continue to identify the potentially most hazardous chemicals and complete thorough investigations into the impacts that xenobiotic compounds can have in the environment.

The Oslo-Paris Commission Report (OSPAR) on PCBs (2001), highlighted new directives being drafted by the European Parliament and the Council on Waste Electrical and Electronic Equipment (WEEE), for manufacturers to take responsibility for contamination problems. Once agreed, manufacturers must bear recycling costs and use less hazardous substances in order to stop feeding the waste stream with toxic chemicals. Enforcement of such legislation should herald a start of reducing the release of synthetic chemicals into the environment and is due to commence in the UK in July 2006 (DTI, 2005). Finally, the EU Water Framework Directive states that by 2015 all European surface waters should be of "good ecological quality". For this to be achieved in the UK, the environmental and health consequences of toxic chemicals, their impacts and disposal must be addressed.

6.4 The future for toxicology

Mesocosm studies using contaminated sediments sourced from the environment together with multiple test species are perhaps the most realistic toxicological, chemical-mixture studies. The contaminants present can be measured and the effects detected on the study species can, not only be compared to data from individual chemical exposure studies such as this, but probably also extrapolated with great accuracy to predict field effects. It is only by integrating results from all possible fields of investigation that the full extent of xenobiotic contamination can be understood.

The concept of eco-epidemiology utilises data from wildlife and lab studies as well as knowledge of the mechanisms of hormone action to aid decision making (Colborn et al., 1996). Promoters of eco-epidemiology suggest that pragmatic judgements should be made based on the weight of evidence rather than small discrete areas of scientific proof (Colborn et al., 1996). Whilst this is not strict scientific convention it has been suggested that it would be conceited to assume that biology and science itself must always follow the rules imposed on it by the human mind (Colborn et al., 1996). In some respects, the precautionary principle is based around this concept whereby even if it is not 100% certain that a chemical might pose a serious risk to the health of ecosystems its use should be prevented or restricted until further evidence becomes apparent.

6.5 Further work

Throughout this thesis, areas for further work in and improvement of examining xenobiotic effects have been identified. The summary below outlines specific areas that should be of further interest with respect to PCBs and chemicals of emerging concern.

- Exposure studies should aim to cover as wide a range of concentrations as possible. Where experimental options are limited, priority should be placed at the low end of the range if data are lacking.
- Investigation of effects early in development is paramount given the increased sensitivity of early life stages to endocrine disruption.
- Studies of endocrine disruption should aim to be environmentally relevant with respect to both the concentrations used and the exposure pathway (Holm et al., 1993) i.e. aquatic, dietary of parental exposure.
- Emphasis should be placed on both field and laboratory work as much as is reasonably possible.
- Where possible, multiple species should be incorporated into toxicological studies though care should be taken that they have not been sourced from populations with resistance to the compounds in question or from inbred laboratory strains, both of which could cause confounding results
- Multiple, interdisciplinary parameters should be investigated in studies such as this in order to account for the sensitivity of different endpoints and assist in explaining the observed effects. Behaviour is the integrated result of many physical and physiological processes. As many multiple behavioural and physiological parameters as possible should be measured in order to create an overall picture of an organism's ability to survive in its environment.
- In further experiments with batch spawners, such as the plaice, dosing should take place before the spawning season commences so that changes in egg PCB content and observed effects can be investigated.
- With the possibility of long lag times between exposure and observed effects such as those of methylmercury and Aroclor 1268 on the second generation of mummichogs following maternal exposure (Matta et al., 2001), experimental periods should be extended to evaluate the reproductive capacity of offspring.
- Although PCBs are still a chemical of concern, a large body of evidence now exists against them and more emphasis should be placed on testing new chemicals such as PBDEs or HBCDD the effects of which are far less well documented.

By applying as many of the criteria listed above, toxicological studies of this kind can only improve our understanding of xenobiotic effects in the environment.

6.6 Conclusions and final comments

The work presented in this thesis has added to the weight of evidence against PCBs and their potential to cause deleterious effects in the aquatic environment. Behavioural and physiological performance and survival of the early life stages of fish can be placed at risk by maternal transfer of polychlorinated biphenyls and it is likely that other toxins are also transferred to the oocytes in this way. Studying ecotoxicology is a complex issue and no one single piece of work is likely to be able to predict the impacts of a particular compound on all species. However, the endocrine system has remained relatively unchanged for millions of years. Similarities rather than differences observed between species in wildlife and the laboratory should be paid closer attention (Colborn et al., 1996) and caution should be applied with regard to developing the next generation of synthetic compounds. Only in this way can we stand a good chance of avoiding the problems arising from the production of compounds such as PCBs or DDT and ultimately protect the health of humans as well as that of the environment.

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"So long, so long, and thanks for all the fish…"

The dolphins, *The hitchhiker's guide to the galaxy*,
Douglas Adams