GASTRO-INTESTINAL MAGNIFICATION AND DIETARY BIOAVAILABILITY OF CHLORINATED ORGANIC CONTAMINANTS/XENOBIOTICS: IMPLICATIONS FOR BIOMAGNIFICATION

by

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Abstract

In this thesis, two studies were performed. The purpose of the first was to determine if the results of a dietary exposure experiment using rainbow trout were consistent with those predicted using a thermodynamically based biomagnification model. To do this, adult rainbow trout were exposed to 2,2',4,4',6,6'-hexachlorobiphenyl (HPCB) through their diet for a period of 72 days. At regular intervals during the experiment, fish were euthanized and analysis was done on fish tissue and chyme (digested food) from several sections of the gastro-intestinal tract. Analysis was done for HPCB fugacity, HPCB concentration, lipid content, organic carbon content, chromium-III-oxide (an indigestible internal marker) content and sample mass.

Experimental results were consistent with a thermodynamically based biomagnification model. Data showed a 1.8-fold increase in HPCB fugacity in the anterior section of the intestine which occurred as a result of a 4-fold decrease of the chyme HPCB fugacity capacity. The decrease in fugacity capacity occurred concurrently with a 6-fold decrease in lipid content, suggesting that although lipid content is the major determinant of gut-content fugacity capacity, it is not the only determinant. This study suggests that undigested, non-lipid organic carbon (OC) also contributes to the overall fugacity capacity of the
chyme and presents a model describing the relative contribution of lipid and non-lipid OC to the fugacity capacity.

The justification for pursuing the first study was to better understand the process of biomagnification so that improvements could be made to existing chemical mass transfer models used in proactive environmental management.

The second study tested ethylene vinyl acetate (VA) films as a potential technical improvement for measuring the fugacity of persistent organo-halides. VA films were found to be an improvement over head-space analysis for the measurement of semi-volatile fugacities. They were typically more sensitive and had better accuracy than head-space analysis. Uptake curves of PCBs and chlorobenzenes by VA from a spiked sample revealed that an equilibrium (complete partitioning) can be achieved in a couple of days. It was also shown that the time to equilibrium was a function of both the VA film thickness and the molecular weight of POC being absorbed.
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1. General Introduction

1.1 INTRODUCTION

Persistent organo chlorines (POCs) are a group of chemicals typically consisting of a large carbon backbone and having one or more bonding sites occupied by a chlorine molecule. By nature of their chemical structure, they have been linked to a number of environmentally damaging effects. POCs have been attributed to several forms of cancer (Bertazzi et al. 1993), abnormal sexual behavior (Hunt et al. 1977, Fox et al. 1978), poor reproductive success and embryological defects (Gilbertson 1983, Kubiak et al. 1989, Guillette et al. 1994), suppression of the immune system (Davis 1988, Aguilar et al. 1994) and a declining sperm count in humans (Auger et al. 1995). Despite their use being predominantly restricted or severely curtailed in North America since the late 1970s, global transport combined with the persistence of POCs has made them the most ubiquitous xenobiotics on earth. Biological concern has arisen as a result of their toxicity, ability to accumulate in biological organisms and resistance to metabolism (Tanabe et al. 1987, Woodwell 1967).

Common examples of POCs are dichlorodiphenyltrichloroethane (DDT), dioxins, polychlorinated biphenyls (PCBs) and chlorinated benzenes (Figure 1). They
have been used as heat stable additives for transformers and heavy machinery lubricant (e.g. PCBs), as pesticides (DDT, 2,4,5-T, Chlordane, Mirex), as anti-sap stain agents (chlorobenzenes), and have been produced as unwanted byproducts in the production of other industrial chemicals (dioxins and furans).

![Chemical structures]

**Figure 1:** Chemical structure of some common persistent organo-halides.

Accumulation of POCs by organisms, called bioaccumulation, occurs principally as a result of the high fat solubility (or lipophilicity) of these chemicals. Lipophilicity causes POCs to preferentially partition into compartments which either have a high fat or organic carbon content (Goerke 1984). The extent of lipid affinity is often expressed in terms of log $K_{ow}$, which is the log of the partition coefficient of a given xenobiotic between octanol and water (Isnard and Lambert 1988). $K_{ow}$ is determined by dividing a chemical's observed concentration in octanol by the concentration in an adjoining water
compartment when the net transfer of chemical between compartments is zero (equilibrium has been achieved) and where neither compartment is saturated.

Because many chemicals which partition into fat have very high $K_{\text{ow}}$'s, convention is to express these values in logarithmic format. PCBs, for example, have $K_{\text{ow}}$'s ranging from about $10^{4.45}$ (for 2-monochlorobiphenyl) to $10^{8.18}$ (for decachlorobiphenyl) (Hawker 1988).

There are two types of bioaccumulation, the first occurs as a result of uptake of chemical from water and sediment and is referred to as bioconcentration (Barron 1990). Bioconcentration occurs predominantly via the gills and skin of aquatic organisms. The second type of chemical accumulation occurs as a result of chemical uptake via the diet and is called biomagnification (Bruggeman 1981). Biomagnification is most noticeable within long food chains such as those found within many aquatic trophic systems (Gobas and Morrison 1997).

A fish residing within contaminated water will bioconcentrate xenobiotic via the gills and skin with time until a steady state of chemical concentration is achieved between the fish and the surrounding water (Figure 2) (Branson 1975). The ratio of fish tissue concentration to water concentration is called the bioconcentration factor, BCF. If regressed against log $K_{\text{ow}}$ for several PCB congeners, a relationship having a high $r^2$ value is often achieved (Figure 3) (Gobas 1989, Neely 1974, Bruggeman 1981, Konemann 1979). The linear
relationship suggests that chemical bioconcentration in fish is very similar to the diffusive partitioning of lipophilic chemical between water and octanol. Thus, it has been concluded that uptake of organic contaminants occurs via simple diffusion (Mackay 1979, Mackay 1982, Isnard and Lambert 1988). The concentration achieved in the each of the fish and water compartments at

\[ C_t = \text{Concentration of xenobiotic in fish body.} \]

\[ C_w = \text{water concentration of xenobiotic} \]

**Figure 2:** Illustrative Example of the Concentration of POCs in Fish Tissue (\(C_t\)) as a Function of Time as a Result of Being Exposed to POC Contaminated Water with Concentration \(C_w\) (Figure Adapted from Branson et al., 1975).

equilibrium depends upon the relative chemical absorbing capacity (dissolving ability) of both the fish and water as it does for the partitioning of chemical between octanol and water.
The significantly higher concentration of POCs achieved in the fish at equilibrium is expected as the solubility of POCs in water is known to be much lower than the solubility in the fish which contains significant amounts of lipid. The matrix property which determines how well a chemical dissolves in the matrix will be referred to as the "absorbing capacity" of the matrix or the "fugacity capacity" of the matrix (MacKay 1979). Fugacity capacity will be discussed further in future sections.
If an uptake curve is plotted depicting exposure of xenobiotics via the diet, an equilibrium is attained by which the predator fish has a concentration of xenobiotic greater (Connolly & Pedersen 1988) than that of the prey. If passive diffusion is the primary transporting mechanism, it would be expected that the predator fish would have a greater absorptive capacity than the prey and thus would have a higher body fat concentration. In most cases, however, the larger fish have comparable, if not lower body fat concentrations (Connolly & Pedersen 1988). The question which this thesis addresses is how could a predator attain a higher concentration than it’s prey if it doesn’t have a higher absorbing capacity for the POC? Or in simpler terms, what is the mechanism behind biomagnification? Despite the existence of several theories which will be discussed in chapter 2, the mechanism which results in biomagnification remains largely unknown.

A mechanistic understanding of biomagnification is important for xenobiotic management purposes. Modern environmental practices are becoming increasingly proactive and consequently employ greater use of mechanistic models to predict the biological outcome of chemical exposure scenarios. It was shown above that steady state body burdens due to bioconcentration can be easily predicted due to a clear relationship with $K_{ow}$ (Mackay 1979, Mackay 1982, Isnard and Lambert 1988). Dietary exposure, on the other hand, enjoys no such direct relationship. If dietary assimilation efficiency of POC (the %
absorbed from food) is plotted against log $K_{ow}$, the graph in Figure 4 is produced. The large variability (Gobas 1989) and lack of any well-defined trend indicates that other parameters must be considered in addition to $K_{ow}$ when modeling biomagnification; parameters such as dietary, environmental and species dependent factors (Parkerton 1993). Skin or gill uptake on the other hand, are predominantly a function of $K_{ow}$ and lipid content of an organism.

![Figure 4: POC uptake efficiency plotted as a function of $K_{ow}$. Symbols represent different measuring methodologies. (Figure taken with permission from Ph.D. dissertation of Tom Parkerton, 1993).](image)

A greater understanding of POC dietary exposure in particular is important because it is the primary exposure route for terrestrial animals and for aquatic
organisms when xenobiotics have an extremely high affinity for lipid (Russell 1995, Herbert 1996, Elkin 1995). An "extremely high affinity for lipid" is a feature of chemicals having a log K<sub>ow</sub> greater than 6, hence a million times greater than that for water (Russell 1995, Black 1988, Schrap 1990, Thomann 1989).

In ecosystems having long food chains, incremental increases in concentration between prey organisms and predator organisms (biomagnification) can result in the top predator having POC body concentrations many times greater than those of the organisms at the bottom of the food chain (Norstrom et al. 1978, Connolly & Pederson 1988, Clark et al. 1988). The increase in POC body burden concentration observed ascending a food chain is referred to as trophic amplification and is typically largest in aquatic environments where food chains tend to be very long (Norstrom et al. 1978).

The focus of this thesis is to use fish in order to develop and test a model of POC accumulation resulting from dietary exposure. This thesis consists of three chapters, each being subdivided into sections. Chapter 1 provides the reader with background knowledge pertaining to biomagnification. The first section of chapter 1 introduces the concept of fugacity and fugacity capacity, and discusses their relevance to passive diffusion. Section 2 addresses the mechanism resulting in triglyceride absorption and argues that factors which have an impact on efficient triglyceride absorption may also have an impact on
the degree of xenobiotic absorption. Section 3 talks about the existing knowledge surrounding the topic of gastrointestinal absorption of POCs. Section 4 defines the three major terms that are important in understanding methodologies for fugacity measurement.

Chapter 2 introduces an experiment in which rainbow trout were dosed with hexachlorobiphenyl through their diets and where HPCB concentrations, fugacities, lipid content, food mass and chromic oxide levels were monitored. An argument is made that experimental results show that biomagnification occurs as a result of changes occurring within food as it is being digested. Implications of the digestion model are discussed.

Chapter 3 presents a novel method which is an improvement over conventional methods for fugacity measurement. Existing methods are discussed and desirable attributes listed.

1.2 THEORETICAL CONSIDERATIONS FOR DIFFUSIVE MOVEMENT OF ORGANIC COMPOUNDS

Passive diffusion of chemical mass within and between phases is usually described by using the thermodynamic concept of chemical potential (Mackay 1991). It is possible to use chemical potential to model the diffusive transfer of
chemical. However, it is cumbersome because it is directly proportional to the log of concentration. An alternative way to express chemical potential is the use of fugacity, which is equivalent to the partial pressure of a chemical dissolved in a phase and thus is measured in units of pressure (Mackay 1991). Fugacity is linearly related to concentration and thus simplifies diffusion calculations.

