BIOCONCENTRATION AND BIOTRANSFORMATION OF SELECTED PHTHALATE ESTERS IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

by

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ABSTRACT

Phthalate esters (PEs) are one of the largest groups of industrially produced chemicals and consequently are ubiquitous in aquatic environments. Potential toxic effects such as endocrine disruption and carcinogenesis have made PEs the target of hazard assessment by many regulatory agencies. However, PEs are generally hydrophobic and poorly water soluble, making it difficult to assess their hazards to aquatic organisms. Measured bioconcentration factors (BCFs) are often much lower than theoretical BCFs, possibly as a result of biotransformation and low bioavailability. The first objective was to determine BCFs for several PEs in rainbow trout (*Oncorhynchus mykiss)* using direct measurement of PE concentration. The second objective was to investigate the biotransformation of PEs by identification of phthalate monoesters in the tissues of the trout. To achieve thesis goals I performed a bioconcentration experiment with dimethyl phthalate (DMP), diethyl phthalate (DEP), dibutyl phthalate (DBP), butylbenzyl phthalate (BBP) and di(2-ethylhexyl) phthalate (DEHP) in rainbow trout. BCFs based on total water concentrations BCFs ranged between 1.74 for DMP to 918 for BBP. Values for BCFs based on operationally defined freely dissolved water concentrations ranged between 1.76 for DMP to 50000 for DEHP. The BCFs based on total or operationally defined freely dissolved water concentration in this study were much lower than those predicted by equilibrium partitioning theory and lower than the 5000 CEPA criteria for bioaccumulation. The detection of some monoalkyl phthalate esters in the rainbow trout tissues indicates that PEs undergo biotransformation in the fish. Biotransformation is expected to be a key reason why BCFs of PEs are less than predicted based on equilibrium partitioning. BCFs determined based on calculated freely dissolved water

concentration indicate that the inherent bioconcentration potential of BBP and DEHP may exceed the CEPA bioaccumulation criteria of 5000. This suggests that BBP and DEHP may have a significant inherent capacity to bioconcentrate but that in natural waters sorption of these hydrophobic compounds to organic particulate matter reduces the bioavailability of the compounds to cause a lower realized bioconcentration behaviour.

DEDICATION

To Nora Ratzlaff, Michael M. Bolan and Larry W. Ratzlaff

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BBP - Butylbenzyl phthalate
BCF - Bioconcentration factor
C_{18} - Octadecyl
C_B - Chemical concentration in the organism (g/kg organism)
C_F - Chemcial concentration in the fish (g/kg fish)
C_W - Chemical concentration in the water (g/L)
CEPA - Canadian Environmental Protection Act
DBP - Dibutyl phthalate
DCM - Dichloromethane
DEHP - Diethylhexyl phthalate
DEP - Diethyl phthalate
DMP - Dimethyl phthalate
DOM - Dissolved organic matter
EDC - Endocrine Disrupting Compound
GF - Glass fibre
HPLC - High Performance Liquid Chromatography
k_1 - first order rate constant for uptake from water via the gills (L water/kg organism/day)
k_2 - rate constant for elimination via the gills to the water (1/day)
k_E - rate constant for elimination by fecal egestion (1/day)
k_G - rate constant for growth dilution (1/day)
k_M - rate constant for metabolic transformation of the chemical (1/day)
K_{ow} - Octanol Water Partition Coefficient
LRTAP - Long-range Transboundary Air Pollution Protocol
MBP - Monobutyl phthalate
MBzP - Monobenzo phthalate
MEHP - Mono-2-ethylhexyl phthalate
MEP - Monoethyl phthalate
MMP - Monomethyl phthalate
MDL - Minimum Detectable Level
MRL - Minimum Reportable Level
PCB - Polychlorinated biphenyl
PE - Phthalate ester
UNEP - United Nations Environmental Program

1 INTRODUCTION

1.1 Background

Phthalate esters (PEs) are organic chemicals with a variety of industrial uses. Their main application is as additives in plastics. Plastics are produced at high temperatures and pressures and as a result are often brittle after production. Plasticizers such as PEs are added to reduce the temperature and pressure needed to produce plastic and to increase the flexibility and durability of the final product (Woodward 1988). Other uses include industrial and lubricating oils, paints, inks, insect repellent, cosmetics, perfume fixatives and glue. Often their contribution to the weight of plastics is second only to that of the polymer itself. In some products, PEs make up as much as 55% (Brown and Thompson, 1982) or 67% (Giam *et al.* 1984) of the total weight. PEs are not chemically bound to the polymer, but are dispersed in the matrix of the polymer chains (Mathur 1974). Because they are not chemically bound, PEs readily leach from plastics into the environment.

Approximately 4.7 million tonnes of PEs are produced worldwide, making PEs one of the most industrially produced and used chemicals in the world (Parkerton and Konkel, 2001). Release to the environment can occur during industrial manufacturing and after disposal of plastic products containing PEs, through the leachate from municipal and industrial landfills (Perwak *et al.* 1981, Bauer and Herrmann 1997; Oman and Hynning 1993). Because of their high production rates and ability to leach from plastics, PEs have become ubiquitous environmental contaminants (Woodward 1988).

1.1.1 Current Regulations

Chemicals may be banned from use and production if they meet several criteria. The United Nations Environmental Program (UNEP) long-range transboundary air pollution protocol (LRTAP) and the Canadian Environmental Protection Act, target chemicals that are bioaccumulative, persistent and toxic.

Bioaccumulation refers to the process of accumulation of a chemical substance in an organism, resulting from chemical uptake through all routes of exposure (dietary absorption, transport across respiratory surface, dermal absorption, and inhalation), and typically takes place under field conditions (Gobas and Morrison 2000). The degree to which bioaccumulation occurs is described by the bioaccumulation factor (BAF), which is the ratio of the chemical concentration in the organism to that in the water, as a result of all routes of chemical exposure (water and diet).

Bioconcentration is the process by which the chemical concentration in an aquatic organism achieves a level that exceeds that in the water, as a result of direct chemical uptake from the water, i.e., not including dietary routes. The magnitude of bioconcentration is described by the bioconcentration factor, which is the ratio of the concentration in biota to the concentration in the water. At steady state the BCF can be defined as:

$$\mathbf{BCF=C_B/C_W=k_1/(k_2+k_E+k_M+k_G)}$$

Where C_B is the chemical concentration in the organism (g/kg organism), C_W is the chemical concentration in the water (g/L), k_1 is the first order rate constant for uptake from water via the gills (L water/kg organism/day), k_2 is the rate constant for elimination via the gills to the water (1/day), k_E is the rate constant for elimination by fecal egestion (1/day), k_G is the rate constant for growth dilution (1/day), and k_M is the rate constant for metabolic transformation of the chemical (1/day).

The octanol-water partition coefficient (Kow) refers to the ratio of the concentration of a chemical in octanol to its concentration in water, at equilibrium. Octanol is a surrogate for lipids, thus the Kow is a measure of hydrophobicity and indicates the potential of a chemical to partition into the lipid of an organism and bioconcentrate.

Chemicals are considered to be "bioaccumulative" within the context of the CEPA if they have bioconcentration factors that are greater than 5000 (Environment Canada, 1999). In absence of empirical BAF or BCF data, chemicals may be identified as bioaccumulative if they have a Kow greater than 10^5.

1.1.2 Physical Chemical Properties

PEs are formed when phthalic acid is esterified with various alcohols to produce a variety of diesters which vary in alkyl chain length and branching (Figure 1.1).

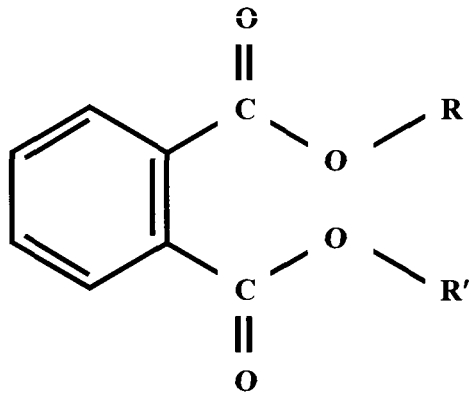

Figure 1.1 Generalized chemical structure for PEs

As a group, PEs have a wide range of physical chemical properties (Table 1.1), some of which make PEs targets of regulatory agencies. While the lower molecular

weight congeners are relatively soluble in water, the higher molecular weight congeners are very hydrophobic and have a log Kow > 5.

Table 1.1 The physical chemical properties of selected PEs (Staples et al., 1997a).

PE Congener		Molecular Weight (g/mol)	Log Kow	Water Solubility (mg/L)	Vapour Pressure (Pa)	Henry's Law Constant (Pa m^3/mol)
Dimethyl phthalate	DMP	194.2	1.61	4200	0.266	0.0124
Diethyl phthalate	DEP	222.2	2.38	1100	0.133	0.0270
Dibutyl phthalate	DBP	278.4	4.45	11.2	0.00359	0.0895
Benzylbutyl pthalate	BBP	312.4	4.59	2.7	0.000665	0.0771
Di(2-ethylhexyl) phthalate	DEHP	390.6	7.5	0.003	0.0000133	1.73

1.1.3 Environmental Fate

PEs are detected in surface and subsurface soils, freshwater, seawater and biota in various locations worldwide. PEs have been detected in landfill leachates (Perwak *et al.* 1981, Oman and Hynning 1993, Bauer and Herrmann 1997; Bauer *et al.*, 1998). As a result, environmental samples regularly contain PEs at levels as high as μg/L in surface waters (Giam 1984, Preston and Al-Omran 1986, Fatoki and Vernon 1990, Law *et al.* 1991, Tan 1995, Huang *et al.* 1999) and sediments (Tan 1995, Vitali *et al.* 1997). PEs were detected in fish collected from Great Lakes harbours and tributaries at levels as high as 32.10 mg/kg (DeVault 1985).

After they enter the environment, PEs do not undergo extensive abiotic degradation. The rates of hydrolysis and photolysis are very low, and therefore are unlikely to play an important role in the fate of PEs in the environment (Staples *et al.* 1997a, Giam 1984). Overall, the principal mechanism for removing PEs from the environment is metabolic breakdown by microorganisms including bacteria (Gibbons and

Alexander 1989, Lewis and Holm 1981), microalgae (Yan *et al.* 2002) and yeasts (Begum *et al.* 2003). However, some studies show biodegradation rates are also low, much slower than the rates of production and release, thus resulting in net accumulation in the environment (Madsen *et al.* 1999).

1.1.4 Toxicological Profile

1.1.4.1 Phthalate Diesters

Concern over PEs in the environment is warranted. Toxic effects may include carcinogenicity, mutagenicity and peroxisome proliferation (Kluwe *et al.* 1982, DeAngelo *et al.* 1986, Rhodes *et al.* 1986, Hodgson 1987, Lake *et al.* 1987, Rao and Reddy 1987, Rao *et al.* 1990). Laboratory studies using many mammalian species show that organ systems including the liver, kidney and thyroid may also be damaged by PEs (Lake *et al.* 1986, Rhodes *et al.* 1986, Busser and Lutz 1987; Dostal *et al.* 1988, Price *et al.* 1987, Marsman *et al.* 1988, Parmar *et al.* 1988, Rao *et al.* 1990, Ganning *et al.* 1991).

PEs are also suspected to be endocrine disrupters (Harris *et al.* 1997). Endocrine disruptors are man-made or naturally occurring substances that can mimic or interfere with the biosynthesis, binding and/or action of natural hormones, and thereby disrupt physiological processes that are under hormonal control (Jobling 1998). Disruption of the endocrine system alters the balance of hormones in the body, thus altering the interactions of hormones with various organs and resulting in deleterious effects on sexual and functional development. Exposure to endocrine disrupting compounds (EDCs) can lead to disruption of cell differentiation and development in younger organisms and damage to reproductive organs resulting in atrophy and cancer (Gray and Butterworth 1980, Parmar *et al.* 1987, Price *et al.* 1987).

Exposure to endocrine disrupting compounds, including a wide variety of chemical compounds, may result in a variety of effects in fish including alterations in plasma hormone concentrations (Khan and Thomas 1998), alteration in gonadal size (Jobling *et al.* 1996) and high levels of the egg yolk precursor vitellogenin in male fish (Jobling *et al.* 1996). *In vitro* studies have shown PEs to be weakly estrogenic in fish (Jobling *et al.* 1995, Knudsen and Pottinger 1999, Tollefsen 2002). However few studies directly related to PEs report adverse effects *in vivo*. Norrgren *et al.* (1999) reported skewed sex ratios in juvenile Atlantic salmon (*Salmo salar*) in waters shown to contain PEs. Survival of fathead minnow (*Pimephales promelas*) embryos was reduced after exposure to dibutyl phthalate (DBP) (McCarthy and Whitmore 1985). While few studies show adverse effects directly related to PE exposure, it is important to note that weakly estrogenic substances such as PEs may act additively with other estrogenic contaminants in the environment resulting in endocrine disruption.

Acute toxicity values are reported in Table 1.2. Unfortunately, there has been an emphasis on obtaining LC50 values rather than investigating the mechanism of action of PEs with respect to fish. Currently, Type II narcosis is the accepted mode of action (Veith and Broderius 1987, Verhaar *et al.* 1992).

Table 1.2 Acute toxicity (measured as 96 hour LC50) of PE congeners to rainbow trout (*Oncorynchus mykiss*)

PE Congener	Solubility	LC50*	Reference
DMP	4200 mg/L	56 mg/L	Adams *et al.* 1995
DEP	1100 mg/L	12 mg/L	Adams *et al.* 1995
DEP	1100 mg/L	>0.5 mg/L	De Foe 1990
DBP	11.2 mg/L	1.5 mg/L	Mayer and Ellersieck 1986
DBP	11.2 mg/L	2.6 mg/L	Mayer and Ellersieck 1986
DBP	11.2 mg/L	1.6 mg/L	Adams *et al.* 1995
BBP	2.7 mg/L	0.82 mg/L	Adams *et al.* 1995
BBP	2.7 mg/L	**3.3 mg/L**	Gledhill *et al.* 1980
DEHP	0.003 mg/L	**>20 mg/L**	De Foe *et al.* 1990
DEHP	0.003 mg/L	**>0.32 mg/L**	Adams *et al.* 1995

*Values in bold indicate LC50 values that exceed the water solubility of the PE

The acute toxicity of PEs appears to be low in fish. Generally the toxicity of PEs increases with increasing alkyl chain length, with the exception of DEHP. The toxicity of DEHP appears to be very low, however this is likely due to the extremely low water solubility of this PE rather than its inherent toxicity. Some LC50 values reported for DEHP are >20 mg/L, an unreasonably high amount because the test concentrations exceed the water solubility of 0.003 mg/L. This high water concentration is often achieved through the use of solvents or the addition of droplets of undissolved PE.

The LC50 values reported for DBP (Mayer and Ellersieck 1986, Adams *et al.* 1995) and BBP (Adams *et al.* 1995) are close to or below 1 mg/L. The inherent toxicity criterion in the CEPA is <1 mg/L for LC50 (Environment Canada, 1999). Therefore some PEs may be classified as inherently toxic under CEPA.

Several sublethal effects have been reported in freshwater species that may reduce the survival of fish in the wild. In a study by Ghorpade *et al.* (2001), acetylcholinesterase activity was reduced in fish exposed to DEP resulting in sluggish, nonmotile behaviour. Wibe *et al.* (2002) showed that shoaling behaviour and bottom-dwelling behaviour in threespine stickle-back, *Gasterosteus aculeatus*, were altered as a result of exposure to BBP. Other behavioural effects may include loss of equilibrium, depressed locomotor activity, loss of startle response and darkened colouration (De Foe *et al.* 1990). DBP and DEHP have been shown to modulate the function of carp phagocytic cells suggesting PEs are also immune modulators (Watanuki *et al.* 2003).

Experimental exposures are often in mg/L (Table 1.2) whereas measured concentrations of PEs in aquatic environments are usually a few μg/L (Giam 1984, Preston and Al-Omran 1986, Fatoki and Vernon 1990, Law *et al.* 1991; Tan 1995, Huang *et al.* 1999). Experimentally defined toxic concentrations generally greatly exceed environmental concentrations, therefore the acute toxicity of PEs appears of little

concern. However, exposure to water concentrations below toxic levels may pose a problem because high internal concentrations may be achieved via the bioconcentration of PEs.

1.1.4.2 Phthalate Monoesters

Few studies have investigated the toxicity of the metabolites of PEs. The LC50 values reported by Scholz (2003) are >1mg/L therefore the acute toxicities appear relatively low (Table 1.3). Some metabolites have been identified as potent testicular toxicants, thus the focus of research has been on testicular toxicity. In particular, the monoester metabolite of DEHP, mono (2-ethylhexyl) phthalate, has been shown to reduce fertility and induce testicular atrophy in laboratory animals (Albro 1987, Lamb *et al.* 1987).

Table 1.3 LC50 values of phthalate monoesters in *Cyprinus carpio* (Scholz 2003)

Monoester		LC50 (mg/L)
MNBP	Mono-*n*-butyl phthalate	133
MEHP	Mono-2-ethylhexyl phthalate	62

1.1.5 Bioconcentration

Bioconcentration is the process by which the chemical concentration in an aquatic organism achieves a level that exceeds that in the water, as a result of direct chemical uptake from the water i.e. not including dietary uptake. Bioconcentration is a concern because organisms may concentrate low levels of contaminant in the water to internal concentrations many orders of magnitude higher than aqueous concentrations. Toxic effects may occur if concentrations at the target site exceed threshold levels. Exposure to chemicals will not necessarily result in an adverse effect. The response

depends on the timing, duration and level of exposure. However, accumulation of high concentrations of PEs in lipids of aquatic organisms can lead to a sustained release which maintains low levels of PEs in the blood (Geyer *et al.* 2000). This results in long-term, continuous exposure to EDCs that may be effective in stimulating hormonal responses. In addition, elevated contaminant levels in fish can adversely impact higher trophic levels in the food chain such as birds, mammals and humans.

The octanol water partition coefficient (Kow) is a measure of the hydrophobicity of a substance. Substances with a high Kow, i.e., most of the substance partitions to octanol, are hydrophobic. In aquatic environments, hydrophobic contaminants can bioconcentrate as they remain in the lipids of organisms to "escape" the water. As a result of this phenomenon, several relationships between Kow and BCF have been reported that describe bioconcentration as a chemical partitioning process (Hamelink *et al.* 1971, Neely *et al.* 1974, Veith *et al.* 1980, Mackay, 1982; Meylan *et al.* 1999). One model predicts the BCF as L*Kow where L is the lipid content of the fish in kg lipid/kg wet weight and Kow is the octanol water partition coefficient (Gobas and Mackay 1987).

PEs have log Kows that range from 1.61 to 10 (Staples 1997b). Therefore, given the high Kow of many PEs, there is great potential for bioconcentration. Predicted BCFs for 5 PE congeners are found in Table 1.4. The predicted BCFs for DEHP exceeds the CEPA definition of a bioaccumulative substance of 5000.

Table 1.4 Predicted BCFs for several phthalate esters in fish based on 5% and 15% lipid contents.

PE Congener	Log Kow	Kow	Predicted BCF (5% lipid content)	Predicted BCF (15% lipid content)
DMP	1.61	40	2	6
DEP	2.38	240	12	36
DBP	4.45	28184	1409	4227
BBP	4.59	38905	1945	5835
DEHP	7.50	31622777	1580000	4740000

There are several factors that can affect bioconcentration and result in measured BCFs that differ from the predicted BCFs. These include growth, metabolism and bioavailability.

1.1.6 Biotransformation

Biotransformation of PEs has been well documented in mammalian species. The first step is ester hydrolysis to form the mono-deesterified metabolite, for example mono-ethylhexyl phthalate (MEHP) from DEHP (Albro and Lavenhar 1989). This is followed by extensive oxidation of MEHP and ultimately excretion. Barron *et al.* (1995) demonstrated that biotransformation of PEs such as DEHP by rainbow trout proceeds similarly to that of mammals and that there are no metabolites unique to rainbow trout. Metabolites are excretable, so biotransformation may play an important role in the elimination of PEs and may result in measured BCFs that are much lower than predicted. BCFs predicted based on Kow do not take biotransformation into account therefore experimentally derived BCFs should therefore be preferred over predicted BCFs.

1.1.7 Bioavailability

Bioavailability can be defined as the fraction of a chemical in a medium that is in a form which can be absorbed by the organism. At the molecular level, chemical substances can cross biological membranes via simple molecular diffusion, facilitated diffusion, mediated transport or a combination of these processes (Stein, 1981). Organic chemicals tend to cross membranes by simple molecular diffusion due to their lipophilic nature. In aquatic environments, hydrophobic chemicals tend to associate with particles or dissolved organic matter (DOM) in the water. Once adsorbed to a particle,

molecules cannot cross a membrane by simple diffusion. Thus it is only the molecules that are not attached to particles, or "freely dissolved," that are bioavailable and able to bioconcentrate. Due to the tendency of hydrophobic organic molecules to associate with dissolved organic material (DOM), bioavailability of these chemicals is reduced in aquatic environments (Gschwend and Wu 1985, Park and Erstfeld 1999, Akkanen and Kukkonen 2003).

BCFs predicted using Kow are based on lipid partitioning theory which assumes that all chemical is available for uptake. In reality, a fraction of the test chemical is not bioavailable and as a result, many studies report BCFs orders of magnitude lower than predictions based on Kow (McCarthy 1983, Servos *et al.* 1989, Haitzer *et al.* 1998, Akkanen and Kukkonen 2001). If it is assumed that all PE detected in the water is bioavailable, the BCF will be underestimated because the concentration in the organism will result from only the bioavailable concentration. Therefore bioavailability is an important factor controlling BCFs.

1.2 Bioconcentration of PEs

Overall, the bioconcentration information for PEs is sparse. A summary of measured BCFs for 5 PE congeners is found in Table 1.5. Although there is a wide range of BCFs listed for each congener, generally BCFs are higher for higher Kow congeners. Several linear relationships between BCF and Kow have been reported (Mackay 1982, Meylan *et al.* 1999). However, for chemicals with a log Kow greater that approximately 6, the linear correlation is lost resulting in a parabolic relationship (Gobas and Morrison 2000, Meylan *et al.* 1999).

Table 1.5 Summary of BCFs for several PEs in fish species

PE Congener	Exposure Duration (days)	Fish Species	Total BCF mL/g wet weight	Reference
DMP	21	*Lepomis macrochirus* (Bluegill Sunfish)	57	Barrows *et al.* 1980
DMP	1	*Cyprinodon variegates* (Sheepshead Minnow)	6	Wofford et al. 1981
DEP	21	*Lepomis macrochirus* (Bluegill Sunfish)	117	Barrows *et al.* 1980
DBP	1	*Cyprinodon variegates* (Sheepshead Minnow)	12	Wofford et al. 1981
BBP	21	*Lepomis macrochirus* (Bluegill Sunfish)	663	Barrows *et al.* 1980
BBP	17	*Lepomis macrochirus* (Bluegill Sunfish)	188	Heidolph and Gledhill 1979
BBP	3.27	*Lepomis macrochirus* (Bluegill Sunfish)	449	Carr *et al.* 1997
DEHP	35	*Lepomis macrochirus* (Bluegill Sunfish)	112	Macek *et al.* 1979
DEHP	21	*Lepomis macrochirus* (Bluegill Sunfish)	114	Barrows *et al.* 1980
DEHP	5	*Leuciscus idus melanotus* (Golden orfe)	40	Freitag *et al.* 1985
DEHP	4	*Oncorhynchus mykiss* (Rainbow Trout)	8.9	Tarr *et al.* 1990
DEHP	1	*Cyprinodon variegates* (Sheepshead Minnow)	14	Wofford et al. 1981
DEHP	1	*Gambusia affinis* (Mosquito Fish)	11	Metcalf *et al.* 1973

The literature on the bioconcentration of PEs in fishes is generally unreliable for a variety of reasons involving the experimental design and documentation of methods. The experimental design of previous studies is often flawed in the duration, exposure methods and measurement and interpretation of the fish and water concentrations. Further, often the documentation of the study does not include details on the experimental design making it difficult to assess the reliability of these studies.

Consistent BCFs can only be determined if the uptake process is followed until the concentration within the fish reaches a steady state. Often, the exposure duration of

BCF studies is too short to achieve steady state and the resulting are BCFs underestimated. For example, studies by Freitag *et al.* (1985), and Carr *et al.* (1997) are only 3 days in duration.

Physical chemical characteristics such as water solubility and Kow present several difficulties when working with PEs, especially in simulating environmentally relevant conditions. Water solubility is an important factor to consider when determining the bioconcentration of hydrophobic substances. For PEs, water solubility decreases with increasing molecular weight. Thus water solubilities range from 4200 mg/L for DMP to 0.003 mg/L for DEHP (Staples 1997a). This low solubility of the higher molecular weight PEs is a source of experimental difficulty because it is difficult to achieve water concentrations that do not include PE emulsions. Further, working with low solubility compounds often leads to unreliable results because it is often assumed that the PEs added to the water are completely bioavailable, an inaccurate interpretation of the water concentration. Higher molecular weight PEs are highly hydrophobic and as a result adsorb to various materials. Sullivan *et al.* (1980) showed that some PEs were very strongly sorbed to clay particles and organic matter in aquatic samples. Depending on their Kow, a large portion of PEs will be associated with organic matter including particulate organic matter and dissolved organic matter (POM and DOM), some of which was introduced by the fish in the tank. Association with organic matter in the water reduces the bioavailablity of hydrophobic substances such as PEs. This phenomenon can lead to erroneous estimates of the amount of dissolved PEs in the system because it is often assumed that the concentration measured is completely bioavailable when in fact the actual freely dissolved, bioavailable water concentration is much lower. Thus care must be taken to determine the freely dissolved concentration rather then total PE concentration which includes PEs sorbed to POM and DOM. Measurement of the total water concentration inflates the amount of PE truly available for uptake by the fish. Thus

the BCF will be underestimated if the freely dissolved concentration is not distinguished from the chemical concentration that is particulate bound. Given these problems, current extraction methods do not effectively measure the freely dissolved concentrations.

In addition to problems in achieving freely dissolved water concentrations, it is also difficult to achieve constant PE concentrations in the tank water. Once PEs enter the tank, their hydrophobic properties will cause them to partition out of the water and bind to organic matter in the water or the glass of the tank. As a result, fish are exposed to much lower concentrations than intended. A flow through system replaces tank water thus replenishing the PEs and maintaining a relatively constant water concentration. Therefore the use of a flow through system to supply the tank is preferred over a static system. However, throughout the literature, static methods have been used. For example, studies by Metcalf *et al.* (1973) and Wofford *et al.* (1981) use static exposure systems. This difficulty in maintaining exposure to a known concentration can result in uncertainty when calculating the BCF. In particular if the authors use the relationship: $C_f = C_w * k_1/k_2(1-e^{-k2t})$ in their derivation of the BCF as it is based on the assumption that C_w is contstant.

Some BCF studies involve the use of radiolabelled PEs to measure the total PE residue within the fish. This can be a very useful method because it shows directly how much of the labelled PE is concentrated by the organism. However, often the total radioactivity in the organism is measured. Measurement of total radioactivity includes metabolites and parent compound. While some metabolites may still contain the radioactive label, they have a different chemical structure and do not contribute to the overall BCF of the parent PE. Thus including these metabolites in the BCF calculation would lead to an over estimation of the BCF.

Given these problems with conducting bioconcentration studies with PEs, it is necessary to conduct careful experiments that take these issues into consideration. Some problems may be very difficult to address, but changing some aspects of experimental design and execution can make great improvements in the reliability of the resulting BCFs.

1.3 Objectives

The **first objective** was to determine BCFs for several PEs in rainbow trout (*Oncorhynchus mykiss)* using direct measurement of PE concentration. The **second objective** was to investigate the metabolism of PEs by identification of phthalate monoesters in the tissues of the trout and by comparison of PE tissue concentrations to PCB tissue concentrations of similar Kow.

1.4 Experimental Design

This study was conducted in collaboration between Simon Fraser University (SFU) and the Institute for Ocean Sciences (IOS). The chemical extraction and analysis of the water and fish samples was conducted at IOS by Joel Blair and Natasha Hoover (GC-MS and LS-ESI/MS machine analysis). Measurements of organic carbon were conducted at IOS by Linda White. The work at IOS was done under a grant to Dr. Frank Gobas, SFU.

2 METHODS

2.1 Overview

Juvenile rainbow trout (*Oncorhynchus mykiss)* weighing 1 to 3 grams were obtained from a local hatchery and acclimatized to laboratory conditions for a minimum of 1 week. Three replicate tanks of 20 fish (total 60 fish) were exposed to freshwater contaminated with dimethyl phthalate (DMP), diethyl phthalate (DEP), dibutyl phthalate (DBP), butylbenzyl phthalate (BBP), di(2-ethylhexyl) phthalate (DEHP), hexachlorobenzene, decachlorobiphenyl, 2,2,4,4,6,6-hexachlorobiphenyl, and 2,2,5,5,-tetrachlorobiphenyl. To obtain background concentrations and control for mortality or other adverse effects, one tank was kept under identical conditions except for the absence of PEs and PCBs. Trout were exposed to the contaminated water for 61 days to ensure steady state concentrations within the fish were reached; the trout were then moved into clean water to measure the elimination rate of the contaminants. On days 0, 2, 4, 7, 10, 21, 44 and 61 of the uptake period, water samples were collected and one fish from each tank was euthanized using a blow to the head. Fish were also euthanized on days 0, 2, 4, 7, 12, 21, 51 and 60 of the elimination period. Water samples were extracted at SFU laboratories and the chemical analysis of fish and water samples was completed at the Institute of Ocean Sciences (IOS). BCFs were then determined using steady state concentrations. The metabolism of PEs was investigated by monoester analysis.

2.2 Experimental

2.2.1 Materials

Dimethyl phthalate (DMP), Diethyl phthalate (DEP), Di-n-butyl phthalate (DBP), Butylbenzyl phthalate (BBP), and Di(2-ethylhexyl) phthalate (DEHP) were purchased from Aldrich (Milwaukee, WI). The isotope-labelled compounds: d^4-DEP, d^4-DBP and d^4-BBP used as method internal standards and d^4-DEP and d^4-BBP used as method performance standards were purchased from Cambridge Isotope Laboratories (Andover, MA). Individual standard stock solutions were prepared at various concentrations in toluene and the spiking solutions were prepared in acetone. All solutions were kept at 4°C in the dark. Solvents including acetone, toluene, hexane, dichloromethane (DCM) and iso-octane were purchased from EMI Science. Reagent water was high-purity HPLC grade (Burdick and Jackson, MI). Alumina (Neutral) was purchased from ICN Biomedicals (Germany). Sodium acetate and anyhdrous sodium sulfate (granular) was purchased from Aldrich. The 3 polychlorinated biphenyls (PCBs) 2,2',5,5' tetrachlorobiphenyl, 2,2,',4,4',6,6' hexachlorobiphenyl, and 2,2',3,3',4,4',5,5',6,6' decachlorobiphenyl were obtained from Accu Standard, (New Haven, CT).