Fugacity is an important concept to the environmental sciences as it gives an investigator the ability to predict the environmental fate of a POC after a dosing event (Mackay 1991). On a toxicological basis, fugacity enables the scientist to model the extent to which a xenobiotic can be absorbed by an organism residing within or in contact with a contaminated matrix.

To understand fugacity on a conceptual basis, consider a glass of water open to the air (Figure 5). Water molecules will continually diffuse from the surface of the water to the ambient air until all the water has evaporated. The reason for the constant and continual flux of water is the existence of a fugacity gradient. Unless the ambient air has a 100% relative humidity, the fugacity of water molecules in the water will always be larger than the fugacity water molecules in the ambient air, thus producing a fugacity gradient favoring the evaporation of water. Now consider putting an air-tight cap on the glass of water. At first, diffusion will occur from the water surface to the air in the glass with the same flux as the previous
example, but this net flux will soon decrease to the point where there is no longer a net evaporation of water. What is happening is that the air volume is no longer essentially infinite and thus the concentration of water molecules has a chance to increase in the air phase. The concentration of water molecules in the air increases to the point where no further increase of water vapor in the air is observed. Equilibrium is said to have been achieved which means that the flux of water molecules leaving the water for the air is equal to the flux of water molecules leaving the air for the water. Thus there is no net flux. Determining the magnitude of flux from each of the water and air phase is the fugacity of water in each of the two compartments. If equilibrium is achieved, the fugacities of water in each compartment are equal and thus, there is no net transfer of water molecules. Although fugacity is proportional to concentration, it is important not to think of diffusion occurring as a result of a concentration gradient. The concentration gradient is the sole determinant of diffusion only within a single matrix type. In the environment, it is common to have diffusion between compartments with vastly different physical properties.

Consider the uptake of dissolved O₂ from water through the gill of a fish: The concentration of O₂ in the blood circulating through gill capillaries is always larger than those in the ambient water, yet there is still diffusive uptake across the gill endothelium; thus it appears that diffusion is occurring against a concentration gradient! Physiologists would explain that the partial pressure of
$f_{vapour} = \text{fugacity of water in the air compartment.}$

$f_{liquid} = \text{fugacity of water in the liquid compartment.}$

$G$

FIGURE 5: FUGACITY EXPLAINED CONCEPTUALLY
O\textsubscript{2} in water exceeds that of the blood and that it is the difference in partial pressure which results in diffusion. The difference in partial pressure comes about as a result of a difference in O\textsubscript{2} absorbing capacity; blood having hemoglobin tends to absorb O\textsubscript{2} to a much greater extent than does water. The preceding example illustrates that diffusion is caused by a partial pressure gradient which is a function of both the concentration in the two matrices and the ability of each matrix to absorb the diffusing chemical. In thermodynamic terms, the partial pressure and absorbing capacity above would be referred to as fugacity (\(f\)) and fugacity capacity (\(Z\)), respectively.

When a fish is placed in an aqueous environment containing a fat soluble xenobiotic, equilibrium partitioning of the chemical occurs principally between the water and the lipid of the organism. Consider an example of equal concentrations of PCB in adjacent water and oil compartments (Figure 6). The concentration gradient between the two compartments would be zero, yet there would be a very strong fugacity gradient favoring the diffusion of PCB from the water and into the oil. Thus, fugacity (\(f\)) is a function of both concentration (C) and an other term, fugacity capacity (\(Z\))

\[ f = \frac{c}{Z} \]

"\(Z\)" can be thought of as the ability of a compartment to "store" a chemical.
Although the terms fugacity and fugacity capacity may be unfamiliar, most scientists already understand the principles involved and their importance in determining chemical partitioning between two compartments. Take for instance, the pharmaco- toxicokinetic partitioning of a fat soluble chemical between two tissues. Realizing that lipid dissolves lipophilic chemicals to a much greater extent than other biological molecule groups, lipid tends to determine how well a tissue is able to "hold on" to a lipophilic chemical. Consequently, many papers have expressed the chemical concentration in each tissue as mass of contaminant per mass of lipid found each tissue. These concentrations are said to be lipid-normalized and are directly proportional to the tissue chemical fugacity. Therefore, two different adjacent tissues having different contaminant...
concentrations based on total tissue mass and different lipid contents will realize no net diffusion of chemical between them if, and only if, the lipid-normalized concentrations in each of the tissues is equal:

Fugacity capacity plays a pivotal role in determining the concentration of contaminants in different environmental compartments once a contaminant is emitted into the environment (Mackay 1991). If a fish is residing within polluted water, chemical will be absorbed by the fish and will continue to be absorbed until a steady state has been achieved. Steady state occurs when influx equals the out-flux and the concentration of contaminant in an organism remains constant. Unlike equilibrium, however, a fugacity gradient will still exist. Instead of having diffusive out-flux as the sole chemical loss process, steady state entails chemical dilution due to growth, metabolic losses of chemical and/or energy dependent removal of chemical (Mackay 1991).

At steady state, the fish contaminant concentration \( C_f \) divided by the water contaminant concentration \( C_w \) is defined as the BCF (bioconcentration factor).

\[
BCF = \frac{C_f}{C_w}
\]

If the above concentrations are expressed in terms of fugacity and fugacity capacity the above formula becomes:

\[
BCF = \frac{f_fZ_f}{f_wZ_w}
\]
where:

\( f_F \) is the contaminant fugacity of the fish.

\( f_W \) is the contaminant fugacity of the water.

\( Z_F \) is the fugacity capacity of the fish.

\( Z_W \) is the fugacity capacity of the water.

It can be seen in formula 1-3 that the fugacity capacities of both matrices have an important role in determining the ratio of concentrations in the fish and the water in which it resides.

In cases where metabolism and active transport processes are absent, as is the case with POCs, and if growth is negligible, this steady state will also represent a state of equilibrium. Equilibrium exists only when adjacent compartments have equal fugacities and thus will cancel out in the formula above.

Thus:

\[
BCF_{\text{equilibrium}} = \frac{Z_F}{Z_W} \tag{1-4}
\]

Thus, at equilibrium, the greater concentration of POC achieved within fish tissue compared with water is due solely to the greater fugacity capacity of the fish tissue.

For simple hydrophobic organic chemicals that are not metabolized and which have log \( K_{ow} \) 's less than 8, BCF is linearly related to \( K_{ow} \), indicating that
Diffusive partitioning is occurring between the fish and water (Geyer 1995, Goerke 1979, Earnest 1971, Keck 1979).

Similarly, BMF, the biomagnification factor, is the ratio at steady state of the concentration of a xenobiotic in an organism to the concentration in its diet. In thermodynamic terms this ratio can be expressed as follows:

\[ \text{BMF} = \frac{Z_f}{Z_d} \]

Where the subscript "d" represents diet. Because triglycerides contribute essentially all the POC absorbing capacity of biological tissue (Paterson & Mackay 1987), \( Z_f \) and \( Z_d \) can be approximated by measuring the lipid content of the fish and its diet respectively. If the fish has either a similar or a smaller lipid concentration than its diet, as is often the case (Henderson & Tolcher 1987, Tarr et al. 1990), it would be expected that fugacities in the fish must be higher than those in the diet in order to have BMFs greater than one. It appears, then, that absorption is occurring against a fugacity gradient. Potentially this finding could be used as a powerful argument against absorption of POC via passive diffusion.
1.3 POC UPTAKE VIA THE DIET

1.3.1 AN OVERVIEW

Ingested POCs will predominantly be dissolved in the lipid fraction of food and are thought to follow lipids and lipolysis products into micelles. The micelles shuttle the POCs across the polar, unstirred layer of the intestinal lumen to the brush border where they are absorbed. Once inside the enterocyte POCs either partition into "forming" chylomicrons, or diffuse independently through the lamella propria to blood and lymph vessels (Gobas 1993, Vetter et al. 1985).

With any epithelial layer there are two ways by which a chemical can pass, either by going through epithelial cells (transcellular movement) or by passing between two epithelial cells (paracellular movement). For a chemical to take a paracellular pathway, it must be small enough to pass through the tight junctions joining cells. Molecules that utilize this pathway are small water soluble molecules and ions.

Transcellular requires the movement of a substance across cellular membranes. There are essentially 2 ways which this can happen, through either a non-energy requiring process (diffusion or facilitated diffusion), or by an energy requiring process (active transport or pinocytosis). In section 2, these
mechanisms will be discussed in the context of biomagnification. What follows here is a brief overview of the topic.

Membrane diffusion is the process by which a molecule is able to squeeze between several membrane phospholipids and temporarily dissolve within the non-polar region of the membrane before emerging from the other side of the membrane. No energy is expended in this form of molecular transport through membranes and molecular movement is always in the direction of a chemical potential gradient. In order for a molecule to diffuse through a membrane, it must be sufficiently small and be relatively non-polar, such that it can displace phospholipids and dissolve in the non-polar middle region of the phospholipid bilayer. Illustrating the importance of molecular size, the rates of diffusion of non-polar molecules are proportional to their molecular weights.

Facilitated diffusion permits the passage of molecules which are either too large or too polar for passive diffusion to move across a cellular membrane in the direction of a chemical potential gradient. The process usually involves a protein imbedded into the cellular membrane which specifically binds to the diffusing chemical. The process may also be regulated.

Active transport processes are mediated by large protein complexes imbedded within membranes, which bind specifically to a molecule and then use energy to
move the molecule across a cellular membrane against a chemical potential gradient.

Due to their small molecular size and high lipophilicity, most POCs can easily diffuse through cellular membranes. Consequently an active transport process specific to them would be redundant and thus be highly unlikely (Gobas 1993).

Pinocytosis involves the physical capturing by the membrane of extra- (or intra-) cellular fluid into a vacuole. Like active transport it requires energy, but is obviously less specific about what is being transported. Like active transport it can also be considered redundant as a POC transport mechanism due to the high membrane permeability of POCs.

If diffusion is the predominant mechanism of absorption via the enterocytes, then dietary uptake efficiency would logically be a function of absorption rate and chyme contact time to absorbing surfaces. Fick's law states that for a given chemical, the absorption rate is determined by three parameters; the size of chemical potential gradient (restricted to a concentration gradient when dealing with a single fugacity capacity), resistance to molecular diffusion and diffusive area (Chang 1981, Berne 1988). Resistance to molecular diffusion is a function of the molecular weight of the diffusing molecule, the absolute temperature and the viscosity of the matrix being diffused through (Berne 1988, Chang 1981).
1.3.2 Physiochemical Factors Affecting Absorption Efficiency

Several authors have recorded decreasing dietary absorption efficiencies with increasing $K_{ow}$'s of xenobiotics with log $K_{ow}$'s greater than 3. (Houston 1974, Parkerton 1993). The prevailing theory is that the uptake efficiency is not an issue of lipid solubility, but rather one of molecular size (Niimi, 1988). For PCBs, chlorobenzenes, dioxins and furans, an increase in molecular weight is the result of another chlorine atom replacing a hydrogen.

Research has shown that the diffusion rate of lipophilic xenobiotics through cellular membranes decreases with increasing molecular weight (MW) (Leatherland & Sonstegard 1982, Hilton 1983). This observation appears to be consistent with Stokes law which states that the rate of diffusion is inversely proportional to the molecular radius; the molecular radius being roughly proportional to the cube root of molecular weight (Berne and Levy 1988). Some authors believe that the relationship between diffusion rate and MW is even more pronounced in the intestine, as the intestinal micro-villar membrane is one of the most viscous, highest melting point membranes in vertebrates. Larger molecules likely have to displace (or push aside) phospholipid molecules to a greater degree (Houston 1973, Brasitus 1980).
Gobas (1989) presented evidence to suggest that membrane permeability is not the rate limiting process in the diffusive uptake of super lipophilic substances ($K_{ow} > 6$) across cellular membranes. He found that chemical permeability actually increased with increasing $K_{ow}$ and suggested that the extremely low water solubility of lipophilic substances in the aqueous medium on either side of the cellular membrane is what limits cellular uptake of this group of chemicals. The higher the $K_{ow}$ of the POC, the less water soluble it is.

1.3.3 Physiological Factors Affecting Absorption Rate

Physiological differences between organisms and within individual organisms over a period of time may have profound effects on dietary absorption efficiency. Parkerton (1993) calculated the absorption efficiencies from the experiments of Leatherland & Sonstegard (1982) and Hilton et al. (1983) in which rainbow trout and coho salmon were fed identical diets of contaminated coho salmon carcasses. He found that trout had a higher assimilation efficiency than coho. At this point it is not known what combination of factors to attribute the differences in uptake efficiency. However, it is likely that differences in at least one of chemical potential gradient, membrane resistance, gastro-intestinal transit time or intestinal surface area play an important role.
1.3.4 DIETARY FACTORS AFFECTING PCB ABSORPTION RATES

The type of diet has been found to have profound effects on the extent of POC absorption efficiency. Fisher et al. (1983) fed blue crabs diets of both keptone-contaminated fish and oyster and found that the assimilation efficiency of keptone was higher for the fish diet. The fish-fed crabs also had a growth rate nearly four-times that of those crabs fed the oyster diet. This indicates that the extent to which a diet is digested/absorbed is an important factor in determining the extent of POC assimilation.

1.3.4.1 Level of Lipid on absorption efficiency of contaminant

Research has shown that when lipophilic contaminants are administered along with a digestible oil, the absorption efficiency of the contaminant increases. (Vetter 1985). A consequence of orally introducing a chemical without a digestible oil is the absence of intestinal lipid metabolites, that are required to produce functioning liposomes and micelles. Micelles and liposomes increase the water solubility of lipid metabolites and consequently increase the concentration of lipid metabolites diffusing through the unstirred aqueous layer of the intestinal brush boarder (Dietschy 1971, Westergaard 1976, Wilson 1971). It has been theorized that liposome and micelle transport also assists the flux of lipophilic chemical contaminants across the unstirred layer (Vetter 1985). Thus, if xenobiotics have not partitioned into micelles, movement across the
aqueous unstimred layer of xenobiotic may become significantly retarded and transit time of the intestine may not permit an adequate time for diffusive mass transfer to the brush border.

Vetter's (1985) observations appear to be stating that lipid soluble xenobiotic absorption should increase with increasing dietary lipid concentration. Unfortunately, such a direct relationship does not exist. Gobas et. al (1992) revealed that uptake efficiency of POC with log \( K_{ow} \)'s greater than 6.3 are greater in food with low lipid (%) concentrations than those with high (%) concentrations. The dependence on lipid content appears to be due to the low lipid food having a lower POC fugacity capacity and thus higher fugacity even after digestion (Gobas 1993). The higher fugacity, as mentioned in previous sections, increases the fugacity gradient and results in both a larger chemical flux.

By considering the works of both Vetter and Gobas, it is possible to conclude that an optimal absorption of lipid soluble xenobiotics occurs at a low dietary lipid concentration. Below the optimal concentration micelle structure is limiting, above the optimal concentration, the fugacity gradient is limiting.

1.3.4.2 Other Food Components

If the high fiber content of an experimental food exceeds the digestive capacity of an organism (e.g., plant material fed to an carnivore), the intestinal motility
rate will increase and lipids may not be completely digested (Meienberger 1993, Granger 1985). Poor digestion of lipid can contribute to a poor contaminant fugacity gradient being formed.

The type of protein found in a diet may also have some effect on the POC absorption efficiency. Some blood proteins, for example have shown various degrees of affinity for lipophilic chemical contaminants (Vetter et al. 1985, Streit & Sire 1993, Borlakoglu et al. 1990).
2. Biomagnification: the underlying mechanism

2.1 Abstract

Hexachlorobenzene (HPCB) was administered to adult rainbow trout through the diet for a period of 73 days. Over the duration of the experiment, intestinal content (chyme) at various locations in the digestive tract were analyzed for fugacity, concentration and total mass. Various fish tissues were analyzed for the concentration of HPCB. Analysis of results showed a 1.8-fold increase in HPCB fugacity in the anterior section of the intestine which occurred as a result of a 4-fold decrease of the fugacity capacity of the diet resulting from food absorption and digestion. The decrease in fugacity capacity occurred concurrently with a 6.6-fold decrease in lipid content, suggesting that although lipid content is the major determinant of gut content fugacity capacity, it is not the only determinant. This study suggests that undigested, non-lipid organic carbon (OC) also contributes to the overall fugacity capacity of the chyme. A model is presented describing the relative contribution of lipid and non-lipid OC to fugacity capacity.
2.2 INTRODUCTION

Little work has been done addressing how biomagnification can occur and specifically how a predator can achieve a concentration of contaminant greater than that in its diet. One theory, proposed by Woodwell et al. (1967), stated that biomagnification occurs as a result of a reduction of biomass with increasing trophic level. In other words, organisms absorb 100% of their diet; some of the diet is metabolized, some is used for tissue maintenance, while the remainder including the xenobiotic if not eliminated, concentrates with time. Thus, the dietary lipid matrix will be metabolized once within the organism, but contaminants, unmetabolized and not excreted, will accumulate. A flaw in this theory is that it does not suggest how lipophilic contaminants can be absorbed from the gut lumen against a lipid normalized concentration gradient, i.e., their chemical potential gradient. The significance of a lipid normalized concentration gradient was discussed previously.

Biomagnification has been accounted for by active transport of highly lipophilic chemicals. POCs are believed to be coassimilated along with lipids and fatty acids within vesicles produced in the brush border (Roth and Wilson 1993). The active transport theory does not address the fact that the same lipophilic chemicals diffuse easily through cellular membranes, following the direction of lower chemical potential. Back diffusion would therefore circumvent biomagnification via active transport (Houston et al. 1973). A possible exception
to this would be high molecular weight compounds that do not move easily through biomembranes. However, most of the xenobiotics that bioaccumulate in the environment do not fall into this category.

Another hypothesis, originating from experiments in guppies and goldfish (Gobas 1993), the analysis of field data (Clark et al. 1988) and theoretical work (Gobas 1989), suggests that gastro-intestinal digestion and food absorption in the GIT could work together to raise the thermodynamic activity of contaminants in the GIT. This theory, called the thermodynamically based theory in this thesis, was used as the basis of a mechanistic model presented by Gobas et al. (1993). In his model, Gobas used fugacity measurements and the presence of a fugacity gradient to explain how the GIT tract creates an environment favoring the biomagnification of POCs.

All chemicals are known to diffuse from an area of high chemical fugacity to an area with lower fugacity. Fugacity, as with partial pressure, represents the chemical potential of a contaminant, or in simple terms, the desire of a chemical to leave a compartment. The fugacity \( f \) of a chemical is related to its concentration in a given compartment \( C \) through the following formula:

\[
f = \frac{C}{Z}
\]

where \( Z \) is the ability of a compartment to hold onto or absorb a given chemical, the fugacity capacity.
The thermodynamically based theory (Connolly et al. 1988, Gobas 1993) centers around the suggestion that digestion and absorption of food in the alimentary tract progressively lowers the fugacity capacity. This, in turn, results in a higher contaminant fugacity of the gut content (chyme) relative to the ingested food (formula 2-1). The elevation of fugacity resulting from the effective increase in chyme POC concentration and drop in fugacity capacity have the effect of driving the uptake of contaminant to a level higher than food. The increase of contaminant fugacity within the GIT has been coined gastrointestinal magnification by Gobas (1993).

Previous work in the area of fugacity-driven intestinal diffusion of lipophilic contaminants was done with guppies and goldfish dosed with a mixture of chlorobenzenes and polychlorinatedbiphenyls. Analysis consisting of fugacity and concentration determination of food, fecal matter and fish tissue showed that the fugacity of PCBs were greater in the fecal matter than in the fish food, while the fugacity capacity in the fecal matter was 4-to 5-times smaller than that for the food (Gobas 1991,1993). The conclusion drawn was that digestion and absorption of food acts as a "fugacity pump", raising the PCB fugacity in the intestine above that of the consumed food. The elevated fugacity which was found to be due to an effective increase in sample PCB concentration and
decrease in fugacity, ultimately results in biomagnification and food chain amplification.

By using small fish Gobas (1993, 1993a) was able to work with a large number of individual organisms thus enhancing the statistical significance of the results. However, only fecal matter and not the gut contents could be analyzed for fugacity and concentration. The drawback of collecting fecal matter from water is the concern of analyte loss to the much greater volume of water. To date no one has tested the thermodynamically based theory by analyzing contaminated food in different stages of digestion in fish.

The objectives of this research were three-fold. First, to determine if the fugacity of the contaminant increased as food was digested, as predicted by the thermodynamically based theory. Second, to determine where in the GIT this increase in fugacity occurred. Third, to determine the contribution of food digestion and food absorption to the increase in fugacity.

For this experiment, rainbow trout was chosen because of the availability of larger individual fish and the very extensive physiological data base available for this species.
The hypothesis presented in this chapter is that digestion of food results in a chemical potential gradient favoring the passive diffusion of chemicals into an organism such that the lipid normalized POC concentrations in an organism exceed those of its diet (ergo biomagnification).

The above hypothesis was tested by determining the fugacity \( (f) \) and fugacity capacity \( (Z) \) of food in various stages of digestion. If \( Z \) decreased and \( f \) increased during digestion, this would agree with my hypothesis. The decreasing \( Z \) indicates that digestion is decreasing the ability of the food to hold onto the POC while the increasing \( f \) indicates that, if diffusion were occurring, the lipid normalized concentration achieved in the GIT of the organism would be greater than that in its food. Hence, the organism would be able to achieve a concentration that is greater than that in its diet due to simple diffusion controlled uptake in the GIT.

Our hypothesis would be disproved if no change in \( f \) in the GIT was apparent. If \( f \) of the food was the same before and after digestion then a diffusion gradient could not exist and an organism could not attain a lipid normalized POC concentration greater than its food via passive diffusion.
2.3 Methodology

Fish:
Forty rainbow trout (Oncorhynchus mykiss) from West Creek trout farm (Langley, BC, Canada) (weighing 439 g ± 64 g; lipid content: 8% ± 2%), were placed in a flow-through aquarium one month prior to start of experiment. The flow-through aquarium consisted of a 3,000 L tank (divided in two compartments with plastic mesh), an inflow of fresh, uncontaminated water (4 L/min.), a fecal collector and a water filter consisting of fine mesh, filtering wool and activated carbon. The water had a temperature of 7°C, and a pH of 6.1 to 6.3. The tank was partitioned into two equal volumes such that twenty control fish were to be exposed to the same water as the experimental fish.