2.2.2 Fish Care

Juvenile Rainbow trout (*Oncorhynchus mykiss*) were obtained from Sun Valley Trout Farms in Mission BC approximately three weeks prior to the commencement of the experiment. The fish, weighing 1 to 3 g, were maintained at a mean temperature of 14°C with a 12 hour photoperiod. Fish were maintained in glass tanks supplied with dechlorinated city water with a pH of 6.43 and a hardness of 6. Tanks were supplied using a flow-through system with a replacement rate of 360 L/hour. For the duration of the experiment, food (Nutra Plus Moore Clark, 50% protein, 23% fat) was supplied daily.

Remaining food and feces were vacuumed from the tanks a few hours after feeding. Fish were euthanized with a blow to the head, then stored frozen at -5°C until analysis.

2.2.3 Fish Lipid Analysis

Lipid analysis was completed using protocols provided by IOS. Frozen whole fish were thawed and ground with a mortar and pestle until homogeneous. Approximately 2 g of homogenized tissue was mixed with 50 g of sodium sulphate. The mixture was transferred to a 1 cm X 1 m glass column and eluted with 50 mL of a 1:1 DCM/hexane mixture. The sample was reduced to approximately 1 mL using a Rotovap then blown down further using high purity nitrogen gas. The remaining lipid was placed in a vented oven overnight then cooled completely in a desiccator before weighing. The weight was recorded and the lipid content calculated.

2.2.4 Preparation of Equipment

PEs are ubiquitous laboratory contaminants and contamination from solvents, glassware and other laboratory materials presents an obstacle to accurate quantification of PEs (Giam, 1984). Experimental equipment was therefore cleaned extensively to prevent contamination. Glassware, aluminium foil and pipets were rinsed once with acetone, once with toluene, once with hexane and once with DCM, then baked at a minimum of 350°C for 6 to 8 hours and rinsed twice with iso-octane, once with hexane, once with DCM, twice with methanol, and once with DCM. Clean glassware was finally rinsed once with 1:1 DCM/hexane. This final rinse was collected and analysed by GC to confirm that the residual PE levels were negligible. Once dry, glassware was covered with baked and solvent rinsed aluminium foil. Glass fibre (GF) filters and octadecyl (C_{18}) extraction disks were cleaned by three successive 15 minute sonications in iso-octane, doubly distilled toluene, and 1:1 DCM/hexane.

2.2.5 Water Contamination

Figure 2.1 shows the water contamination unit designed to contaminate water with environmentally relevant concentrations using a method similar to the slow stirring method used to measure Kow (De Bruijn *et al*, 1989). The contamination unit consisted of a glass bottle containing concentrated PEs and PCBs (Figure 2.2). A fraction of the water supply was directed into the unit, mixed with freely dissolved PEs and PCBs within the bottle and reconnected with the remainder of the water supply that flowed directly to the experimental tanks. The flow rate through the contamination unit was adjusted to prevent contaminant emulsions from entering the tanks and ensures the water concentration of the chemicals remains below their water solubility.

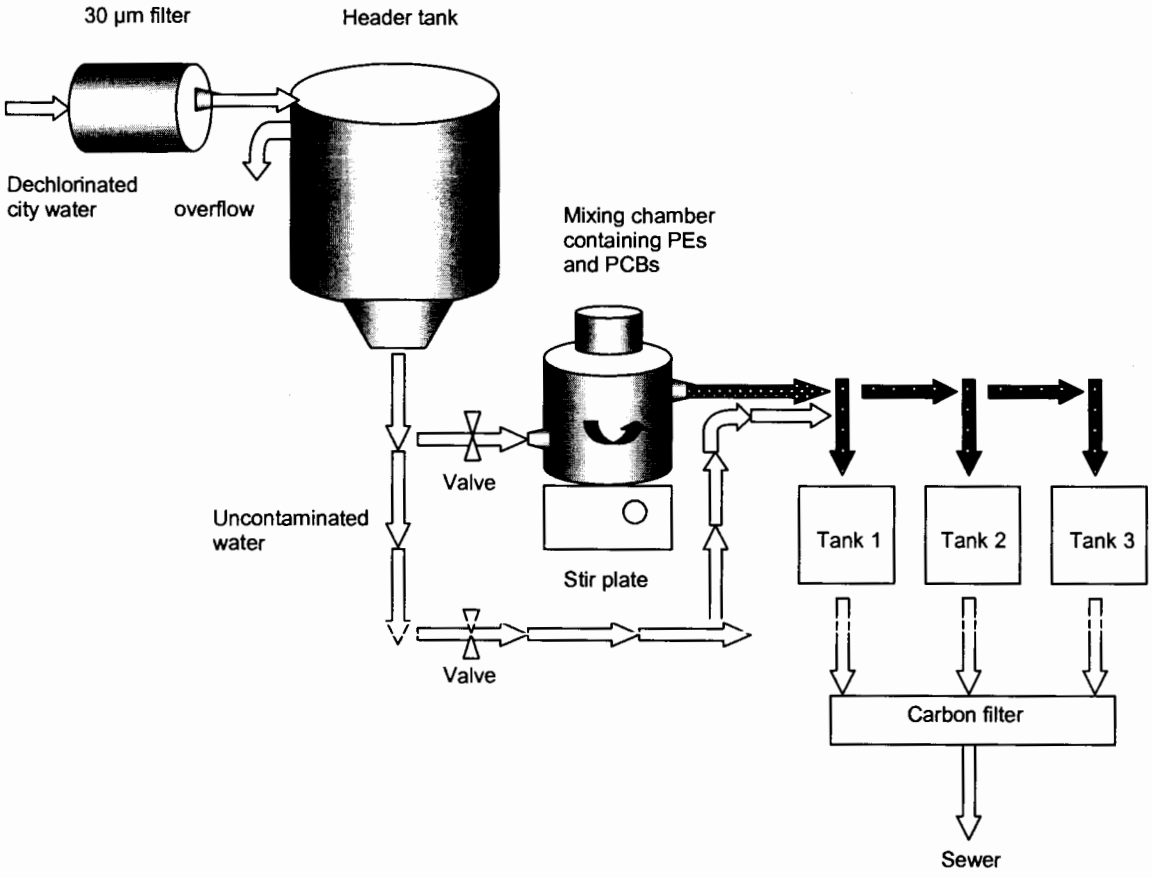

Figure 2.1 Water contamination apparatus set up for bioconcentration of PE and PCB in rainbow trout. Water flowed through the filter into the header tank and supplied the three tanks. A portion of the water flowed through the mixing chamber where it was contaminated with PEs and PCBs then combined with the uncontaminated water before supplying the tanks. Wastewater flowed through a carbon filter to remove contaminants before entering the sewer.

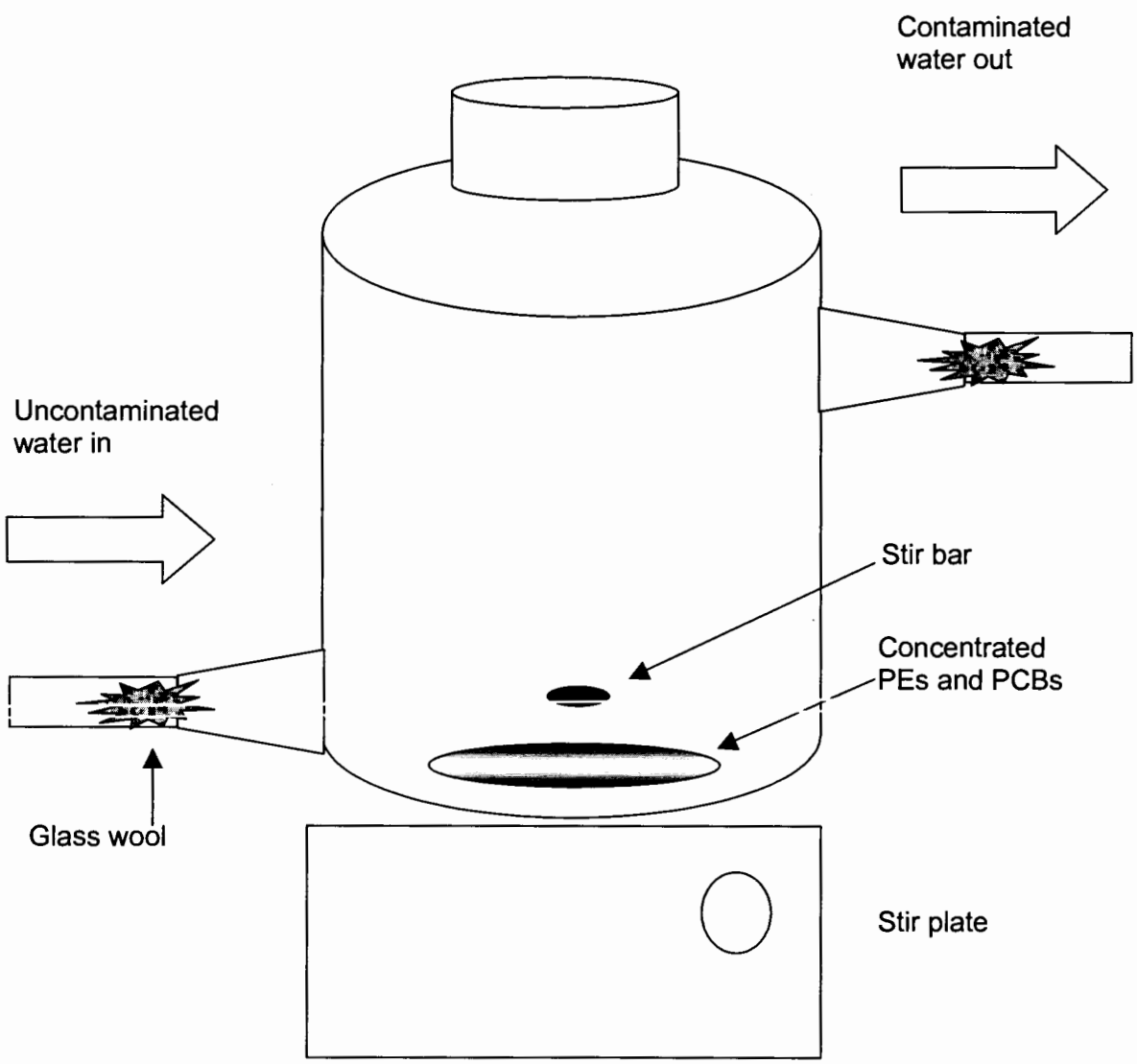

Figure 2.2 Mixing chamber used to contaminate water with freely dissolved PEs and PCBs during a bioconcentration experiment using rainbow trout.

2.2.6 Water Extraction

Approximately one hour before extraction, water samples (1 L) were spiked with 100 ng of internal standards consisting of deuterated PEs and ^{13}C labelled PCBs in the following concentrations: 1 ng/µL of DMP-d^4, 1 ng/µL of DBP-d^4, 1 ng/µL of DnOP-d^4, 1pg/µL of ^{13}C-PCB 209, 44.6 pg/µL of ^{13}C-PCB 52 and 41.5 pg/µL of ^{13}C-PCB 128. To aid in the extraction of PEs and PCBs from the water, 5 mL of methanol was added immediately before the sample was extracted.

The extraction apparatus consisted of a 47mm glass fibre (GF) filter (0.45 µm diameter pore size, from Gelman Laboratory, Pall Corporation, Ann Arbor, Michigan) and two independent 47mm octadecyl (C_{18}) disks (90 % octadecyl bonded silica particles and 10% matrix PTFE by weight, 3M, St. Paul, MN) housed in stainless steel 47mm in-line filter holders connected to a FMI valveless pump (Model RP G50) which pumped water at 8-10 mL/min (Figure 2.3). The GF filter was used to remove PEs associated with particulate organic matter and the two C_{18} disks were used to measure the freely dissolved chemical in the water phase (the operationally defined freely dissolved water concentration).

Prior to filtering the first water sample, the water extraction apparatus was flushed with 100 mL of well water, 200 mL of iso-octane and 200 mL of methanol to remove any residual contaminants from previous extractions. Once the extraction apparatus was flushed, the filters were added and the sample was extracted. Once the entire water sample was pumped through the extraction apparatus, the sample bottle was rinsed with 40 to 50 mL of well water to remove any residual PEs and this was pumped through the filters. The filters were removed using clean metal forceps and

placed in clean 125 mL jars containing 30 mL of a 1:1 mixture of DCM/hexane. The jars were closed with aluminium foil lined metal lids, sealed with Teflon tape, and stored at -4°C.

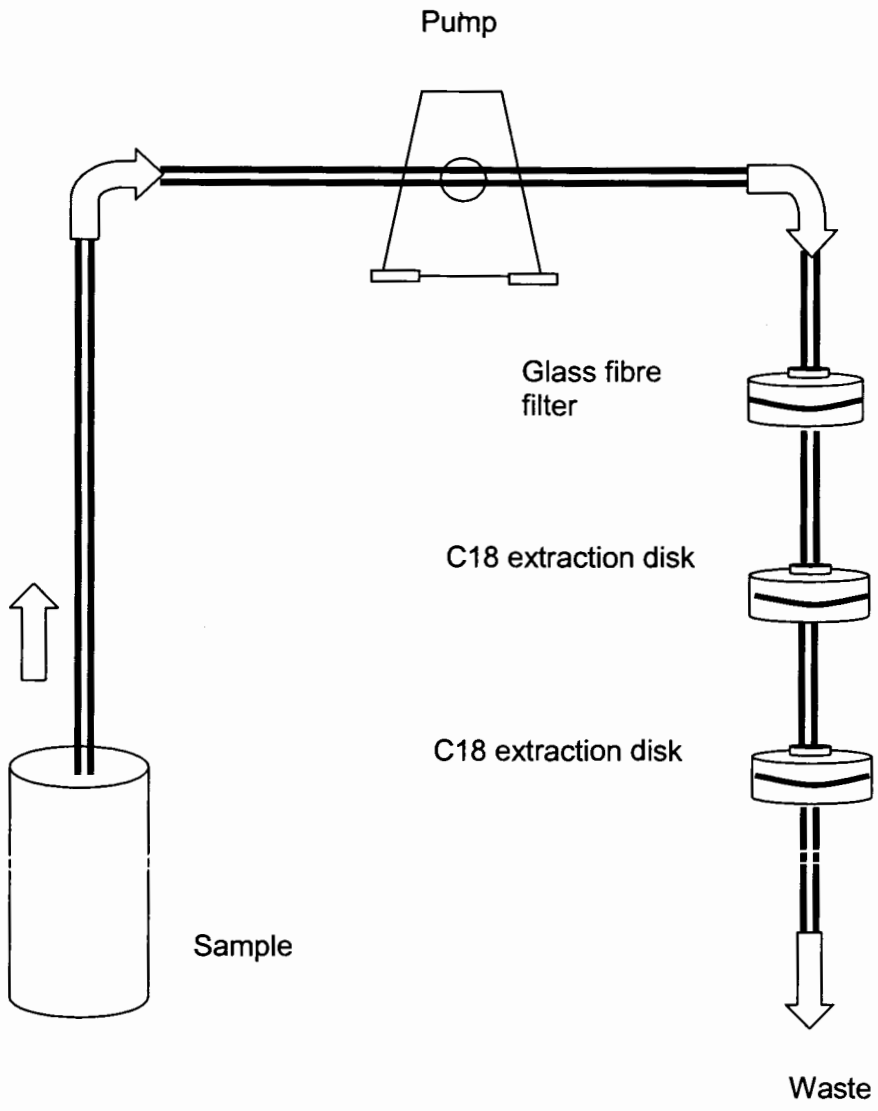

Figure 2.3 FMI valveless pump and filter holders used to extract PEs and PCBs in a bioconcentration experiment with rainbow trout.