Exposure:
Ten grams chromium (II) oxide (Sigma Chemical Co.) and 900 mg 2,2',4,4',6,6'-hexachlorobiphenyl (HPCB) (Accustandard, New Haven, CT, USA) were dissolved in 100 mL of 30-60 bp petroleum ether and then slowly mixed (using an Osterizer Kitchen Center) into 1 kg of ground Silver Cup trout food for a period of 2 hours during which solvent was allowed to evaporate. Water was then mixed in until the food began to take on a sticky consistency. The mixture was then placed in a solvent-rinsed meat grinder and extruded through a die with 2 mm diameter holes. The resulting pellets were then spread out and dried.
in a fume hood overnight. The food for the control fish was prepared in a similar manner but without HPCB.

Experimental and control fish were individually fed an average rate of 4 g of food /fish between 8:30 and 9:30 am every second day. The pelleted food was dispensed onto the surface of the tank water and all fish appeared to participate in feeding. Half an hour after feeding, excess food was removed via suction, sorted, dried and analyzed for mass. The feeding rate was determined as the mass of food dispensed minus the mass of food removed from the tank divided by the number of fish in the tank. The actual feeding rate was 3.45 g/fish every second day ± 0.71 (SD). On days 0, 1, 3, 6, 10, 18, 29, 43 and 73, always 30 hours after feeding, two experimental and two control fish were euthanized via a blow to the head. Within 10 minutes of killing, the fishes GIT were removed and divided into the stomach and four intestinal sections. These sections were approximately 5 cm in length and referred to as "anterior", "anterio-central", "posterio-central" and "posterior" progressing towards the anal vent of the fish (Figure 7). The contents from each of these sections were then removed for analysis. The remaining GIT was gently rinsed with distilled water and then, as much intra-peritoneal lipid was removed (scraped or cut off) as possible. The GIT was then with the rest of the body as part of the whole body analysis.
Thirty hours after feeding was chosen as a sampling time as it was found to be the time elapsed between feeding and initial egestion of the same food. Using this time ensured that all gastrointestinal compartments would have contents for analysis. Initially fish were fed un-altered commercial fish food and then were switched to a diet containing the internal marker, Cr₂O₃. Fecal Samples were then taken from the bottom of the tank at regular intervals until all pellets collected contained Cr₂O₃.

**FIGURE 7: DEPICTION OF GIT SHOWING REGIONS SAMPLED**

The contents of the stomach and intestinal sections were analyzed for (i) concentration of HPCB, (ii) the fugacity of HPCB, (iii) lipid content, (iv) organic carbon content, and (v) chromic oxide concentration. The intra-peritoneal fat,
liver and remaining fish body were analyzed for all the above except chromic oxide concentration. Those fish and chyme samples not immediately analyzed were frozen for future analysis.

Fugacity Analysis:

The fugacity analysis procedure was adapted from the methodology used by Gobas et al. (1993, 1993a). Two 0.5 mL portions of gut content were transferred into 2 mL glass vials. Two drops of 1 M mercuric chloride were added to each vial in order to prevent microbial growth during sample equilibration. In order to prevent oxidative damage of the gas chromatograph column during head-space analysis, atmospheric air was replaced by N₂ and the vial was capped and airtight sealed. Samples were allowed to equilibrate at room temperature for 30 days, after which 80 μl of N₂ was analyzed by gas-chromatography (GC). GC analysis was performed by a HP 5890 series II GC equipped with an on-column injection port. The column used was a HP-1 (Methyl Silicone Gum) Instrument Column, whose dimensions were 5 m x 0.53 mm x 2.65 μm film thickness and having a helium flow of 20 cm/s (at 35°C). The temperature program was 35°C to 270°C at 20°C/min and the concentrations of samples were determined by comparing areas to a regressed calibration curve created using standards prepared from HPCB crystals.
The measured gaseous concentration of HPCB were related to HPCB fugacities through the Ideal Gas Law, i.e., fugacity (Pa) equals the product of concentration (mol / m³), temperature (K) and the gas constant (2-2). After completion of the head space analysis, the vial contents were divided into two equal fractions for HPCB and chromic oxide analysis. The detection limit for HPCB head-space analysis was 0.02 pg in a 60 μl injection.

\[ f = P = nRT/N \]  

2-2

Lipid analysis:

Two grams of gastro-intestinal content was ground with sodium sulfate crystals and then transferred to a 0.02 m x 1 m column containing 18 g of sodium sulfate. The column was eluted with 150 mL petroleum-ether. The eluent was evaporated to dryness using a rotary evaporator (Yamato, RE47, Japan) and then placed in an oven overnight at 35⁰ C. The amount of lipid was then determined by weight.

No special precautions were taken to denature lipase activity as the net Gibbs free energy favoring the enzymatic conversion of triglycerides to metabolites is very small, indicating that any metabolite build up will quickly halt further catalysis (De Silva 1995).
HPCB concentration analysis of chyme (gut content):
The sample from the weigh boat was transferred to a mortar and completely homogenized using 5 g of granular anhydrous sodium sulfate. The homogenized sample was then transferred into a column containing, from bottom-to-top, glass wool, 1 g granular sodium sulfate, 12 g acidified-60 mesh silica gel and 18 g sodium sulfate powder. The column was eluted with 250 mL petroleum ether over a 6 h period with recoveries greater than 90%, determined by elution of spiked samples which had been left for more than 24 h to equilibrate. The eluent was diluted 10x before gas chromatogram (GC) analysis. The detection limit for HPCB concentration analysis was 0.02 pg in a 1 μg injection.

Chromic Oxide analysis:
The sample was air-dried for two days, then weighed, ashed and digested following Fenton and Fenton (1979). The resulting samples were made up to 5 mL and spectrophotometrically analyzed at 440 nm (Perkin-Elmer uv/vis absorbance spectrometer).

Tissue Analysis:
Tissue analysis was conducted on the liver, intra-peritoneal fat and whole fish (minus the liver and intra-peritoneal fat). Each fish was ground using a hand powered meat grinder and the resulting ground tissue was further homogenized by placing it in a 500 ml beaker and vigorously mixing with a spatula. From this
homogenate, 10 g was ground in 20 g anhydrous Na₂SO₄ and then placed in 0.02 m x 1 m column containing 5 g anhydrous Na₂SO₄. This column was allowed to sit over night with 30-60 bp petroleum ether and was eluted the next day to a total of 250 mL 30-60 bp petroleum ether. Ten mL were withdrawn, extracted and analyzed for HPCB as described earlier. The remaining 240 mL were analyzed for lipid using the lipid analysis procedure above. A further 1 - 2 g of homogenate was removed for moisture content analysis which was determined by weighing the sample before and after it has been allowed to sit for two days at room temperature. The sample was spread thinly on the weigh boat to aid drying. Moisture content values were required in order to calculate concentrations in terms of dry mass of sample.

Liver tissue was ground finely and divided as follows; 0.5 g for PCB analysis, 2 g for lipid analysis and 0.3 g for moisture analysis. Intra-peritoneal fat was ground finely and divided into 0.2 g for PCB analysis, 0.5 g for lipid analysis and 0.3 g for moisture analysis. PCB and lipid analysis were performed using the same procedure as for GIT content and moisture content was performed as per whole fish homogenate.

**Organic Carbon Analysis:**

A 2 g dietary sample was transferred to a ceramic crucible, air-dried over a period of 48 h and then heated in a muffle furnace at a temperature of 550 °C.
After cooling, the sample ash mass was determined and compared to the mass of the same sample dry. It was assumed that inorganic carbon levels were very small in all samples measured.

2.4 DATA ANALYSIS AND STATISTICS

Chromic Oxide, food ingestion and egestion:
Chromic oxide was used as an internal marker in this experiment. When placed in the fish diet, chromic oxide is not absorbed as it passes through the GIT (Fenton and Fenton 1979). Its mass can easily be compared to those food components that are absorbed.
Chromic oxide concentration can be used to determine the egestion rate as follows:

\[ E = I \times \left[ \frac{[CrO_2]_d}{[CrO_2]} \right] \]

\[ E = \text{egestion rate} \]
\[ I = \text{ingestion rate} \]
\[ [CrO_2]_d = \text{Chromic oxide concentration of the diet} \]
\[ [CrO_2]_t = \text{Chromic oxide concentration of the chyme in the anterior section of the intestine.} \]
Fugacity Data:

Fugacity was determined from the head-space analysis results by using the ideal gas law:

\[ PV = nRT \]

where:

- \( P \) is the partial pressure of the HPCB in the vial, thus the fugacity.
- \( V \) is the volume of gas in the vial.
- \( n \) is the number of moles of HPCB
- \( R \) is the gas constant
- \( T \) is the temperature in degrees Kelvin.

The ideal gas law was converted into the following more convenient form:

\[ f = P_{\text{HPCB}} = C_{\text{HPCB-v}}RT \]

Where:

- \( f \) is the fugacity of HPCB in the vial in Pa.
- \( C_{\text{HPCB-v}} \) is the head-space concentration in moles/m\(^3\).

Once fugacity values had been determined from each head-space measurement, the mean and SD were determined using all fugacity measurements taken from each sample vial. The mean values were visually compared across sampling dates. It was apparent that the HPCB fugacity of the
various gut content samples did not elicit statistically significant changes over time. To arrive at this conclusion, the fugacity data were regressed against sampling date and the zero slope null hypothesis could not be rejected ($p > 0.05$). The mean fugacity measurements for each independent sample was then determined. The mean, standard deviation and standard error of the fugacity were then determined for all the independent samples. Thus each fish generated 2 samples of each sample type. Dietary HPCB fugacity values in the stomach, anterio-central region, posterio-central region and posterior region of the intestine were compared using a one way ANOVA (Devore 1987).

**GIT Concentration data:**

A single concentration analysis was performed on each independent sample vial. As with the fugacity data analysis, gut content concentrations were regressed across sampling dates and again the slope null hypothesis could not be rejected ($p < 0.05$), thus justifying the pooling of data across time. The mean, standard deviation and standard error values were determined from the concentration value derived from each individual sample vial. Dietary HPCB concentration values in the stomach, anterio-central region, posterio-central region and posterior region of the intestine were compared using a one-way ANOVA (Devore 1987).
Fugacity Capacity Determination:

The fugacity capacity was calculated by taking the ratio of the HPCB concentration (mol/m$^3$) and the HPCB fugacity (Pa) of any GIT content sample. i.e.

\[ Z = \frac{C_{HPCB}}{f} \]

Where:

- $C_{HPCB}$ is the sample HPCB concentration.
- $f$ is the fugacity of HPCB in the vial in Pa.

Whole Fish Uptake Curve:

Whole body HPCB concentration of each fish was determined by adding the mass of HPCB in liver, intra-peritoneal fat, and fish (missing liver and intra-peritoneal fat) and then dividing by the dry fish mass. The concentration values were then regressed against time (sampling date).
Calculation of HPCB Uptake Efficiency:

HPCB uptake efficiency was calculated in two ways. The first way was to compare the mass of HPCB in food and fecal matter. The difference represented the amount absorbed and, when divided by the mass in food and multiplied by 100, the HPCB uptake efficiency was determined.

i.e.