2.3 Analytical

2.3.1 Water Analysis

As stated in the overview of this section, staff at the Institute for Ocean Sciences (IOS) conducted the chemical analysis and organic carbon measurements. Samples were analysed according to the methods developed and described in Lin *et al,* (2003). However, because of their importance, a description of the methods is included.

The PEs extracted from the glass fibre (GF) filter represented the particulate bound PE. The PEs extracted from the two C_{18} extraction disks were the freely dissolved. The GF filter and the two C_{18} extraction disks were extracted separately 3 times for 20 minutes in a Branson 5210 ultrasonic water bath with 15 mL of a 1:1 mixture of DCM and hexane. The extracts were concentrated to 3 to 5 mL under a gentle stream of high purity nitrogen, which was then quantitatively transferred to a 15%-deactivated neutral alumina column for cleanup. After analysis, the combined amounts of the test chemicals measured on the GF filter and C_{18} extraction disks were used to determine the total water concentration.

The alumina column was packed with 15 g deactivated alumina (15% H_20, w/w) that had 2 cm of anhydrous Na_2SO_4 on its top layer. Approximately 15-20 mL of doubly distilled hexane was run through the column before the extract was loaded onto the column. Elution was with 30 mL of doubly distilled hexane. This first fraction contained PCBs. The second fraction consisted of 30 mL of 1:9 DCM:hexane and was discarded. Finally the column was eluted with 30 mL of 1:1 DCM/hexane and allowed to run dry. The third fraction, which contained the target analytes, was collected and concentrated to about 100 µL. Then 50 ng of recovery standards in the form of deuterated DEP and

DBP were added. Sample vials were capped with clean aluminium foil-lined septa. After GCMS analysis, these samples were evaporated to dryness under a gentle stream of high purity nitrogen, and dissolved with 0.3mL HPLC grade methanol.

2.3.2 Particulate Matter

GF filters were air dried for 24 hours and then weighed to determine the mass of particulate matter in the sample. Analysis of particulate organic carbon was performed at IOS. The filters were fumed with concentrated HCl to remove inorganic carbon, and analyzed on a Leeman's 440 Elemental Analyzer. Organic carbon content was expressed on a dry weight basis (g OC/g dry particulate matter).

2.3.3 Fish Analysis

The chemical analysis of the fish samples was completed according to the methods developed and described in Lin *et al.* (2003). Whole fish were homogenized in a Sorvall Omni-Mixer. Before use and between samples the homogenizer was taken apart and scrubbed with tap water and a brush. After scrubbing it was reassembled and solvent-rinsed with HPLC grade solvents from EM Science including: once with acetone, twice with iso-octane, once with hexane, once with dichloromethane, twice with methanol and once with dichloromethane. This procedure was also applied to all spatulas, knives or scalpels that were used. Homogenized samples were stored in clean jars and stored at –20°C until further analysis.

Samples were thawed, dried by grinding with prebaked sodium sulfate, and spiked with internal standards consisting of 1 ng/μL of DMP-d^4, 1 ng/μL of DBP-d^4, 1 ng/μL of DnOP-d^4, 1pg/μL of ^{13}C-PCB 209, 44.6 pg/μL of ^{13}C-PCB 52 and 41.5 pg/μL of ^{13}C-PCB 128. The samples were then extracted in a Branson 5210 ultrasonic water bath with a 1:1 solution of DCM/hexane and concentrated to 2 to 3 mL under N_2. The

extract was cleaned up with an alumina column and eluted in 3 fractions. Fraction I consisted of 30 mL of hexane containing the PCBs. Fraction II, consisting of 30 mL of a 1:9 solution of DCM/hexane, was discarded as waste. Fraction III consisted of 30 mL of a 1:1 solution of DCM/hexane containing the PEs.

2.3.3.1 PCB Analysis

Fraction I from the alumina column elution was concentrated under N_2 to approximately 1 mL. DCM was added to make the solvent composition 1:1 DCM/hexane. The extract was loaded into acidic/basic silica columns and eluted with 60 mL of 1:1 DCM/hexane, then concentrated to near dryness and resuspended with 2 mL of hexane. Further clean up was performed using a column packed with approximately 10 g of dry alumina. The sample was loaded and eluted with 25 mL of hexane. This fraction was discarded. The sample was then eluted with 60 mL of 1:1 DCM/hexane and the fraction collected was concentrated to 0.1 mL. The extract was spiked with 30 μL of the PCB recovery standard (^{13}C-PCB-111) and then analysed using GC/HRMS analysis.

2.3.3.2 PE Analysis

Fraction III from the alumina column elution was concentrated under N_2 to approximately 0.1 mL. The samples were transferred to autosampler vials and 50 ng of PE recovery standard was added to each sample. To prevent contamination, GC vials were capped with clean aluminum foil under the seal. Samples were analysed by GC/MS. After GC/MS analysis, samples were evaporated under N_2 to dryness and 0.3 mL of methanol was added to the vial. Samples were then analysed using LC/MS.

2.3.4 Monoester Analysis

Staff at IOS analysed six fish samples for monoester metabolites. Fish were chosen from days 2, 4, 7, 10 and 21 of the uptake phase for monoester analysis. Fish samples consisting of 2 to 4 g of homogenized tissue (whole body) were spiked with ^{13}C-labelled mono butyl phthalate (MBP) and mono ethyl hexyl phthalate (MEHP) internal standards. The samples were extracted 3 times by sonication extraction in 15 mL of 1:1 DCM/acetone solution for 15 minutes. The extract was concentrated under N_2 to dryness and then resuspended in 1 mL of acetonitrile and 5 mL of pH 2 buffer and cleaned up using SPE Oasis cartridges (6 cc, 500 mg). The extract was eluted with 5 mL of acetonitrile and 5 mL of ethyl acetate then concentrated to dryness and resuspended in a 1:1 mixture of DCM:hexane. Further clean up was performed with a GPC column. Extracts were eluted with 300 mL of 1:1 DCM:hexane and reduced in a Rotovap to approximately 1 mL, transferred to centrifuge tubes and concentrated to approximately 250 µL. This extract was concentrated under N_2 to dryness and resuspended in 100 µL of methanol. The extract was then spiked with 50 µL of deuterated recovery standard (MiNP-d^4 RS) and analysed using LC/MS analysis.

2.4 Data Analysis

2.4.1 Water Data

2.4.1.1 PE Experiments

Total water concentrations were determined by adding the measured concentrations on the GF filter and C_{18} disks. To determine the recovery of the PEs throughout the extraction and clean up process, water samples were spiked with internal

standards consisting of deuterated PEs including: DMP-d^4, DBP-d^4 and DOP-d^4. The measured concentrations on the GF filter and C_{18} disks were corrected based on the recovery of the internal standards. There was a linear relationship between the recovery and log Kow of the internal standard, therefore the recoveries for each congener were adjusted based on this relationship and the concentrations were recovery-corrected.

The total water concentration was blank-corrected and screened against a minimum detection limit (MDL) to ensure that concentrations reported in the water were not a result of contamination during the chemical analysis. Blanks consisted of 1 L samples of well water processed in the same way as the experimental water. The MDL was determined as the mean blank concentration (n=14) plus 3 standard deviations above the mean concentration of all blanks. Only concentration data above the MDL were considered.

The concentration measured by the C_{18} disks was used as the operationally defined freely dissolved concentration. However the C_{18} disks may not only capture freely dissolved PEs but also those sorbed to small diameter (i.e. less than 0.45 μm) particulate matter. This was confirmed by a slight brown tinge to the disks after filtering the water samples. Therefore the following model was used to assess the feely dissolved water concentration in absence of a true measure:

$$\mathbf{F_{DW} = 1/ [1 + \beta_{SDSM} \cdot Kow + \beta_{LDSM} \cdot Kow]}$$

Where $\beta_{SDSM} = 0.35 \cdot OC_P \cdot \Phi_P \cdot \delta_P$ and 0.35 is a constant (L/kg), which represents the ratio of K_{OC} to K_{OW}, OC_P is the organic carbon content (kg organic carbon/kg suspended matter), Φ_P is the concentration of small diameter suspended matter (kg small diameter suspended matter/L water), and δ_P is the degree of chemical disequilibrium between the small diameter suspended matter and the water.

2.4.1.2 PCB Experiments

The PCB data were derived at IOS using methods outlined in Ikonomou *et al.* (2001).

2.4.2 Fish Data

Quantified PE and PCB concentrations were corrected for background contamination using Na_2SO_4 blanks run during the analysis of the fish tissues. Samples were blank corrected and screened against a MDL consisting of the mean PE concentrations in the Na_2SO_4 from each sample analysed plus 3 times the standard deviation. Samples that fell below the MDL were not considered.

3 RESULTS AND DISCUSSION

3.1 PE Experiments

3.1.1 Water concentration

The MDL was determined as the mean blank concentration (n=14) plus 3 standard deviations above the mean concentration of all blanks (Table 3.1). Overall, the total and operational freely dissolved water concentrations were above the MDL. Generally, the water concentration decreased and plateaued by day 20 of the uptake phase with the exception of the more hydrophobic compounds BBP and DEHP, which increased initially before plateauing (Figure 3.1 to Figure 3.5).

Table 3.1 Mean well water blank concentrations (n=14) and minimum detection limits for PE water concentrations

PE	Mean Blank Concentration (ng/L)	MDL (ng/L)
DMP	2.85	6.71
DEP	28.34	29.31
DBP	128.25	133.53
BBP	12.65	18.53
DEHP	121.86	128.15

There is little difference between the total, operationally defined freely dissolved and predicted freely dissolved water concentrations for low Kow congeners (Figure 3.1). However, operationally defined and predicted freely dissolved water concentrations are, in many cases, orders of magnitude lower than total concentrations for congeners with higher Kows, for example BBP and DEHP (Figures 3.4 and 3.5). This is further

illustrated in Figure 3.6 where the fraction of freely dissolved PE, as measured by the C_{18} disk, is highest for the low Kow congeners. For example, DMP (log Kow 1.61) is almost 100% freely dissolved whereas DEHP (log Kow 7.5) is only 6%.

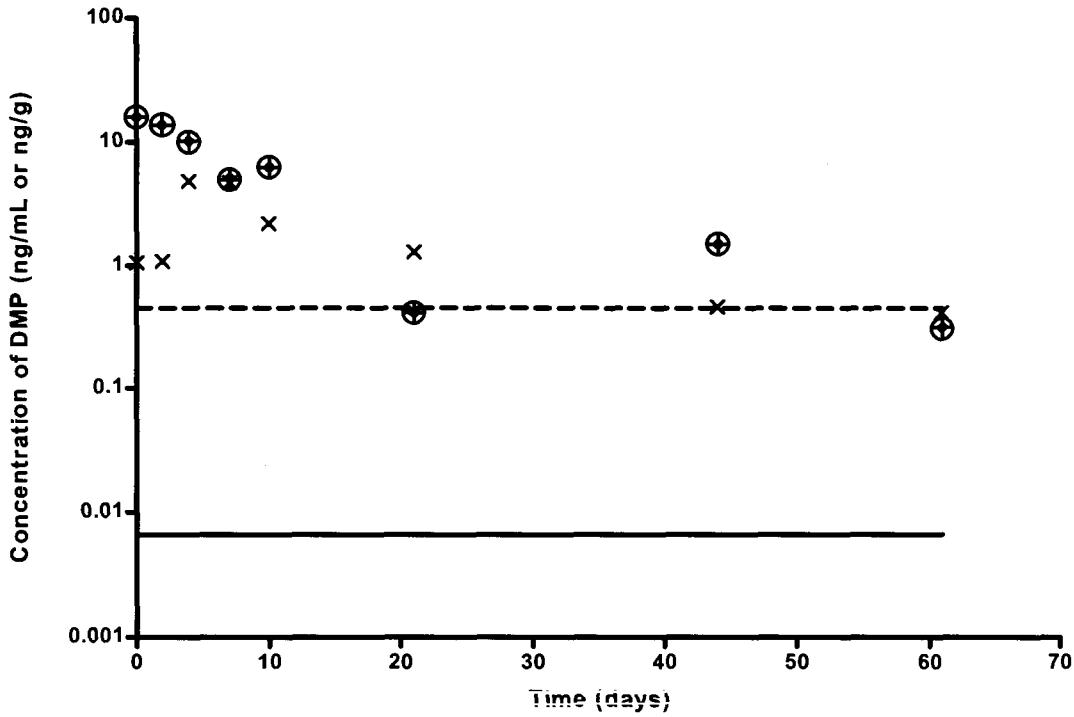

Figure 3.1 Average DMP concentrations in fish (rainbow trout) and water during the 60 day uptake period of a bioconcentration experiment . Total water concentration (♦), Operationally defined freely dissolved water concentration (+), Predicted freely dissolved water concentration (O), and Fish concentration (x). MDL for water concentrations (——), MDL for fish concentrations (------)

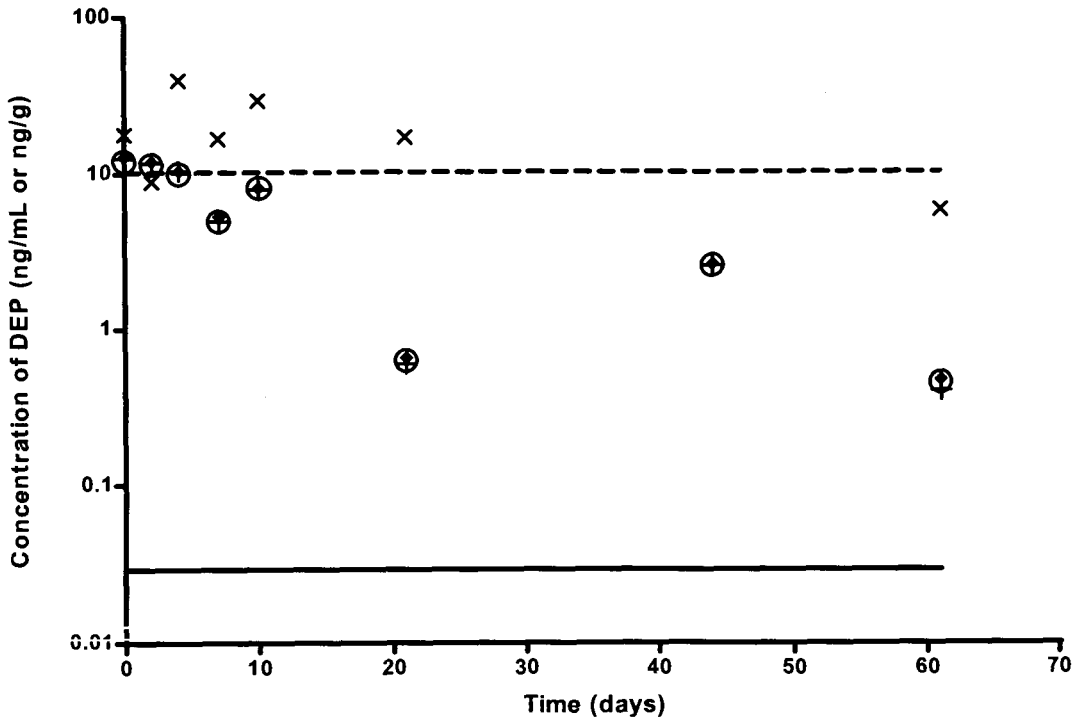

Figure 3.2 Mean DEP concentrations in fish (rainbow trout) and water during the 60 day uptake period of a bioconcentration experiment. Total water concentration (♦), Operationally defined freely dissolved water concentration (+), Predicted freely dissolved water concentration (O), Fish concentration (x), MDL for water concentrations (——), MDL for fish concentrations (------).

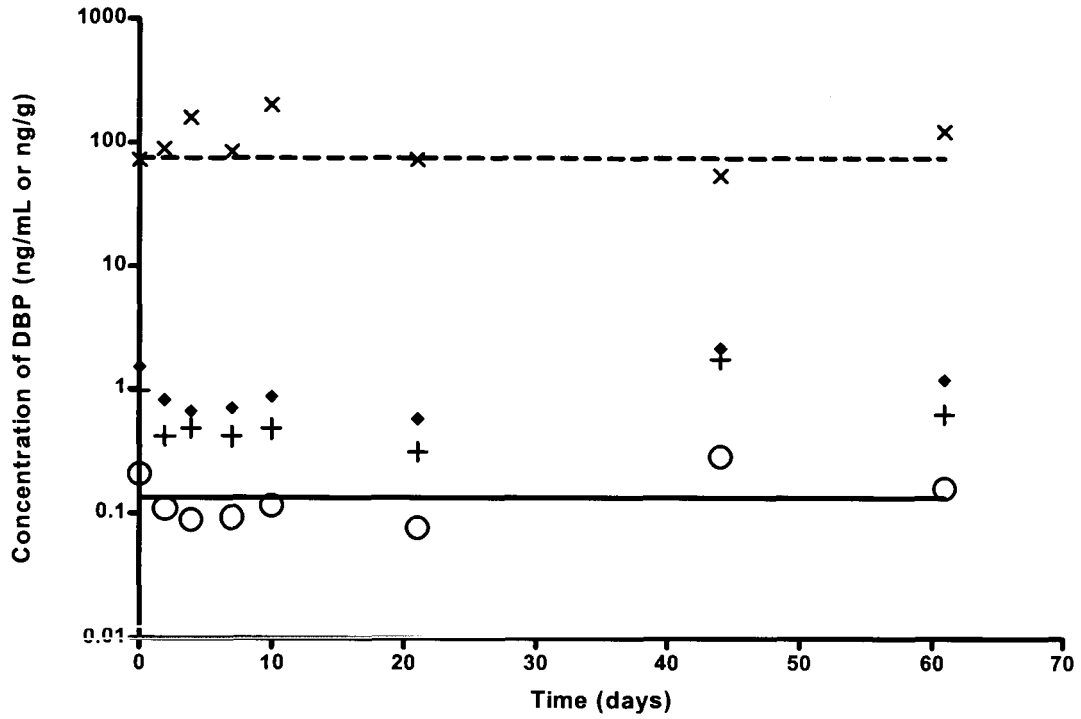

Figure 3.3 Mean DBP concentrations in fish (rainbow trout) and water during the 60 day uptake period of a bioconcentration experiment. Total water concentration (♦), Operationally defined freely dissolved water concentration (+), Predicted freely dissolved water concentration (O), Fish concentration (x), MDL for water concentrations (——), MDL for fish concentrations (------).

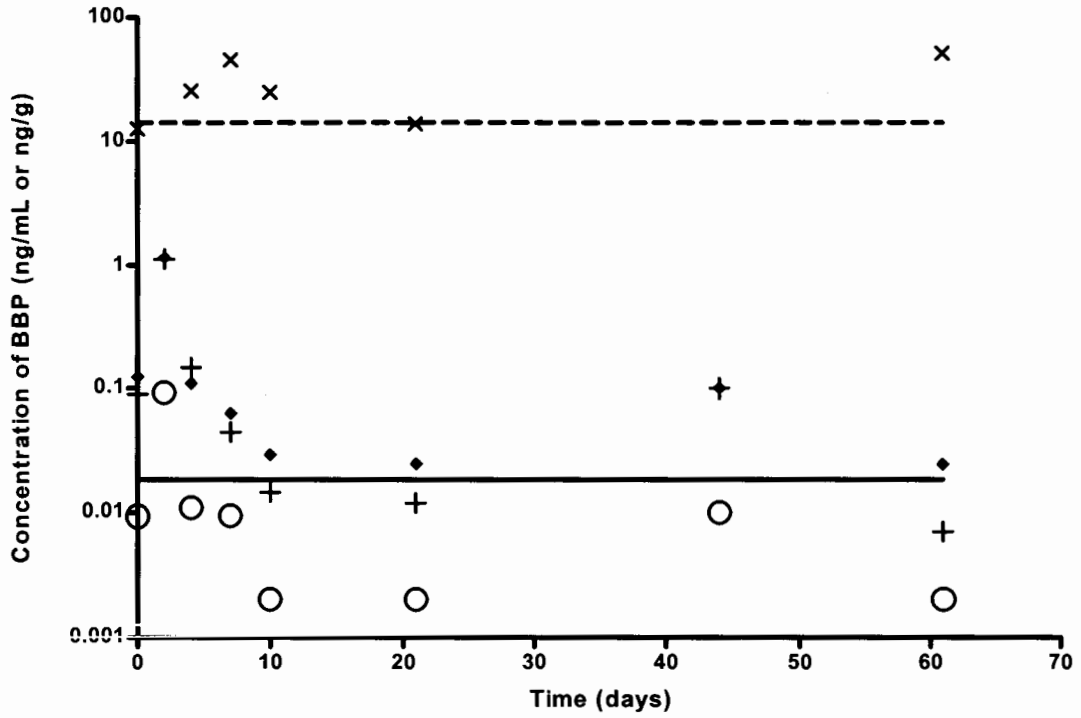

Figure 3.4 Mean BBP concentrations in fish (rainbow trout) and water during the 60 day uptake period of a bioconcentration experiment. Total water concentration (♦), Operationally defined freely dissolved water concentration (+), Predicted freely dissolved water concentration (O), Fish concentration (x), MDL for water concentrations (——), MDL for fish concentrations (------).

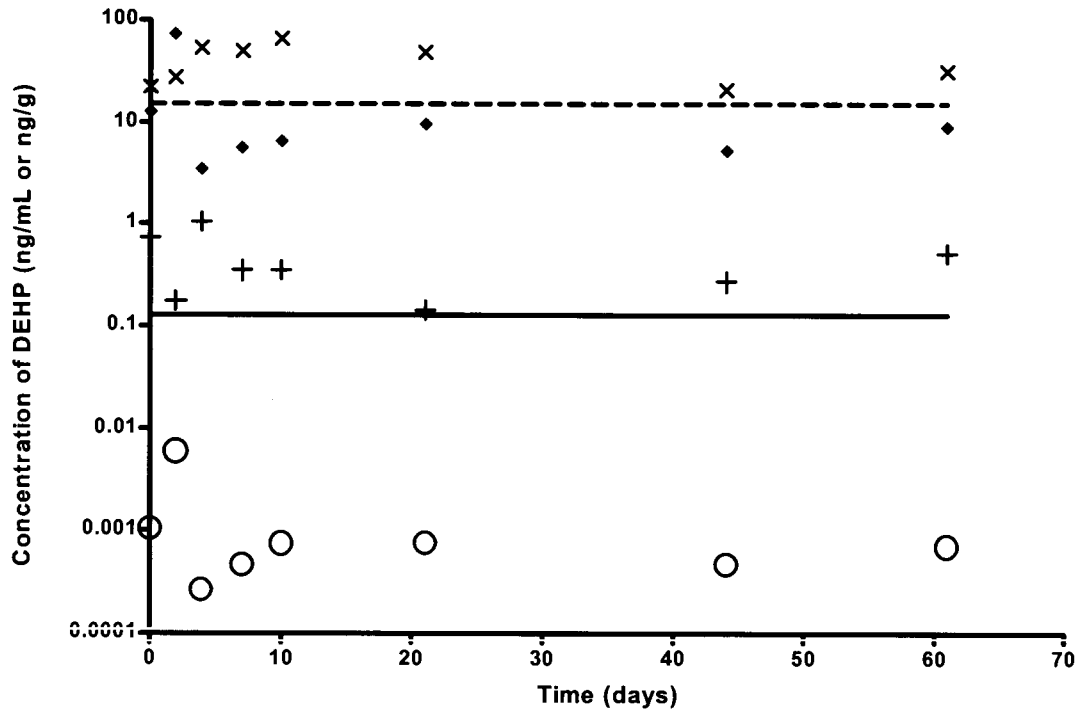

Figure 3.5 Mean DEHP concentrations in fish (rainbow trout) and water during the 60 day uptake period of a bioconcentration experiment. Total water concentration (♦), Operationally defined freely dissolved water concentration (+), Predicted freely dissolved water concentration (O), Fish concentration (x), MDL for water concentrations (——), MDL for fish concentrations (------).

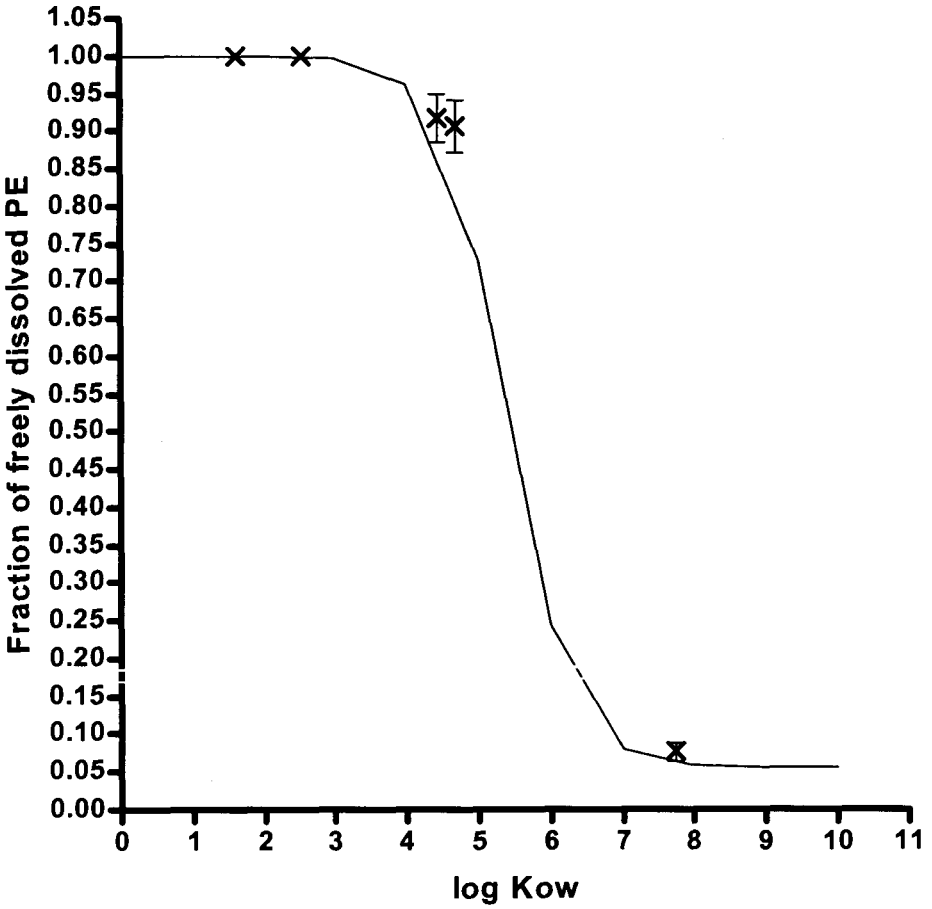

Figure 3.6 Fraction of operationally defined freely dissolved PE versus Kow. The fraction of freely dissolved PE is based on the operationally defined freely dissolved concentration as measured by the C18 disk.

3.1.2 Fish concentration

The MDL was determined as the mean blank concentration plus 3 standard deviations above the mean concentration of all blanks (Table 3.2). The majority of the fish concentrations were above the MDL. Generally, the concentration of each PE congener in the fish increased during the first 10 days of the uptake period then followed the water concentrations for the rest of the uptake period (51 days) (Figure 3.1 to 3.5). After the initial increase in concentration, the whole body tissue concentrations generally followed the decline in the water concentrations, possibly indicating that a steady state is achieved rapidly. The latter is further illustrated in Figures 3.8, 3.9 and 3.10 which show the measured BCF, i.e. the ratios of the measured concentrations in fish and water, over the uptake period. After an initial increase, the BCFs appear to reach a maximum which indicates steady state. However, it is important to note that the concentration of DEHP in fish appears to drop after day 21 which may be due to enzyme induction.

Lipid contents are reported in Table 3.3. The lipid content of the fishes decreased as the fish grew larger (Figure 3.7). The mean lipid content was 15.9±4.5% (n=11) for the whole fish.