Uptake Efficiency =

\[
\frac{(\text{HPCB mass ingested} - \text{HPCB mass egested})}{\text{HPCB mass ingested}} \times 100
\]

Where:

HPCB mass ingested was calculated as \( I \times C \) where \( I = 3.42 \) g and \( C \) is the concentration of analyte (HPCB) in the food.

HPCB mass egested was calculated as \( E \times C \) where \( E \) was calculated as shown in equation 2-3 and where \( C \) is the concentration of analyte (HPCB) in the chyme collected from the anterior intestine.

The second approach taken was to determine the increase in total fish HPCB mass with time. The slope from the regressed HPCB uptake curve (above) was multiplied by the average fish dry mass. The resulting slope (units of mg/day) was then divided by the known HPCB dosing rate and the resulting value was the uptake efficiency.

Uptake Efficiency =

\[
\frac{\text{Gastro-intestinal HPCB uptake rate}}{\text{Fish HPCB ingestion rate}} \times 100
\]
The above calculation is based on the assumption that there is no significant HPCB loss through the gills or skin, no HPCB metabolism and no dilution of HPCB due to fish growth. The first two assumptions are reasonable considering HPCB's extremely slow elimination kinetics (Bruggerman 1983) derived a $k_2$ value of $0.004 \pm 0.004 \text{d}^{-1}$ for male guppies for 2,2',4,4',5,5' - hexachloro biphenyl, a chemical having similar properties to HPCB (Bruggerman 1983, Opperhuizen 1985, Gobas et al. 1993). Also, the growth rate was found to be negligible during the period of HPCB dosing.

Comparison of IP fat and body tissue concentrations:

The regressed HPCB concentration uptake curves for IP fat and body tissue concentrations were tested for co-linearity by creating a dummy variable model describing both lines. The equation containing the dummy variable was referred to as the "full model". A reduced model was also created which did not include a dummy variable, but was used to regress both sets of data pooled. A F statistic was then generated by comparing the residual SS of both the full and reduced models (Kleinbaum et al. 1987). Due to the small number of fish used in this experiment IP fat and body concentration values were often taken from the same fish and thus cannot be considered completely independent.
2.5 RESULTS

In all experimental and control fish, the anterior intestinal segment contained a small amount of liquid that was insufficient for analysis. The other sections of the intestines were filled with digested food in all cases. HPCB concentrations and fugacities, as well as chromic oxide concentrations, lipid and organic carbon content in the various sections of the GIT did not show significant (p < 0.05) changes over the duration of the exposure period. As a result, measurements of concentrations and fugacities in the various sections of the GIT were pooled with respect to time and analyzed statistically.

Chyme HPCB Concentration and Mass:

Analysis of the gut contents revealed that HPCB concentrations of the stomach contents was not significantly different (p < 0.05) from the original food. However, the HPCB concentration did decrease 1.8-times between the stomach and the anterio-central portion of the intestine (Figure 8b). The mass of HPCB, when expressed as a fraction of the mass of HPCB in the ingested food of the fish, decreased from 99.8 ± 10% in the stomach to 29.3 ± 3.8% in the fish's rectum (Figure 8c), corresponding to a HPCB gastro-intestinal absorption efficiency of 70.5%.
Figure 8. Various measurements taken in food and chyme at different locations in the intestinal system. Roman numerals represent the following: I = food before ingestion, II = food (now chyme) in stomach, III = chyme from anterior intestine, IV = chyme from antero-central intestine, V = chyme from posterio-central intestine and VI = chyme from posterior intestine. Error bars represent one standard error, sample size given in Appendix 2.
Fugacity ($f$) and Fugacity Capacity ($Z$):

The decrease in HPCB concentration occurred concurrently with a 1.8-times increase in fugacity, thus creating a fugacity gradient between the intestinal lumen and fish body favoring the diffusive uptake of HPCB (Figure 8d). The increase in fugacity indicated a 3.9-fold reduction in the fugacity capacity of the GIT contents below that of the ingested food (Figure 8e).

Lipid and Organic Carbon:

Because lipids are known to have much higher $Z$ for HPCB than other dietary components, lipids in the gastro-intestinal contents were expected to account for essentially all of the HPCB fugacity capacity in the GIT (Peterson & Mackay 1987). Thus, the decrease in the fugacity capacity along the GIT was expected to match the decrease in lipid content due to lipid absorption. However, the lipid content of the GIT content fell 6.6-times (Figure 8g), while the fugacity capacity decreased only 3.9-times.

To investigate whether the drop in fugacity capacity upon digestion was more closely related to a change in the organic carbon content rather than the lipid content of the gastro-intestinal contents, the organic carbon content in the various sections of the GIT were measured. The organic carbon, expressed on a dry weight basis, decreased from 95% in the stomach to 83% in the second section ('anterio-central region') of the intestine (Figure 8i).
Chromic Oxide and Food Mass:

Chromic oxide concentrations increased as food proceeded through the intestine, indicating that the total dry mass of the food was decreasing progressively until, in the posterior region, it reached 50% of the original food mass. Food digestion also resulted in net absorption efficiencies of 92% for dietary lipid and 57% for organic carbon (Figure 8h).

While the total mass of the food continued to drop as it approached the anal vent, the HPCB concentrations, fugacities and fugacity capacities remained constant throughout the intestine. This observation suggests that HPCB absorption occurred throughout the intestine.

Fish HPCB Concentration:

Fish concentration data revealed an increasing HPCB body burden with time (Figure 9). Variability between fish make it difficult to say if a line fit to the data should be linear or sigmoidal. However, previous studies strongly suggest that a linear fitted line is the most appropriate (Bruggeman 1983, Gobas 1993). The HPCB uptake curves for guppies had a $t_{1/2}$ of 175 days. As the trout used in this thesis were many times larger than guppies and larger animals tend to have longer equilibration times (Barron 1990), it is expected that the trout would have
a much larger $t_{1/2}$ value; hence fitting a linear curve between day 0 and 73 is warranted.

**Figure 9:** Dry-weight-based concentrations of HPCB in fish (mg/kg fish) during dietary exposure to HPCB as a function of time (in days). Regressed line was forced through the origin and had a slope of 25.4 µg/g/day, $R^2 = 0.90$ and $N = 13$.

A first order regression was done on intra-peritoneal fat and body (liver + intra-peritoneal fat removed) concentration data. An analysis of covariance showed no statistically significant difference between the slopes of the two regression lines ($p > 0.05$) indicating that chemical in the lipid of these two compartments was in equilibrium (Figure 10).
The liver had an initial lipid weighted HPCB concentration approximately 10x greater than the rest of the body but after 45 days the liver and fish body concentrations became statistically identical (Figure 11)(see data analysis and statistics).
Uptake Efficiencies:

The uptake efficiency calculated by measuring HPCB loss from food was found to be approximately 70%. Uptake efficiencies calculated from measurements of fish HPCB body burden over time were found to be approximately 50%.

![Graph showing the ratio of HPCB lipid weight-based concentrations in the liver and whole fish as a function of time.](image)

**Figure 11:** Ratio of HPCB lipid weight-based concentrations in the liver and whole fish as a function of time.

2.6 Discussion

The results of this experiment support the hypothesis that gastro-intestinal digestion is able to elevate the activity (thermodynamic potential) of dietary HPCB as measured in our experiment by the fugacity of HPCB. This supports an earlier hypothesis based on fugacity measurements in guppies and goldfish.
earlier hypothesis based on fugacity measurements in guppies and goldfish (Gobas 1993). The results of this experiment show that the increase in HPCB fugacity is achieved in the anterior intestinal segment, which is an area known to play a crucial role in food digestion. Moreover, lipid digestion and absorption take place in the same anterior section of the GIT. However, lipid and HPCB appear to be absorbed independently. Without independent absorption, no increase in HPCB fugacity would have been expected. Overall, the efficiency of lipid absorption (92.5%) was greater than HPCB absorption (70.5 %), indicating that the HPCB concentration in the GIT increased on a lipid weight basis during the digestive process. The increase of HPCB on a lipid weight basis means that the activity or fugacity of HPCB also increased and that the amount of lipid in the gastro-intestinal content is likely an important factor controlling HPCB's ability or potential to be absorbed.

The significance of the increase in xenobiotic activity during digestion is that the tissues of a predator can potentially reach a level of xenobiotic activity that is greater than that in the prey consumed. This could occur without any specific active transport mechanism and thus may explain why chemical activities and related concentrations have been observed to increase with trophic levels within food-chains. In addition, the increase in xenobiotic activity suggests that Woodwell's (1967) original explanation for the food-chain bioaccumulation of contaminants may not be correct. Our research indicates that concentrations of
chemical substances increase with trophic levels not because of the transformation of contaminant containing biomass into energy, but because preferential digestion / absorption of food over a xenobiotic in the GIT raises the chemical’s activity. Hence bioenergetic models for the description of the food-chain accumulation of contaminants need to be interpreted with great care as they do not describe the process of chemical biomagnification correctly (Norstrom 1976, Weiniger 1978).

2.6.1 THE ROLE OF LIPID AND OC IN DETERMINING THE FUGACITY CAPACITY (Z)

In thermodynamic terms, the drop in lipid concentration with digestion decreases the intestinal content Z for HPCB. However, the 6-fold decrease in lipid levels only produced a 4-fold drop in Z, suggesting that other undigested, non-lipid organic carbon components of the food may contribute to Z. If the undigested organic carbon components have a significant fugacity capacity for HPCB, then they may contribute to a larger degree than expected Z of the fecal matter. The organic carbon levels were also compared to the change in Z. The large effect which lipid digestion has on Z suggests that the degree to which a particular organism is able to digest and absorb fats has a large bearing on the degree to which that organism would absorb POC’s through its diet.

Insights into the mechanism of food-chain accumulation provide an opportunity to develop methods for the assessment of chemical accumulation in food-webs
and to improve methods for the experimental measurement of biomagnification factors. A dietary bioaccumulation model that is consistent with the observations presented in this study was proposed earlier (Gobas 1993, 1993a).