Table 3.2 Na_2SO_4 blank concentrations and minimum detection limits for PE tissue concentrations in rainbow trout

PE	Mean Blank Concentration (ng/g)	MDL (ng/g)
DMP	0.27	0.45
DEP	5.78	10.17
DBP	43.06	74.81
BBP	4.04	14.08
DEHP	5.84	14.88

Table 3.3 Lipid content (% by weight) of fish sampled throughout the uptake phase

Time (days)	Lipid content (% by weight)
0	25.9
0	19.1
31	18.1
31	16.1
31	19.4
31	14.1
164	9.9
164	13.6
164	12.7
164	12.8
164	12.8

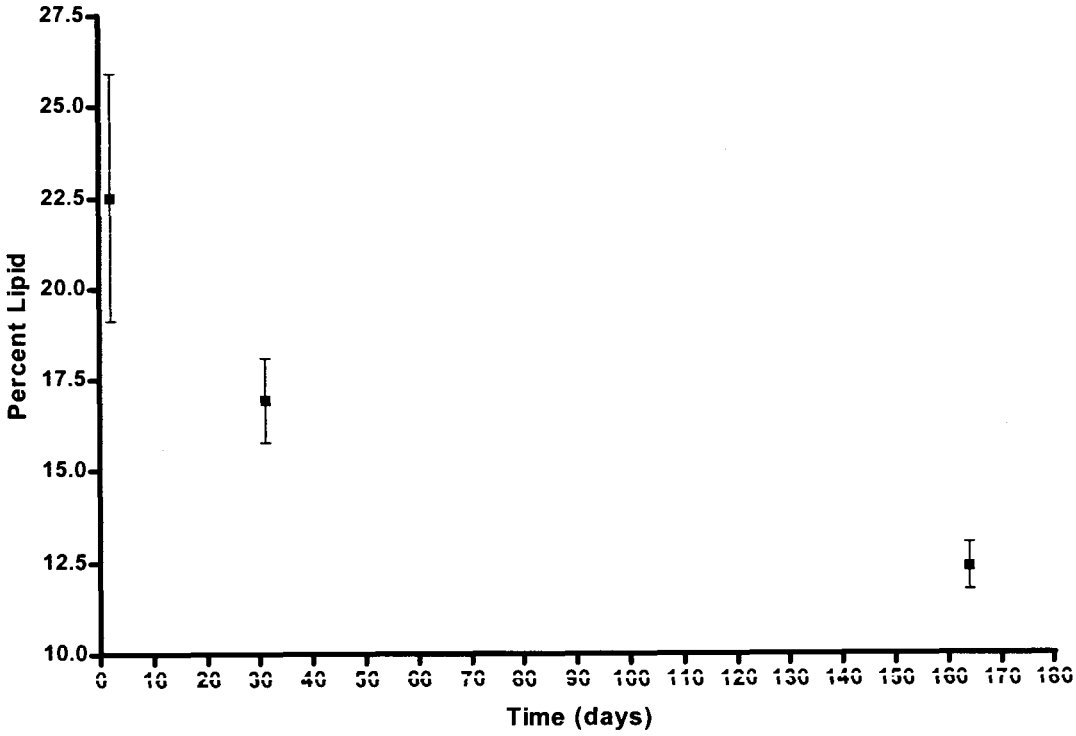

Figure 3.7 Lipid content in rainbow trout versus time. Percent lipid was determined on a weight per weight basis for the whole body.

3.1.3 Monoester Metabolites

Two monoesters, monobutyl phthalate (MBP) and mono-2-ethylhexyl phthalate (MEHP) were detected in the whole body fish samples (Table 3.4). The other metabolites were not detected above the MDL which is quite high due to the relative insensitivity of this new technique.

Table 3.4 Mean monoester concentrations detected in whole body rainbow trout samples.

Monoester		Concentration ng/g						
		MDL	Day 2 Tank 1	Day 4 Tank 1	Day 7 Tank 1	Day 7 Tank 3	Day 10 Tank 1	Day 21 Tank 3
MMP	monomethyl phthalate		ND	ND	ND	ND	ND	ND
MEP	monoethyl phthalate	32.2	ND	ND	ND	ND	ND	ND
MBP	monobutyl phthalate		304.6	191.7	108.6	42.5	59.6	46.4
MBzP	monobenzyl phthalate	31.3	ND	ND	ND	ND	ND	ND
MEHP	mono-2-ethylhexyl phthalate	37.0	56.5	ND	ND	ND	ND	ND

3.2 PCB Experiments

The difficulty and limitations of working with hydrophobic chemicals is further illustrated by the PCB data. The majority of the PCB concentrations in the water were non detect (ND) therefore there is no further analysis of these data. Due to the lack of measurable water concentrations, the bioconcentration and partitioning behaviour of PCBs could not be examined.

3.3 Bioconcentration factors

BCFs were calculated as C_f/C_w where C_w is the concentration in the water (ng/mL) and C_f (ng/g) is the concentration in the fish. BCFs were derived using the measured total water concentration, the operationally defined freely dissolved water concentration (as measured by the C_{18} disks), and the predicted freely dissolved concentrations based on the 3 phase sorption model. Measured concentrations and measured BCFs for each tank over time are reported in Appendix B. Mean BCFs are tabulated in Table 3.5.

Table 3.5 Summary of mean BCFs (± SEM) based on measured concentrations of phthalate esters in rainbow trout (n=3)

PE	Kow	Theoretical BCF 15%lipid * Kow	BCF Total Water Concentration	BCF Operational Freely Dissolved (C_{18} Disk)	BCF Predicted Freely Dissolved Concentration
DMP	40.7	6.1	1.74±0.25	1.76±2.21	1.76±0.27
DEP	347	52	19.0±10.8	20±11.40	21±12.56
DBP	28200	4230	102±15.36	149±19.74	759±114.54
BBP	50100	7515	918±88.48	1890±3.72	11500±1164.30
DEHP	53700000	8055000	5.06±2.81	202±111.02	50000±19573

* Standard error of the mean calculated using the statistical software JMP (SAS Institute)

The BCFs increased over the duration of the uptake period and then reached a maximum value after day 21 (Figure 3.8). This indicates that the system is at steady state. BCFs based on total water concentrations at steady state are reported in Table 3.5. BCFs ranged between 1.74 for DMP to 918 for BBP. The BCFs derived in this study are generally an order of magnitude lower than those reported in the literature. For example, Barrows *et al.* (1980) reported a BCF of 57 for DMP in the bluegill sunfish whereas the BCFs derived in this study ranged from 0.39 to 1.37.

Figure 3.9 shows that BCFs based on operationally defined water concentrations showed a similar relationship with time as the BCFs based on total water concentration. Values for the BCF ranged between 1.76 for DMP to 1890 for BBP. BCFs based on predicted freely dissolved water concentrations also showed a similar relationship with time as the BCFs based on total water concentration (Figure 3.10). Values for the BCF based on calculated freely dissolved water concentration ranged between 1.76 for DMP to 11500 for BBP.

Equilibrium partitioning theory predicts that the BCFs of high Kow PEs will exceed the CEPA criteria for bioconcentration of 5000. However, the BCFs based on total water concentrations measured in this study were much lower than those predicted by equilibrium partitioning theory. Further, this discrepancy between theoretical and measured BCF increases with Kow (Figure 3.11). Possible reasons for the discrepancy may include bioavailability of the PEs, biotransformation and growth dilution.

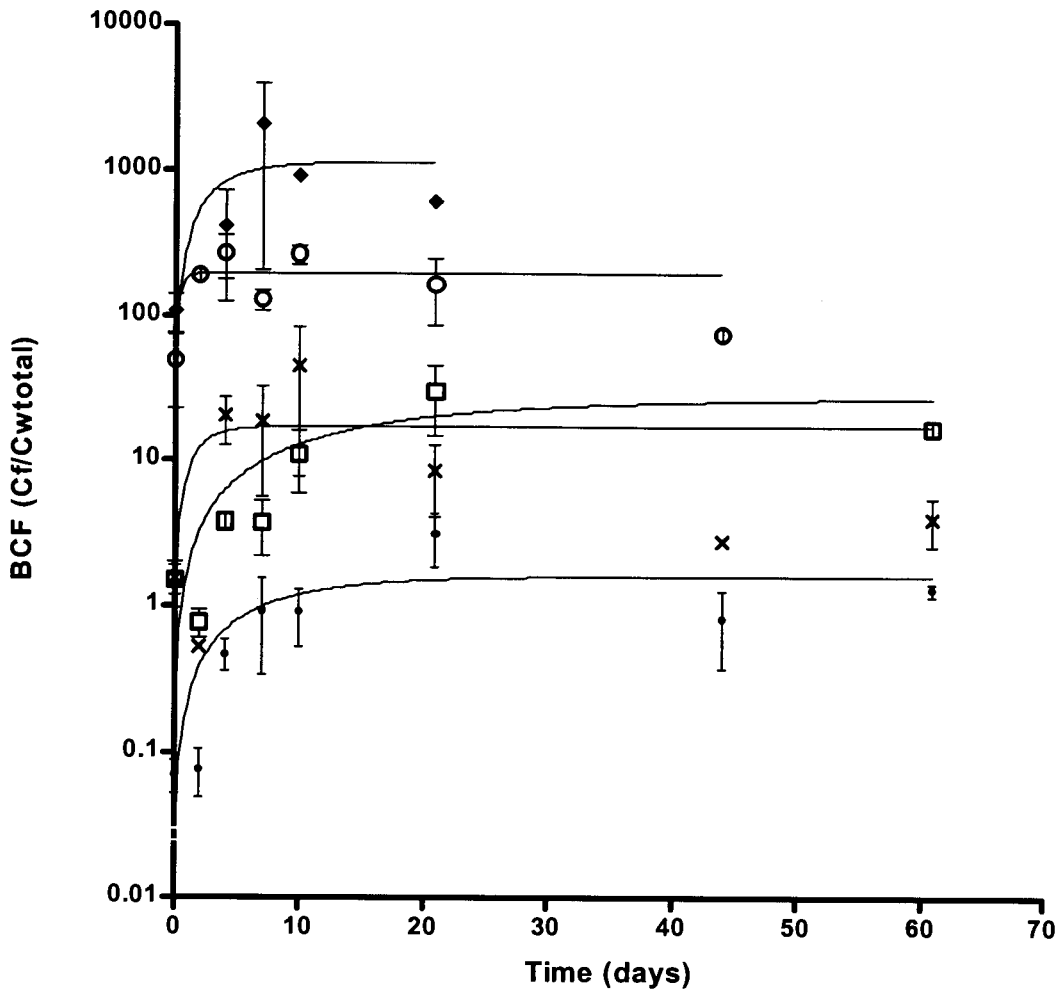

Figure 3.8 BCF of 5 phthalate esters versus time. BCF is determined as the wet weight concentration in the fish divided by the total water concentration. Dimethyl phthalate (DMP) (●), diethyl phthalate (DEP) (□), dibutyl phthalate (DBP) (○), benzylbutyl phthalate (BBP) (◆), and di(ethylhexyl) phthalate (DEHP) (×). Note: Data for BBP (◆) is unavailable after day 44.

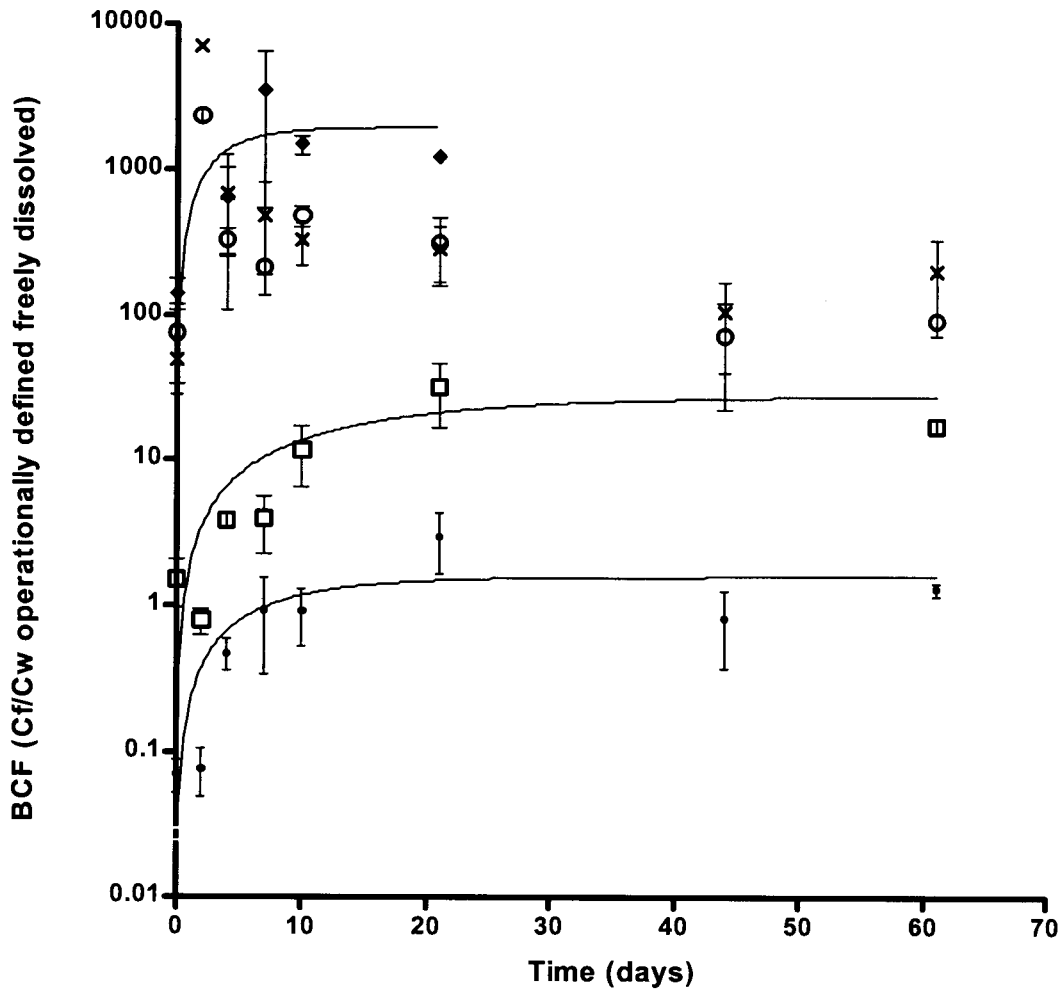

Figure 3.9 BCF of 5 phthalate esters versus time. BCF is determined as the wet weight concentration in the fish divided by the operationally defined freely dissolved water concentration. Dimethyl phthalate (DMP) (●), diethyl phthalate (DEP) (□), dibutyl phthalate (DBP) (○), benzylbutyl phthalate (BBP) (◆), and di(ethylhexyl) phthalate (DEHP) (×). Note: Data for BBP (◆) is unavailable after day 44.

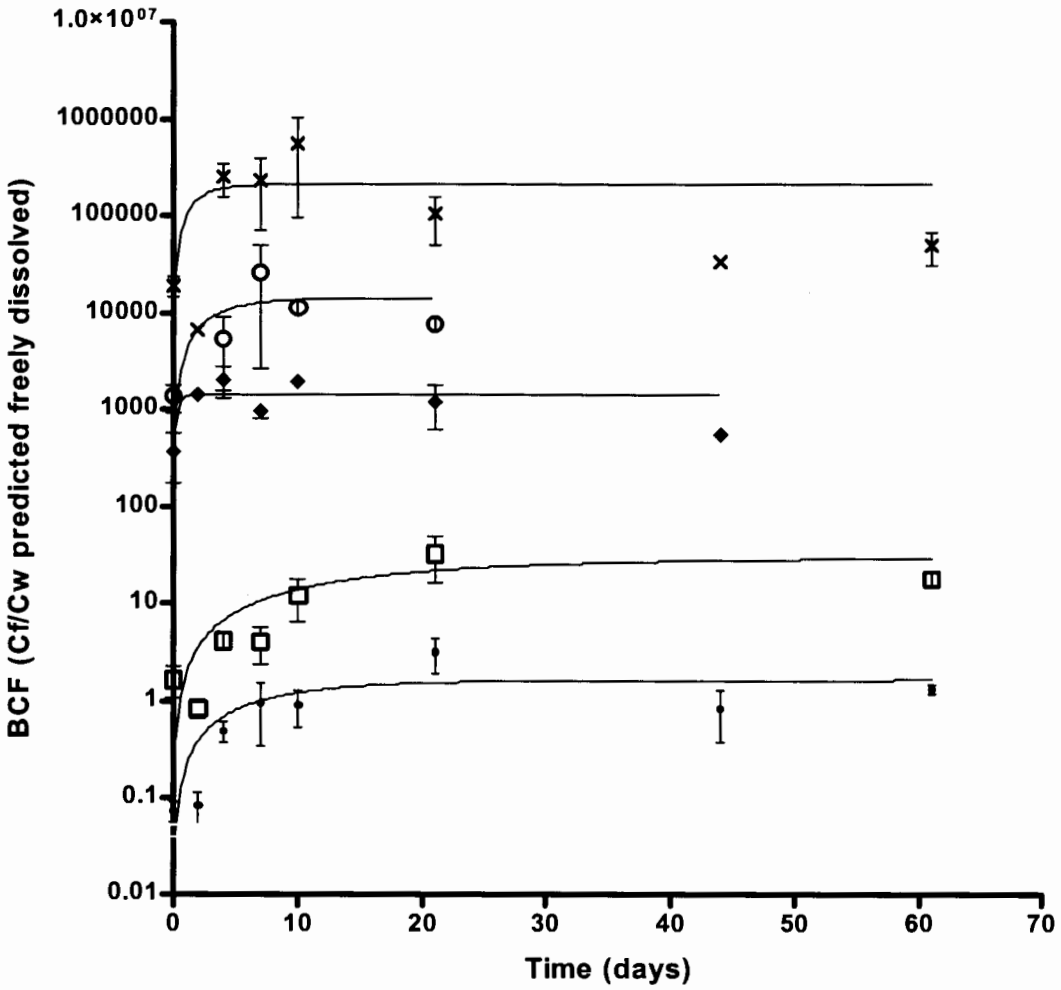

Figure 3.10 BCF of 5 phthalate esters versus time. BCF is determined as the wet weight concentration in the fish divided by the predicted freely dissolved water concentration. Dimethyl phthalate (DMP) (●), diethyl phthalate (DEP) (□), dibutyl phthalate (DBP) (○), benzylbutyl phthalate (BBP) (◆), and di(ethylhexyl) phthalate (DEHP) (×). Note: Data for BBP (◆) is unavailable after day 44.

3.4 Bioavailability

In this experiment, the BCFs derived using the total water concentration appear to follow a linear correlation with Kow, except for DEHP which has a log Kow of 7.5 (Figure 3.10). The BCFs derived using the operational and predicted freely dissolved water concentrations and the predicted freely dissolved water concentrations generally follow the same pattern however, the BCF for DEHP approaches the theoretical BCF (Figure 3.10). The difference in BCFs derived for DEHP using the operationally defined freely dissolved concentrations versus the total water concentrations suggests that DEHP is poorly bioavailable. High Kow compounds are hydrophobic and will adsorb to dissolved and particulate organic matter (DOM and POM). Thus total water concentrations that incorporate DOM and POM appear much higher than the freely dissolved alone (Figures 3.1 to 3.5). This difference in concentrations suggests that PEs with log Kows greater than 6 are poorly bioavailable from the water.

However, it is important to note, for chemicals with a log Kow greater that approximately 6, the linear correlation is lost resulting in a parabolic relationship which is explained by a reduced membrane permeability of high Kow chemicals such as DEHP (Gobas and Morrison 2000, Meylan *et al.* 1999). Therefore the theoretical BCF is lower than predicted using lipid partitioning alone.

BCFs calculated using the operational freely dissolved water concentration (as determined by the C_{18} disks) are higher than those calculated using the total water concentration (Figure 3.11). Further, the difference in BCFs increases with Kow (Figure 3.11). It is widely expected that only the freely dissolved chemical can be absorbed via the respiratory surface of aquatic organisms such as fish (Stein, 1981, Black and McCarthy 1988, Landrum *et al.* 1985, McCarthy and Jimenez 1985, Gobas and Zhang

1994, Gobas and Russell 1991). This implies that, for the high Kow PEs, the actual water concentrations to which aquatic organisms are exposed via their respiratory surfaces are much lower than the measured total water concentrations. As a result, the BCFs derived using operationally defined freely dissolved or predicted freely dissolved water concentrations are higher.

The freely dissolved concentrations measured using the C_{18} extraction disk are likely inflated. This is because the disk measured suspended matter that is smaller than 0.45 µm, which may include organic material such as algae, bacteria, dissolved organic material such as macromolecules, and very fine particulates. Therefore the organic matter may contain PEs that are not truly freely dissolved but are included in the estimate of the operationally defined freely dissolved water concentration. Given the difficulties of visible organic matter being caught on the C_{18} disks, it is expected that the operational freely dissolved water concentrations of higher Kow PEs are substantially greater than the true freely dissolved water concentration. If this is the case, the true inherent bioconcentration potential of these PEs may be higher than measured in this study.

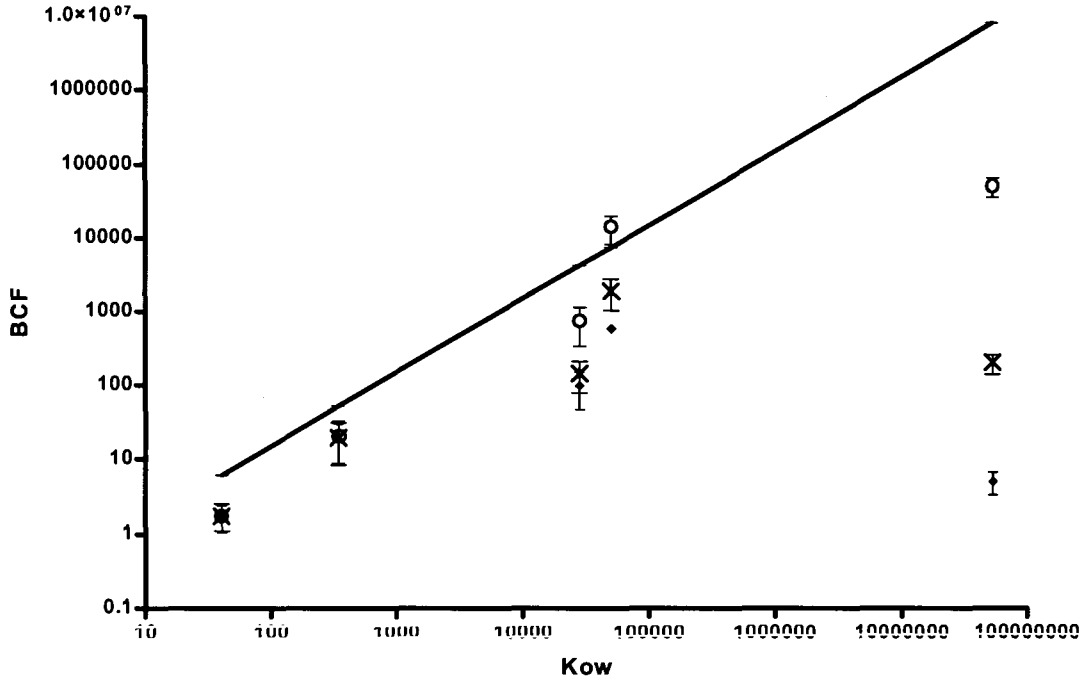

Figure 3.11 Mean BCFs versus Kow. Mean BCFs were determined using the PE fish and water concentrations measured in this experiment. BCFs determined using total water concentration (♦), operationally defined freely dissolved water concentration (x), and predicted freely dissolved water concentration (O). Theoretical BCF (—) was determined using a 15% lipid content * Kow.

3.5 Biotransformation

Previous studies have shown that rainbow trout are able to metabolize PEs (Barron *et al.* 1995). In this study, monoester metabolites of DBP and DEHP were detected in the tissue of fish exposed to PEs (Table 3.4). Biotransformation of PEs produces monoesters that are more water-soluble than PEs, thus increasing elimination from the body and decreasing the concentration of parent PE in the fish.

To estimate the uptake and elimination rate constants (k_1 and k_{Total} respectively), I fitted the BCF data to the following equation:

$$\mathbf{BCF = BCF_{MAX} \cdot (1 - e^{-kTotal' \cdot t})}$$

where k_{Total} is assumed to be an approximation of the total elimination rate constant of the PE from the fish including k_2, k_G and k_M.

Over the course of the uptake and elimination phases, the fish grew substantially. The increase in mean fish weight is shown in Figure 3.12. Fish weights for each tank are reported in Table 3.6. The growth rate constant, k_G, was determined as the change in weight over time normalized by the weight of the fish using a linear relationship between mean fish weight and time from the three tanks over the course of the uptake period. The k_G was determined throughout the experiment and varied from 0.05 to 0.0125 d^{-1}. The mean k_G was 0.02 d^{-1}. Estimates of the metabolic transformation rate constants, k_M, were derived by comparison of kinetic analysis and comparison of theoretical BCF to measured BCFs. Mean rate constants are tabulated in Table 3.7.

Elimination rate constants, k_2, were generally orders of magnitude greater than k_G. This implies that PEs are quickly eliminated and that k_G is insignificant and does not have a substantial effect on the BCF. Compared to the growth rate constant and gill elimination rate constant, the metabolic transformation rate constant, k_M, is considerably

higher (Table 3.7). Biotransformation is likely a key process causing the high rates of elimination as expected gill elimination exchange rates are much lower than the apparent combined elimination rate constants. As a result, the BCFs are lower than those predicted by equilibrium partitioning.

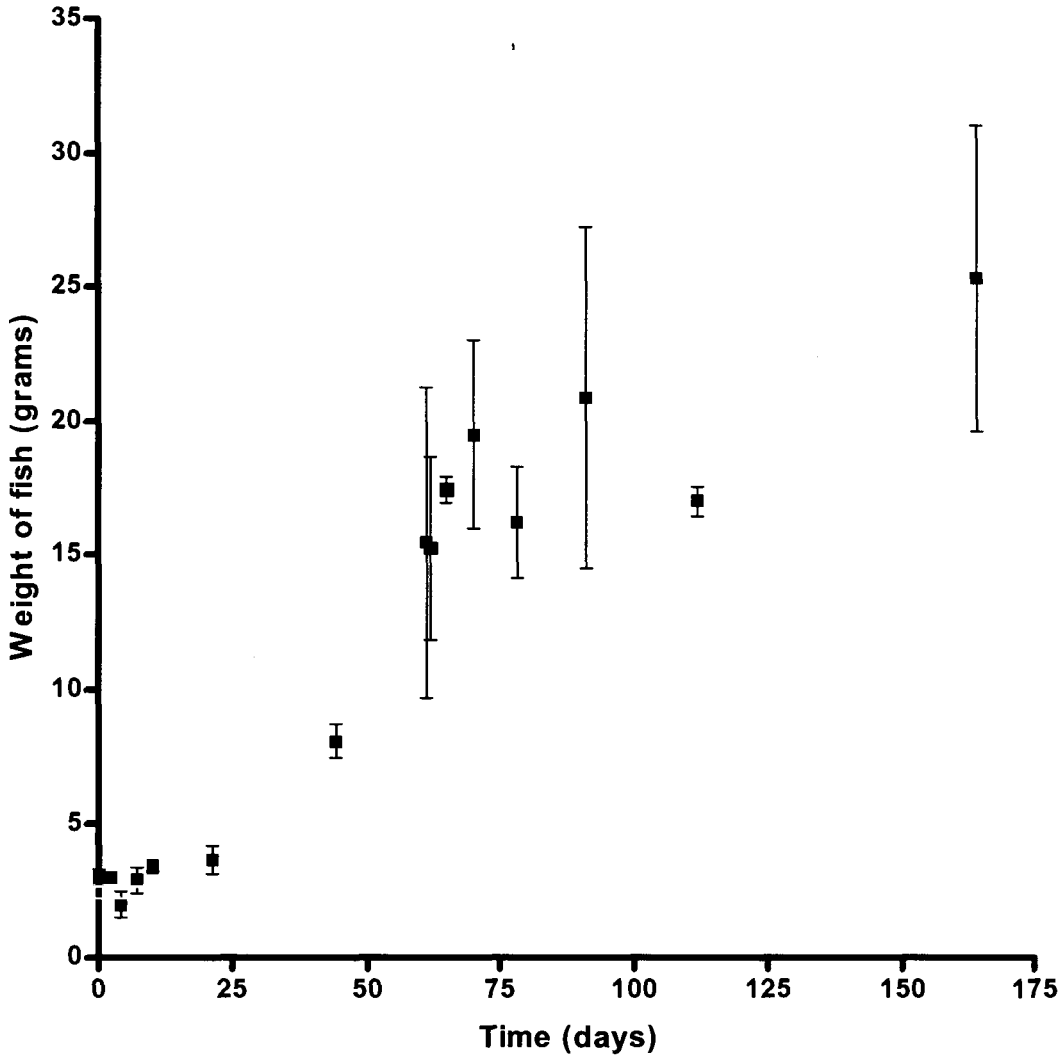

Figure 3.12 Increase in mean rainbow trout weight during the uptake phase of phthalate ester bioconcentration experiment

Table 3.6 Summary of fish weights during the uptake phase of a bioconcentration experiment with phthalate esters in rainbow trout

Time (days)	Whole Body Weight in grams		
	Tank 1	Tank 2	Tank 3
0	3.53	2.44	3.08
2	3.14	2.67	3.25
4	1.25	2.86	1.90
7	3.76	2.09	2.87
10	3.67	3.57	2.99
21	2.73	3.63	4.64
44	6.94	9.17	8.08
61	10.20	9.16	26.99
62	9.08	20.86	15.74
65	17.38	16.61	18.27
70	12.69	24.32	21.42
78	12.53	16.37	19.70
91	9.05	30.91	22.60
112	17.52	N/A	16.45
164	30.98	N/A	19.60

Table 3.7 Growth, gill elimination and metabolism rate constants for 5 PEs during the uptake period of a bioconcentration experiment with rainbow trout

PE Congener	k_G (d^{-1})	k_2(d^{-1})	k_M(d^{-1})
DMP	0.02	0.10	0.09
DEP	0.02	0.02	0.05
DBP	0.02	0.32	0.29
BBP	0.02	0.18	0.21
DEHP	0.02	0.17	0.31

3.6 BCFs as an Indicator of Bioaccumulative Potential

In the absence of bioaccumulation factors (BAF), BCFs can be used to evaluate the bioaccumulative potential of a substance. BAFs are considered better predictors of bioaccumulation potential because they consider uptake from all routes of exposure

whereas BCFs only consider uptake from the water. However bioaccumulation data is often unavailable and costly to collect. Collection of bioconcentration data is relatively straightforward and less expensive for the reason that bioconcentration experiments are conducted in a laboratory setting. While BCFs do not account for uptake through dietary routes, the use of BCFs to evaluate bioaccumulative potential is beneficial because it confirms uptake of a substance into the organism and takes into account biotransformation.

The necessity of measured water concentrations to determine the BAF or BCF of a substance presents a unique problem when evaluating substances with high Kow. Such substances are prone to low bioavailability in aquatic environments, which can drastically affect the bioaccumulative potential. This is demonstrated in this experiment where BCFs calculated using the operationally defined freely dissolved water concentration were higher than those calculated using the total water concentration. As it is only the freely dissolved, i.e. bioavailable, substance that can be bioconcentrated, BCFs should be determined using freely dissolved concentrations. Thus new protocols should be developed which incorporate the use of freely dissolved water concentrations to quantify bioconcentration in aquatic organisms.

The difficulty in measuring freely dissolved concentrations of hydrophobic substances such as PEs is well illustrated in this experiment. Due to a lack of sufficient techniques for measuring freely dissolved water concentrations, it is expected that the measured freely dissolved water concentrations of higher Kow PEs are substantially greater than the true freely dissolved water concentration. If this is the case, the true inherent bioconcentration potential of these PEs may be higher than measured in this study.