The applicability of the model relies on the accurate assessment of (i) the "digestibility" of the diet, which can be expressed as the ratio of the dietary fugacity capacity \( Z_D \) to the fugacity capacity in the GIT \( Z_F \), i.e., \( Z_D/Z_F \), and represents the change in contaminant fugacity capacity due to food digestion, and (ii) the degree of "food absorption", which can be expressed as the ratio of the dietary ingestion \( G_D \) and fecal egestion rates \( G_F \). The results of this experiment indicates that the "digestibility" of the food, i.e., \( Z_D/Z_F \), is approximately 4. If lipid digestion was the sole determinant of this change in contaminant \( Z \), as widely believed, then the ratio of lipid mass in the diet and anterior intestine, i.e., \( L_D/L_F \), should also be 4. The ratio was 6, indicating that "digestibility" would be overestimated if lipid digestion is only taken into account. The results indicate that components other than lipids contribute significantly to the fugacity capacity of the food and fecal matter. Both the food and fecal composition are therefore expected to play an important role in determining the size of final fugacity gradient in the gut and hence the biomagnification factor in the fish.
Dietary organic carbon, other than lipids are known to have some capacity for absorbing POCs, albeit not as great as that of lipid (Mackay 1991, Paterson & Mackay 1987, Thomann 1989). When the change in total organic carbon content (including lipid) occurring in the GIT is compared to the "digestibility" above, it can be concluded that OC as a whole is a poor predictor of the GIT magnification factor. This is likely the result of the fact that both digestible and non-digestible matter in the experimental food predominantly consist of organic carbon. Based on the observed changes in food composition, it is possible to estimate the extent to which lipids and non-lipid organic carbon (i.e., carbohydrates, proteins, fibers and others) contribute to the fugacity capacity of the diet and intestinal content, assuming that (i) the change in the composition of the non-lipid organic carbon fraction does not affect its fugacity capacity for HPCB, and (ii) chromic oxide and other inorganic substances do not contribute significantly to the fugacity capacity of the diet and intestinal content. Under these reasonable assumptions, the Z's of chyme collected from the stomach and the second posterior intestinal fraction can be represented as:

\[
Z_{\text{stomach}} = 0.18.ZL + 0.77.Z_{oc} = 105 \text{ mol/m}^3.\text{Pa} \tag{2-9}
\]

\[
Z_{\text{posterior}} = 0.025.ZL + 0.81.Z_{oc} = 29.104 \text{ mol/m}^3.\text{Pa} \tag{2-10}
\]

Solving the equations suggests that fugacity capacity of the lipids is approximately 30-times greater than that of the non-lipid organic carbon. This
difference appears reasonable given reported fugacity capacities for lipids, proteins and carbohydrates with similar proportions (Paterson & Mckay 1987). This suggests that in our experimental diet, dietary lipids provide approximately 88% of the total fugacity capacity of the diet in the stomach, whereas it provides 49% of the total fugacity capacity in egestable fecal matter. Based on these findings, the following tentative model can be proposed for estimating the contribution of food digestion to the gastro-intestinal magnification factor:

\[ \frac{Z_D}{Z_F} = \frac{(L_D + 0.035.OC_D)}{(L_F + 0.035.OC_F)} \]  

(2-11)

where \( L_D \) and \( L_F \) are the lipid contents of the diet and the fecal matter and \( OC_D \) and \( OC_F \) are the non-lipid organic carbon contents of the diet and the fecal matter, respectively (all values expressed in terms of mass). This simple model suggests that in food-webs, lipid rich prey-items will not only result in a larger exposure of the predator to substances that bioaccumulate, but also result in larger biomagnification factors and hence higher concentrations of substances that bioaccumulate in the predators. On the other hand, organisms consuming a diet that is lipid-poor, but rich in non-lipid-organic-carbon are expected to experience smaller biomagnification factors. It is therefore evident that the application of universal biomagnification factors or food-chain multipliers, as well as the extrapolation of empirical biomagnification factors from one ecosystem to another, in exposure assessment could be associated with substantial errors.
Another implication of the experimental results is that animal experiments that involve dietary exposure of a chemical substance in 100% oil or lipids will substantially over-estimate the chemical’s dietary uptake efficiency and biomagnification factor in the environment. The influence of the dietary matrix on chemical uptake kinetics and biomagnification may explain the large degree of variability that has been reported in measurements of the dietary uptake efficiency of substances which bioaccumulate (Parkerton 1993).

2.6.2 Mass balance analysis of experimental fish

The uptake efficiency calculated by measuring HPCB loss from food (71%) appears to correspond poorly to the uptake efficiency calculated from measurements of fish HPCB body burden over time (50%). This difference is likely a result of some dispensed food not being ingested. If a lower ingestion rate was used to calculate uptake efficiency, a value greater than 50% would be realized.

Lipid normalized HPCB concentrations of the fish body were found to be statistically identical to those of intra-peritoneal fat. The identical HPCB concentrations (Figure 10) indicate that the proximity of the intra-peritoneal fat to the GIT did not mean that contaminant absorption would be greater in this tissue, rather, and as expected, that the xenobiotic passes directly to the liver via
the circulatory system and was then distributed uniformly throughout the body fat deposits (Borlakoglu 1990, Vetter 1985).

Lipid normalized HPCB concentrations in the liver were initially over 10x the concentration in the fish body, but decreased exponentially to the concentration of the fish body in 45 days. These results suggest, as expected, that HPCB initially partitions into the liver from the circulatory system after intestinal uptake, but over time is distributed throughout the rest of the body.

2.6.3 Expected Affect of Steady State on Contaminant Absorption

The body burden results indicated that HPCB absorption was measured under non-steady state conditions. If steady state was being approached, it is likely that some of the GIT measurements above would be quite different. Under environmental conditions when concentrations in the fish are much greater relative to the concentrations in the fish's diet, the net absorption efficiency of HPCB will be much smaller than the 70.5% measured in this experiment and zero percent at steady-state (Bruggeman 1983). The smaller net absorption efficiency would be due to a considerable loss of HPCB from the fish to the chyme, i.e., a fish-to-GIT flux which reduces the net dietary absorption efficiency (Mackay 1991).
Initial Uptake

Large surface area, high degree of vascularization and thorough mixing of contents aid rapid uptake of PCB in this area.

Fat in food is beginning to be digested and absorbed.

Digestion and absorption of fat increases the intestinal fugacity, thus significantly increasing the uptake of PCB.

Steady State

Fugacity of food equals fugacity of fish.

Fugacity of food is lower than that in fish.

Fugacity of food is higher than the fugacity of fish.

Fat in food is beginning to be digested and absorbed.

Digestion and absorption of fat increases the intestinal fugacity, thus significantly decreasing the egestion of PCB.

Figure 12: Expected POC fluxes through the GIT lining soon after initial exposure and at steady state. The degree of shading is representative of the PCB fugacity within the chyme.
At or near steady-state, the concentration of HPCB would increase in the GIT, rather than decrease as observed in our experiment, because the net HPCB absorption efficiency would become significantly smaller than the food absorption efficiency. The same fish-to-GIT flux of HPCB near or at steady-state will also cause the fugacity in the intestinal tract to increase to values that are greater, i.e., approximately 7 - 8 times the fugacity in the food, than those observed in this experiment. This prediction is based on the approximately 4 fold reduction in fugacity capacity and 2 fold reduction in total food mass within the anterior intestine. The Z being a property of the gut contents, is not likely to vary from the results seen in this experiment. The prediction of a larger chyme PCB concentration of a fish at steady state is supported by a study by Russell et al. (1995) which showed that chyme PCB concentrations increased as a fish reaches steady state. Figure 12 illustrates an overview of the differences expected to be seen between a fish at steady state and one actively taking up chemical.

2.7 SUMMARY

The hypothesis tested in this chapter was that digestion of food results in an increase in the HPCB fugacity of chyme. The hypothesis was accepted after a 1.8 x increase in fugacity was realized between the stomach and the posterior-central region of the intestine.
The results support the thermodynamic explanation of the mechanism behind biomagnification. Digestion and absorption of lipids was found to occur predominantly in the anterior region of the intestine which is the same region where the dietary HPCB fugacity capacity decreased significantly. Lipid was found not to be the sole determinant of dietary fugacity capacity as changes in dietary lipid content were reflected by smaller changes in dietary fugacity capacity. It was concluded that other food components must contribute to the total fugacity capacity to food, albeit to a smaller extent. For instance, in our experimental diet, dietary lipids provides approximately 88% of the total fugacity capacity of the diet in the stomach, where as it provides 49% of the total fugacity capacity in egestable fecal matter. The data also suggests that fugacity capacity of the lipids is approximately 30-times greater than that of the non-lipid organic carbon.

The total dietary dry mass was found to decrease 50% between the stomach and the posterior intestine. The reduction in food mass combined with the decrease in HPCB fugacity capacity was postulated to have resulted in an observed increase in the dietary fugacity in the anterior intestinal region and thus caused gastro-intestinal magnification.
2.8 Future Directions

The fugacity determination used in this experiment, although functional, cannot be considered as a routine procedure. It was very labor intensive and required meticulous attention in order to produce useful results. At the heart of the problem were the low concentrations of HPCB in \(N_2\) which limited the sensitivity of the head space analysis. In addition, interference in the chromatogram occurring as a result of poor sensitivity and possible contamination of the needle within the sample vial created a large amount of variability between replicates. To produce statistically useful results, many replicates had to be taken. If future tests to the thermodynamic explanation for biomagnification are to occur, a more utilitarian fugacity measuring technique is required; one with less variability and requiring fewer replicates.

The purpose of the next chapter of was to explore a new and potentially more useful methodology for fugacity determination. The method introduced would not only be more reproducible and thus allow a time savings, but would be considerably more sensitive for POCs with lower volatilities than chlorobenzenes. The technique could also be used for sample matrices other than food.

3.1 Abstract

A novel methodology for fugacity measurement is presented. The method improves upon existing methods intended for use with small sample sizes. Compared to the head-space technique used in chapter 2, sensitivity was increased 100-fold for HPCB. Also reproducibility was improved such that the coefficient of variation decreased from 37% to 12% for HPCB. Lastly, the time-to-equilibrium was shortened to 2 days from 3 weeks.

3.2 Introduction

3.2.1 Introduction to Fugacity Measurement

As discussed earlier, using head-space analysis for the fugacity determination of semi-volatile xenobiotics has two main shortcomings, namely poor sensitivity and a considerable sample variability. To attain significant results, large numbers of replicates are required and consequently, the technique is time consuming. The large amount of time required restricts the breadth and complexity of experiments. A literature search on the subject revealed few fugacity measuring techniques.
The motivation for producing a tool which quickly and conveniently measures fugacity is two-fold. First, more dietary experiments of the type presented in chapter 2 need to be done. Many questions have yet to be answered before biomagnification can be effectively modeled. Two prominent questions are: (1) What happens to the fugacity of xenobiotic in intestinal chyme when the organism is reaching steady state with its diet, and (2) what is the effect of changing the dietary components of a spiked food on fugacity?

The second reason for developing a tool which quickly and conveniently determines fugacity is to enable environmental managers to make better predictions regarding the fate of POCs in the environment. Knowing the fugacities in several adjacent compartments allows the manager to better predict the partitioning of chemical.

Section 3.2.2 will discuss the desirable attributes of any fugacity measuring technique and Section 3.2.3 will discuss the advantages and drawbacks of the only other technique used for fugacity determination, as well as several existing methodologies that could potentially be used for fugacity determination. The experimental work in this chapter introduces a technique which involves exposing a thin film of VA plastic to the head-space of a small POC contaminated sample and argues its superiority over other potential methods.
3.2.2 Desirable Properties of the Sorbant Phase

The introduction provided a brief overview of the requirements of a fugacity measuring device. What follows here is a discussion of those properties which, although not essential, are desirable in a fugacity measuring methodology.

Absorbance or Adsorbance?

A methodology which relies primarily on absorbance is preferable as the relationship between fugacity and xenobiotic concentration in/on sorbing material tends to be linearly related for a greater concentration range. The linearity of absorbance permits the calculation of a single absorbing capacity number for each absorbing material called fugacity capacity and thus greatly simplifies the determination of fugacity. Another concern with using adsorptive materials is the concern of absorptive site competition with other sample molecules. Water, for instance, is known to actively compete with xenobiotics for absorptive sites on soils, hence moist soils typically have less sorption capacity for xenobiotics than dry soils (Unger 1996). If this is indeed a concern, the researcher may have to actively regulate sample humidity. When deciding on sorption materials it should also be known that methodologies relying primarily on adsorption have the benefit of reaching an equilibrium with xenobiotic in the sample quicker. So if a fast equilibrium time is desirable, a
researcher may put up with the inconvenience of creating identical sample humidities and having to determine fugacity from a non-linear calibration curve.