3.7 Conclusions

BCFs measured in this experiment and reported in the literature are much less than theoretical BCFs. The difference between measured and theoretical BCFs increases as the Kow of the PEs increases. This is because of the biotransformation and bioavailability of PEs.

The biotransformation of PEs by various organisms, including fish, has been well documented. All PEs examined in this experiment were affected by biotransformation resulting in measured BCFs that are lower than theoretical BCFs based on lipid partitioning theory.

In addition to biotransformation, bioavailability may play an important role in the bioconcentration of PEs. The fraction of freely dissolved PE decreases as the Kow increases; high Kow compounds adsorb to POM. Thus water concentrations incorporating the POM appear much higher than the freely dissolved alone. This difference in concentrations suggests that PEs are poorly bioavailable in aqueous environments. Thus BCFs based on water concentrations containing POM, i.e. total water concentrations, underestimate the bioconcentration of a substance.

While bioavailable environmental levels of PEs may be low due to their physical/chemical properties, small amounts may accumulate within organisms to much higher levels through bioconcentration. Current techniques used to quantify bioconcentration in aquatic environments appear to be flawed and underestimate the true BCF. Through the use of freely dissolved water concentrations it is apparent that high Kow PEs such as BBP and DEHP are in fact bioconcentrated.

Chemicals on the Domestic Substances List first must be categorized as persistent, bioaccumulative and inherently toxic before they undergo a screening level risk assessment. The main objective of this categorization is to efficiently evaluate substances by evaluating risk without making a link between the toxicity and exposure of

a chemical. Therefore, chemicals are evaluated based solely on their properties with no consideration of environmental factors. For this reason, BCFs should be calculated using freely dissolved water concentrations. Freely dissolved water concentrations are independent of environmental factors such as organic matter therefore BCFs calculated using the freely dissolved water concentration are most relevant.

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APPENDIX A
QUALITY ASSURANCE/QUALITY CONTROL (QA/QC)

The following QA/QC procedures and protocols for the chemical analysis of water and fish samples were provided by IOS.

All pre-cleaned glassware was rinsed with 1:1 DCM:hexane. Rinses were combined and concentrated on evaporator with a gentle flow of nitrogen and analyzed for PEs. If contamination levels of individual PEs in glassware proof rinses exceeded the detection limit, the glassware was not used.

Before any sample was processed, laboratory capability was demonstrated by conducting triplicate analyses of matrix blanks consisting of pre-baked (450 °C) sodium sulphate spiked with native and isotope-labelled PE standards. Criteria for accuracy and recovery for each of the native PEs was that corrected for surrogated recovery must be 80 to 120% of the spiked value (i.e., accuracy of +/- 20%). For isotope-labelled surrogate recoveries the criterion was 40 to 120%.

Before extraction, each sample was spiked with a mixture of isotope-labelled surrogates to assess the degree of analyte loss during sample workup. If the recovery of any surrogate was outside the range of 40 to 120%, the sample was re-processed and reanalyzed.

Known concentrations of isotope-labelled DEP and DEHP were added to each sample extract immediately before GC/MS analysis. These two compounds served as retention time references for isotope-labelled surrogates and also as the basis for calculations of surrogate recoveries.

A procedure blank sample, consisting of 20 g of pre-baked sodium sulfate for biological tissue samples and 20 L of pre-cleaned well water for water samples, was processed with each batch of up to 12 test samples. In addition, every batch of samples (1 blank + 5 samples) had a blank processed in the same manner as real samples.

Before sample analysis, calibration curves were constructed to verify linearity of MS response for all PEs over the concentration range of 0.3 ppb to 2 ppm for native PEs. The established calibration was verified by analysing the calibration verification standard solution at least once during every 12-hour period in which sample analysis occurred. Using relative response factors from initial calibration runs, the calculated concentration of all native analytes must be within 20% of their true concentration. The calculated recovery of each surrogate standard must be within the range of 75 to 125%. Remedial action is required whenever any native or surrogate compound fails this verification test.

Appendix B: BCFs and Measured water and fish concentrations

APPENDIX B
BCFS AND MEASURED WATER AND FISH CONCENTRATIONS OVER TIME

BCFs and Measured Water and Fish Concentrations of **DMP** over time

Time (days)	$C_{w\ Total}$ (ng/mL)	$C_{w\ C18}$ (ng/mL)	$C_{w\ dissolved}$ (ng/mL)	C_{fish} wet wt (ng/g)	BCF $C_{fish}/C_{w\ total}$	BCF $C_{fish}/C_{w\ C18}$	BCF $C_{fish}/C_{w\ dissolved}$
				Tank 1			
0	19.02	19.00	18.85	0.69	0.04	0.04	0.04
2	12.52	12.46	12.40	0.56	0.04	0.04	0.05
4	8.61	8.57	8.53	4.83	0.56	0.56	0.57
7	5.07	5.05	5.02	1.76	0.35	0.35	0.35
10	1.62	1.61	1.61	1.90	1.18	1.18	1.19
21	0.43	0.43	0.43	0.98	2.26	2.28	2.29
44	0.57	0.57	0.57	0.41	0.71	0.71	0.72
61	0.29	0.29	0.28	0.33	1.15	1.15	1.16
				Tank 2			
0	13.68	13.66	13.55	1.32	0.10	0.10	0.10
2	15.08	15.06	14.94	0.86	0.06	0.06	0.06
4	10.92	10.90	10.82	2.76	0.25	0.25	0.26
7	5.10	5.05	5.05	1.61	0.32	0.32	0.32
10	15.71	15.71	15.57	2.55	0.16	0.16	0.16
21	0.40	0.40	0.39	2.21	5.56	5.56	5.61
44	3.62	3.62	3.59	0.41	0.11	0.11	0.11
61	0.29	0.27	0.28	0.44	1.54	1.65	1.55
				Tank 3			
0	15.55	15.53	15.41	1.16	0.07	0.07	0.08
2	13.70	13.68	13.57	1.83	0.13	0.13	0.14
4	11.09	11.09	10.99	6.79	0.61	0.61	0.62
7	4.93	4.91	4.88	10.52	2.13	2.14	2.15
10	1.53	1.53	1.51	2.13	1.39	1.39	1.40
21	0.41	0.41	0.41	0.62	1.50	1.50	1.51
44	0.34	0.34	0.34	0.56	1.65	1.65	1.66
61	0.38	0.38	0.37	0.45	1.20	1.20	1.21

BCFs and Measured Water and Fish Concentrations of **DEP** over time

Time (days)	$C_{W\ Total}$ (ng/mL)	$C_{W\ C18}$ (ng/mL)	$C_{W\ dissolved}$ (ng/mL)	C_{fish} wet wt (ng/g)	BCF $C_{fish}/C_{W\ total}$	BCF $C_{fish}/C_{W\ C18}$	BCF $C_{fish}/C_{W\ dissolved}$
				Tank 1			
0	15.19	15.09	14.07	8.52	0.56	0.56	0.61
2	8.38	8.16	7.76	7.21	0.86	0.88	0.93
4	8.75	8.52	8.11	37.35	4.27	4.39	4.61
7	5.89	5.63	5.46	15.63	2.65	2.78	2.86
10	2.16	1.88	2.00	27.63	12.80	14.70	13.82
21	0.70	0.61	0.65	14.06	20.15	22.96	21.75
44	2.80	2.65	2.60	reject	N/A	N/A	N/A
61	0.71	0.57	0.66	reject	N/A	N/A	N/A
				Tank 2			
0	10.87	10.83	10.87	26.54	2.44	2.45	2.64
2	15.26	15.13	15.26	7.11	0.47	0.47	0.50
4	9.85	9.63	9.85	31.86	3.24	3.31	3.49
7	3.34	3.12	3.34	22.70	6.80	7.27	7.34
10	20.53	20.30	20.53	30.31	1.48	1.49	1.59
21	0.48	0.48	0.48	28.56	59	59.75	63.74
44	4.83	4.71	4.83	reject	N/A	N/A	N/A
61	0.36	0.30	0.36	reject	N/A	N/A	N/A
				Tank 3			
0	12.12	12.05	11.23	18.48	1.52	1.53	1.65
2	12.22	12.06	11.32	12.17	1.00	1.01	1.08
4	12.77	12.77	11.83	49.97	3.91	3.91	4.22
7	6.46	6.05	5.99	11.31	1.75	1.87	1.89
10	1.55	1.51	1.43	29.23	18.90	19.34	20.40
21	0.80	0.72	0.75	8.20	10.19	11.40	10.99
44	0.26	0.25	0.24	reject	N/A	N/A	N/A
61	0.35	0.34	0.33	5.83	16.57	17.09	17.89

BCFs and Measured Water and Fish Concentrations of **DBP** over time

Time (days)	$C_{w\ Total}$ (ng/mL)	$C_{w\ C18}$ (ng/mL)	$C_{w\ dissolved}$ (ng/mL)	C_{fish} wet wt (ng/g)	BCF $C_{fish}/C_{w\ total}$	BCF $C_{fish}/C_{w\ C18}$	BCF $C_{fish}/C_{w\ dissolved}$
				Tank 1			
0	2.03	1.10	0.27	32.82	16	30	120
2	1.00	0.54	0.13	reject	N/A	N/A	N/A
4	0.98	0.60	0.13	134.45	138	225	1027
7	0.67	0.37	0.09	76.45	114	209	852
10	0.99	0.56	0.13	225.98	227	405	1694
21	0.33	0.17	0.04	79.15	243	463	1815
44	3.41	3.00	0.46	66.36	19	22	145
61	1.21	0.63	0.16	56.37	46	90	346
				Tank 2			
0	1.09	0.90	0.15	32.82	30.15	36.36	225
2	1.03	0.67	0.14	reject	N/A	N/A	N/A
4	0.61	0.43	0.08	134.45	221.34	311.90	1651
7	0.45	0.30	0.06	76.45	168.75	253.81	1259
10	0.75	0.40	0.10	225.98	299.78	561.92	2236
21	0.94	0.50	0.13	79.15	84.23	157.89	628
44	0.90	0.54	0.12	66.36	74.02	122.75	552
61	reject	reject	reject	56.37	N/A	N/A	N/A
				Tank 3			
0	1.50	0.95	0.20	150.77	100.39	159.40	748.68
2	0.47	0.04	0.06	89.22	190.35	2313.77	1419.62
4	0.45	0.45	0.06	202.78	447.92	447.92	3340.59
7	1.00	0.58	0.13	99.72	99.53	172.49	742.31
10	reject	reject	reject	153.26	N/A	N/A	N/A
21	0.46	0.28	0.06	65.74	N/A	N/A	N/A
44	reject	reject	reject	41.91	N/A	N/A	N/A
61	reject	reject	reject	191.49	N/A	N/A	N/A

BCFs and Measured Water and Fish Concentrations of **BBP** over time

Time (days)	$C_{w\ Total}$ (ng/mL)	$C_{w\ C18}$ (ng/mL)	$C_{w\ dissolved}$ (ng/mL)	C_{fish} wet wt (ng/g)	BCF $C_{fish}/C_{w\ total}$	BCF $C_{fish}/C_{w\ C18}$	BCF $C_{fish}/C_{w\ dissolved}$
				Tank 1			
0	0.12	0.09	0.01	reject	N/A	N/A	N/A
2	0.07	0.05	0.01	reject	N/A	N/A	N/A
4	0.21	0.04	0.02	13.38	65.18	320	814
7	0.06	0.02	0.01	13.37	207.16	543	2586
10	0.03	0.01	0.00	21.34	833.37	1528	10400
21	reject	reject	reject	reject	N/A	N/A	N/A
44	0.10	0.10	0.01	reject	N/A	N/A	N/A
61	0.03	0.01	0.00	reject	N/A	N/A	N/A
				Tank 2			
0	0.099	0.080	0.008	14.03	142.36	175.11	1777.29
2	0.081	0.063	0.007	reject	N/A	N/A	N/A
4	0.041	0.030	0.003	41.43	1009.21	1399.98	12599.17
7	0.112	0.104	0.009	reject	N/A	N/A	N/A
10	0.029	0.014	0.002	26.31	896.97	1635.76	11197.90
21	0.025	0.012	0.002	14.88	599.74	1227.45	7487.24
44	reject	reject	reject	reject	N/A	N/A	N/A
61	0.020	0.004	0.002	reject	N/A	N/A	N/A
				Tank 3			
0	0.15	0.10	0.01	11.32	74.12	108.08	925.33
2	3.27	3.24	0.26	reject	N/A	N/A	N/A
4	0.11	0.11	0.01	20.98	187.13	187.13	2336.20
7	0.02	0.01	0.00	77.70	3967.62	6565.26	49532.31
10	0.03	0.02	0.00	26.04	1023.56	1093.94	12778.32
21	reject	reject	reject	12.33	N/A	N/A	N/A
44	reject	reject	reject	reject	N/A	N/A	N/A
61	reject	reject	reject	51.45	N/A	N/A	N/A

BCFs and Measured Water and Fish Concentrations of **DEHP** over time

Time (days)	$C_{w\ Total}$ (ng/mL)	$C_{w\ C18}$ (ng/mL)	$C_{w\ dissolved}$ (ng/mL)	C_{fish} wet wt (ng/g)	BCF $C_{fish}/C_{w\ total}$	BCF $C_{fish}/C_{w\ C18}$	BCF $C_{fish}/C_{w\ dissolved}$
			Tank 1				
0	9.5780	1.4308	0.0008	10.71	1.12	7.48	13759.22
2	71.2903	0.3451	0.0058	reject	N/A	N/A	N/A
4	5.3834	0.2180	0.0004	43.47	8.07	199.40	99365.20
7	6.0288	0.7675	0.0005	34.58	5.74	45.05	70579.60
10	14.5556	0.8257	0.0012	88.97	6.11	107.75	75225.14
21	16.0129	0.2565	0.0013	42.80	2.67	166.85	32896.66
44	8.3839	0.5756	0.0007	22.68	2.70	39.40	33285.15
61	11.5247	0.1724	0.0009	22.24	1.93	129.04	23749.60
			Tank 2				
0	7.3287	0.1477	0.0006	9.40	1.28	63.65	15784.82
2	reject	reject	reject	13.96	N/A	N/A	N/A
4	1.9306	0.0351	0.0002	64.41	33.36	1834.78	410564.38
7	9.4077	0.2174	0.0008	52.00	5.53	239.14	68022.27
10	4.1984	0.0921	0.0003	39.91	9.51	433.44	116969.54
21	2.6530	0.0582	0.0002	44.57	16.80	765.95	206738.13
44	6.7738	0.1139	0.0006	19.19	2.83	168.53	34867.27
61	6.7066	0.1022	0.0005	45.74	6.82	447.41	83939.89
			Tank 3				
0	20.89	0.63	0.0017	47.43	2.27	75.20	27941.84
2	75.02	0.01	0.0061	40.00	0.53	7036.52	6562.71
4	2.92	2.92	0.0002	55.35	18.93	18.93	232959.69
7	1.42	0.06	0.0001	64.27	45.29	1140.84	557398.70
10	0.58	0.16	0.0000	70.22	120.62	431.22	1484465.39
21	10.20	0.11	0.0008	59.01	5.78	522.24	71175.98
44	0.90	0.13	0.0001	reject	N/A	N/A	N/A
61	8.68	1.28	0.0007	28.00	3.22	21.82	39683.79