**Interaction with sample**

A fugacity measuring device should not be affected by nor affect the physical properties of a sample. Some materials, for instance will effectively absorb lipid. Not only will the absorption of lipid change the total absorbing capacity for xenobiotic by the fugacity measuring device, but it may also decrease the total absorbing capacity of a small sample. The sampling device should also not have an appreciable affect on the xenobiotic concentration in the sample. If the sample concentration decreases, so will the measured fugacity, even when the true fugacity is higher.

**Uptake rate**

The fugacity measuring methodology should allow for equilibrium to be achieved in a relatively short time. Work done by Gobas et al. (1992) showed that PCB loss from a sealed 2 mL vial containing an unstirred, water saturated fecal sample becomes significant after about one month. If it is impractical to achieve equilibrium, fugacity can still be determined from the concentrations achieved after a fixed exposure period.
Sensitivity and Reproducibility:

Sensitivity and reproducibility, although different concepts, are not independent of each other. If a method has poor sensitivity and concentrations are close to machine detection limits, the reproducibility will suffer. A poor reproducibility, such as found in the last section using the head-space technique, may result in the requirement of a greater number of sample replicates to show statistically significant results.

3.2.3 Methodologies for Fugacity Measuring

Any methodology which involves placing a non-electrolytic xenobiotic containing test matrix in contact with a sorbant with a known contaminant sorbing capacity can potentially be used for fugacity measurement. Once contact has been made between phases, a period of time elapses so that some of the contaminant can be sorbed by the sorbing compartment before it is analyzed for contaminant concentration. As long as the relationship between concentration and fugacity is known for the sorbing compartment and for the physical conditions existing within a sample vial, sample fugacity can be determined. The relationship will be linear if absorption is the prevailing sorbing mechanism (Unger et al. 1996) and
if absorbed concentrations are low enough that saturation kinetics do not play a role.

Although several methods show promise in the area of fugacity measurement, only two methods have actually been used for this purpose; head-space analysis and dynamic head-space gas-partitioning. Listed below are those methodologies which can be and are used in fugacity measurement accompanied with a short discussion of methodological strengths and weaknesses.

Head Space Analysis

Static head space analysis was probably first used as a fugacity measuring tool by Resendes et al. (1992). As discussed in chapter 2, a sample is placed in a sealed vial such that a significant volume of gas exists above. The gas of preference is usually N₂, because it will not degrade the gas chromatograph column as O₂ does. The sample is left until a state of equilibrium exists between the sample and the N₂, after which a known volume of N₂ is withdrawn for GC analysis. The concentration in the N₂ phase is proportional to the fugacity and can be easily calculated using the ideal gas law (see chapter 2).

For the determination of semi-volatile fugacities, the head space approach has the limitation that a very large volume has to be analyzed due to the typically tiny concentrations found in the gaseous phase. Concerns regarding this technique are contamination from microscopic particles in the sample vial and loss of
sample due to adsorption to the internal surface of the syringe. The syringe must also be extremely clean and free of any solvent or other organic carbon residue.

The head-space volume used in chapter 2 could be considered excessive for injecting directly onto an on-column injection port. If a larger N₂ volume was to be sampled, the use of a purge-and-trap methodology is advisable.

**SPME**

SPME (Solid Phase Micro-Extraction) is a technique employing a glass micro-filament to which has been bonded a liquid phase (or stationary phase) and it is to this phase that fat soluble analytes may sorb. The filament is housed within a modified syringe and is introduced to a sample by injecting the syringe through a septum and then exposing the fiber. After extraction, the fiber is withdrawn and transferred directly to the injector of a gas chromatograph in which the analytes are thermally desorbed and subsequently analyzed (Author 1990, Zhang, 1993).

SPME has a number of attributes which are as follows:

1. By applying the absorbing matrix directly to the GC injection port, all absorbed chemical is introduced into the column and thus little analyte needs to be removed from a sample for effective analysis and the sample equilibrium is less likely to be upset.
2. The small sorbing matrix volume allows for rapid uptake of analyte which is limited only by the sample matrix (Vaes 1996).

3. No solvents are required for extraction.

SPME's fugacity measuring potential has recently been realized by Parkerton et al. (1996). Although Parkerton did not use the word “fugacity”, he did use the method to predict the lipid normalized concentration of fat soluble contaminants in organisms after an exposure event. The lipid normalized concentration is proportional to the fugacity of chemical in any biological matrix.

In his study a biological sample contaminated with several volatile organic carbons (VOCs) was placed in a sealed container and the VOCs were allowed to reach an equilibrium between the sample and gas compartments. A SPME fiber was then introduced and left until a new equilibrium had formed between the sample, gas and fiber. At this point the fiber was removed and desorbed within a gas chromatogram injection port.

Theoretically, direct injection of the fiber into the sample is not recommended for fugacity measurement because organic carbon molecules may compete for sorption sites with the analyte and/or some of the sample may “wick” up into the needle housing the fiber once the fiber is withdrawn (Chai 1993).
From a fugacity measuring perspective, the main assets of this technique are the small mass of analyte required on the fiber for analysis, the short time required to reach equilibrium, the absence of solvent and the relatively few steps required. Precautions which must be taken are to insure equilibrium has been reached throughout the sample vial before removing the fiber, and that full desorption of analyte occurs from the fiber within the heated injection port of the GC.

Even though the technique draws a small mass from a sample when applied to gases, this mass will be a significant proportion of analyte found in a 1-2 mL gas matrix, thus upsetting the sample equilibrium. Sampling PCB in 1-2 mL of water have been found to quickly deplete the PCB mass (Arthur, 1990). As gas has a lower fugacity capacity for PCBs than water, it would follow that gas is even more prone to depletion if presented in a small volume. Having a larger volume of gas available for sampling would remedy this problem. For the water sampling technique, 100 mL of water was recommended if multiple injections are required (Arthur, 1990). By comparing fugacity capacities, 100 mL of water equates to 2,450 mL of gas (volume of air required based on 2,2',4,4',6,6'-hexachlorobiphenyl). Zhang (1993) has shown that gas depletion concerns can be remedied by rapid mixing of aqueous sample. The mixing allows for a more rapid transfer of POC from the sample to the air compartment, thus maintaining the POC fugacity of the air compartment (Potter 1994).
Loss of analyte can occur from the fiber while being transferred from the sample vial to the GC. However this amount is negligible as long as the transfer takes less than about 2 minutes. Volatile chemicals tend to be more prone to premature desorption, which can be minimized if either the housing needle is capped, or the syringe is cooled, or a thicker fiber coating is used (Chai 1993). The use of a thicker coating is also preferable for volatile chemicals, which usually have a smaller fiber coating/sample partition constant and consequently SPME exhibits lower sensitivities towards them compared to conventional head-space analysis (Potter 1994).

Semi-volatiles generally take longer to reach a state of equilibrium with the SPME fiber due to the greater mass of analyte absorbed by the SPME coating and the smaller concentration found in the gaseous compartment. A thin coating will minimize the time-to-equilibrium as well as the time-to-desorption (Chai 1993).

Incomplete desorption of semi-volatiles is probably the most serious draw back of SPME for fugacity measuring purposes. Incomplete desorption will result in “carry-over” of analyte from one injection to the next and consequently yield erroneous results. Potter et al. (1994) showed that the carryover for a polydimethylsiloxane (PDMS) coating was 9% and 23% for trichlorobiphenyl and
pentachlorobiphenyl, respectively, and with Carbopack B as the absorbing phase, the carry-over was 10% and 17%, respectively. The presence of carry over brings into question the accuracy of the technique for semi-volatile fugacity analysis.

Carry over, often called "memory effects", can be minimized (although not eliminated) by desorbing the fiber within the GC injection port for a longer time and at a higher temperature. Unfortunately, higher temperatures reduce the life of the SPME coating (Chen 1995).

Lipid/solvent filled dialysis bags (SPMD)

SPMD has been used for the determination of fat soluble xenobiotics from water. A small volume of solvent or lipid is placed within a non-porous plastic sheath. The sheath is sealed and placed in the environment for a specific duration after which it is removed and the contents analyzed for a particular analyte (Huckins et al. 1993, Huckins et al. 1990 & Johnson 1991). If the analyte partitions into a lipid-filled SPMD, extraction may be completed by using either reverse dialysis into solvent, or solvent extraction of the lipid employing cleanup processes (Huckins et al. 1990). Drawbacks of this technique are that large sample sizes are required and that uptake for larger molecular weight analytes occurs very slowly (Huckins et al. 1990). Increasing the molecular weight of non-polar analytes has been found to be inversely correlated with diffusion rate (Leib &
Stein 1969, Huckins et al. 1990). In addition, there are two concerns attributable to the use of lipids in SPMD; biological growth on the outside surface of the SPMD can reduce the flux of non-electrolytic chemicals through the plastic membrane and lipids in the SPMD are susceptible to oxidation.

A similar technique has been used in the determination of xenobiotic partition coefficients between blood and various tissues for the use in PBPK models (Lin 1982, Murphy 1995). In this procedure a xenobiotic containing tissue is homogenized before being placed into a dialysis membrane. The membrane is immersed into a stirred aqueous solution containing a surfactant and having a pH similar to that of blood. Once the system has reached a state of equilibrium, both the dialysis bag contents and buffered solution are analyzed for xenobiotic concentration. Often concentrations used are so low that radio-labeled analytes are required for analysis.

Dynamic head-space gas-partitioning

Originally used by Hassett and Milicic (1985) to measure the binding of PCB to dissolved humic acid in water, the methodology was adapted by Yin and Hassett (1986) as a fugacity measuring technique for Mirex in water. The methodology called for purging water with a fixed volume of air which was bubbled through the water at such a rate that equilibrium occurred between the water and bubbled air. The air was then passed through an activated carbon trap which was subsequently analyzed for Mirex. Knowing the volume of air purged and the
Mirex concentration found in that air, the ideal gas law was used to calculate the sample fugacity (Yin 1986).

The dynamic head-space gas-partitioning method used by Yin and Hassett was later modified by Horstmann and McLachlan (1992) for the determination of fugacity from solid surfaces. The methodology used by Horstmann, referred to as the fugacity meter, involved pumping air slowly past a POC containing sample such that equilibrium was attained between the parcel of passing air and the sample surface (Figure 13). A glass fiber filter/XAD adsorbent trap was used to extract the total mass of analyte in the pumped air. Knowing the volume of air sampled, the sample fugacity was easily calculated from the analyte mass found on the trap using the ideal gas law. The major benefit of this technique is that extremely large samples can be analyzed and, as a result, is very sensitive. Also, living organisms can be placed in sample chambers, thus reducing errors which may arise from conducting in vivo fugacity measurements (Horstmann 1992, Tolls 1994).

The fugacity meter requires a very large surface area for efficient transfer of mass (for equilibrium) and a large enough sample mass that the overall sample fugacity is not affected. As a consequence of the importance of sample surface area, hence adsorption of POC, humidity must be vigorously regulated as the
The presence of water vapor reduces the number of sample surface adsorptive sites. (Horstmann 1992). The amount of air pumped by the sample is also crucial. If too much air is used, the adsorptive sites will begin to become depleted causing
the sample surface fugacities to drop and the air POC concentration measured will result in the calculation of erroneously low sample fugacities (Horstmann 1992). This concern also applies to dynamic head space gas-partitioning method used for fugacity measurement from water. If too much air is bubbled through the water, the water concentration of analyte will drop and the calculated contaminant fugacity will be erroneously low (Yin 1996).

Although the fugacity meter method does suit the measurement of fugacity in soils and plants, it is likely not so useful in studies involving animals where both surface area and total sample mass are often low. The original method proposed by Yin and Hassett (1986) is also not very practical for animal samples as it again requires a large mass of sample and would also require liquefaction and dilution of samples in water.

**Empore Disk:**

Empore disks have been recently been devised as an alternative extraction method from liquids. Known as a solid phase extraction method, it enables the researcher to extract lipophilic contaminants from aqueous liquids without using large volumes of solvent. Once the sample water has been passed through the disk, a solvent which selectively removes unwanted analytes is passed through before application of the final solvent wash which removes the desired analyte.
Although nothing in the literature suggests it's use for fugacity measurement, it does show promise. Like SPME, Empore™ cartridges contain a bonded liquid phase (C_{18}) capable of sorbing lipophilic contaminants and in theory could be used for head-space analysis.

Unlike the SPME fiber, Empore disks are intended to remove all the analyte from a sample (Krueger 1995, Field 1994). This is not a good fugacity measuring attribute as fugacity measurement demands that the sampling methodology must not appreciably effect the original sample concentration. In order to use empore disks for fugacity measurement, either large sample sizes must be used or the empore disk must be trimmed down.

As the absorbent phase is bonded on to a Teflon fiber support matrix, the surface area is large, thus allowing effective removal of organic chemical from water which is forced through (Krueger 1995). If Empore™ disks were used to analyze the head-space above a sample, there would be little advective flow of air and consequently, the area between absorbent particles would be effectively dead space. Without effective flow, it is likely that Empore™ disks would take a very long time to reach an equilibrium.

Recent work by Verhaar et al. (1995) has provided evidence which suggests that the disks could be used for determining the fugacity of contaminants in water. In his research he exposed 13 mg of Empore disk to 2 L of contaminated water.
Verhaar theorized that the disk would work as a surrogate for a biological organism and the concentration of hydrocarbon contaminants achieved in the disk at equilibrium would be proportional to the internal body burden of an organism exposed to the same hydrocarbon contaminated water. There are a number of problems with the technique as stated (1) the disk removes a significant mass of hydrocarbon from the contaminated water such that the total concentration of the sample decreases (2) equilibrium time for "heavier" hydrocarbons was long; i.e. greater than 10 days for pentachlorobenzene and (3) the method has not been tested with matrices other than distilled water (Verhaar et al. 1995). The technique would likely not work for the determination of PCB's in biological matrices. Placed in direct contact with a biological phase, the disk's Z value would be prone to change and the uptake rate for PCB's would be even slower than that for pentachlorobenzene.

For all the techniques mentioned above, there are a number of sample-related factors which must be kept in mind when performing fugacity determination. Two of the greatest potential impediments to accurate fugacity determination are microbial degradation and oxidation of sample macromolecules, as both these can have profound effects on the molecular make up of a sample and thus change the samples' ability to absorb the xenobiotic (Huckins 1990). Microbial degradation can be retarded by using a very potent antiseptic, HgCl₂, while
replacing $O_2$ with $N_2$ eliminates the threat of oxidation. A third process, residual enzymatic activity, can be of concern when analyzing biological tissues and can be halted by heat shock (a quick microwaving). A concern of microwaving is that this technique may change the molecular structure of heat sensitive macromolecules, such as large proteins, resulting in the possibility of fugacity capacity modification. For the purposes of the trout biomagnification experiment, section 2, residual enzymatic activity of gut content samples was assumed to be negligible.

3.2.4 Objectives for the Development of a New Methodology

In order for a fugacity measuring technique to be of experimental value, a number of requirements must be met. For the purposes of this section, the desired method is one which can be used within a laboratory setting, were concentrations are higher than what would normally be found in nature and where there are negligible interfering GC peaks from other chemicals. The desired requirements are to maximize sensitivity, minimize variability between replicates, minimize the time required for equilibrium to be reached, to ensure that a direct proportionality exists between concentration and fugacity for the range of concentrations used, and to ensure that the POC concentration of the tested compartment is not changed upon sampling.
Sensitivity/Detection limit

The sensitivity should be a several-fold improvement over the head space technique, which was close to the detection limit of 0.05 pg for 2,2',4,4',6,6'-hexachlorobiphenyl in chapter 2.

Variability

Greater degrees of variability increase the uncertainty around a chosen mean value. In order to decrease the uncertainty inherent in a technique, larger numbers of replicates have to be taken resulting in a larger expenditure of time and energy. It is a goal of this experiment to decrease the coefficient of variation of sample replicates significantly.

Uptake Rate

For a fugacity measuring technique to be valid, equilibrium must be reached between sampling device and sample. Counteracting the desire to reach equilibrium is the time allotted by the experimenter and the concern of losing analyte. Uptake curves from previous methods using head space analysis of PCBs in fecal samples of fish indicate that equilibrium is achieved at three weeks, beyond which there is a slow decrease due to analyte loss (Gobas 1993).
Linear Relationship With Sample Concentration

Fugacity is determined by measuring the concentration in a sampling device and converting this number into a partial pressure value. If the fugacity capacity of an absorbing matrix is constant and the concentration achieved in the absorbing matrix is low enough such that the conditions under which Raoult's law applies, there must be a linear relationship between fugacity and concentration in the sampling device. The linear relationship permits the calculation of fugacity by dividing concentration by fugacity capacity.

3.3 Methodology

Ethylene vinyl acetate (Elvax 40W, Dupont, Wilmington, DE ) (VA) was chosen as a potential absorbent phase for fugacity determination mainly because of its pliability, availability and proven ability to absorb PCBs (Duong 1991). Monomers of VA consist of a ethylene group bonded to an acetyl group. Polymerization occurs when the oxygen of the carboxyl group of one VA molecule displaces a H molecule and bonds to the carbon in the number two position of another VA molecule. Being a very soft plastic, it acts much like a very viscous solvent phase, yet it is solid enough to be durable. Originally plastic beads having a diameter of 22 mm were placed in contact with a spike matrix, but it was learned from preliminary experiments that the time required for equilibrium to be reached exceeded 2 months. It was then hypothesized that the
time to equilibrium could be shortened if the thickness of the VA was significantly reduced. The development of the VA coating method started with applying a thin layer of VA to a stainless steel rod by dipping in a solution of VA dissolved in methylene chloride. Subsequently, the cover-slip coating method was investigated which required dropping a small volume of VA dissolved in methylene chloride on a glass microscopic cover-slip and then spinning excess solution off.

3.3.1 Stainless steel rod dip methodology

Rod Preparation

Stainless steel rods, 2.5 cm long with a diameter of 3 mm, were washed with water, hexane and dichloromethane (DCM) and set aside. A solution of 10 parts per thousand VA and 1 part per thousand Sudan IV in DCM was prepared. Using forceps only, the stainless steel rods were dipped individually into the VA solution such that 1.5 cm of the rods were covered, the rods were removed and then placed in a drying rack.

Food Preparation

One hundred mL of 30-60 bp reagent grade petroleum ether containing tetrachlorobenzene (10 ppm), pentachlorobenzene (10 ppm),
hexachlorobenzene (10 ppm), 2,2',5,5'-tetrachlorobiphenyl (10 ppm),
2,2',4,4',6,6' hexachlorobiphenyl (100 ppm), decachlorobiphenyl (10 ppm) and
Mirex (10 ppm) was added to 100 g ground Silver Cup trout chow in a 500 mL
round bottom flask (chlorobenzenes were from Aldrich Chemical Co., Milwukee,
WI, USA; PCBs and Mirex were from Accustandard, New Haven, CT, USA).

Rotating slowly on a rotary evaporator (RE47, Yamato, Japan) without suction,
the petroleum ether was allowed to slowly evaporate from the round bottom
flask. After 6 hours of slow mixing, the round bottom flask was removed, capped
and placed within a 5 °C refrigerator for a period of about 8 months.

The food was then placed within a mortar and mixed with 50 mL distilled water
containing 5 ppm sodium metabisulphate (Aldrich). The resulting food had a
paste like consistency. This paste was transferred to a jar and stored at 5 °C.

Sample Vial Preparation

The paste-like, spiked food was packed into a 5 mL modified syringe. Using this
syringe, 0.5 mL of spiked food was added to each of several 4 mL air tight vials.
To the same vials, 0.5 mL of distilled water was added before the addition of a
VA-dipped rod. Each rod was equipped with a metal clip such that the rod would
not come into contact with the spiked sample. All air in each vial was then
replaced with N₂ and the vials were sealed air tight using the provided screw-on
top and hot wax. Vials were then placed upon a turntable having a 20° incline
and rotated at 33 rpm. The purpose of the turntable was to produce gentle and continual mixing within each sample.

Dipped rod sampling and analysis
Once exposed for a set period of time (i.e., 4, 11, 28, 57 days), the vials were removed from the turn-table, opened and the rods removed. The rods were washed with a stream of water and wiped gently with a methanol-soaked tissue in those areas not coated with VA. The rod was then placed within a 5 mL test tube to which 3 mL of Dichloromethane (DCM) was added. This solution was analyzed spectrophotometrically (Lambda 3B, Perkin-Elmer UV-Vis Spectrometer) using an absorbing wavelength of 550 nm; the absorbance being dependent upon the level of internal marker (Sudan IV) and thus determines the total mass of VA on each rod. Sudan IV was used as an internal marker because it doesn't leach out of VA. The solution was then quantitatively transferred back to a 5 mL test tube and concentrated to 1 mL before 200 µL of 2-octanol was added. The sample was further concentrated to remove the remaining 1 mL of DCM. Two mL of methanol was added to the test tube to precipitate out the VA polymers, while allowing the analytes to remain in solution. The test tubes were then centrifuged (Size 2, Model K, International Equipment Company, MA) at 30,000 rpm (2000 g) in order to create a clear supernatant. It was this supernatant which was transferred into 2 mL vials and subsequently analyzed using a gas-
chromatograph (Hewlett Packard model 5890) with an ECD detector, as described in chapter 2.

Reproducibility (variability) Experiment

The procedure described above was followed except that five VA dipped stainless steel rods were added to each of five, 4 mL vials. After two weeks of exposure to spiked food, the rods were sampled. A comparison of analyte concentrations in VA for each dipped rod gave an indication of the reproducibility of the technique.

Uptake Kinetics Experiment

Thirty, 4 mL sample vials were prepared containing one dipped rod and spiked food each. Four vials were sampled after 7, 14, 27, 45 and 60 days of exposure and the dipped rod analyzed for analyte content. It was expected that this would produce an uptake curve.

Sensitivity experiment

At the same time the uptake experiment was being prepared, five vials were sealed with out dipped rods, but similar in every other manner. These same five vials were sampled by head space analysis at day 45 and the results were compared to the analysis of the dipped rod at day 45.
3.3.2 High speed cover slip spin methodology

In response to potential concerns regarding the low surface area to volume ratio combined with the variability in the thickness of VA coating with the rod technique, a cover slip spin methodology was used. A solution containing 4.25 parts per thousand VA was made up in 100% DCM. A pre-cleaned 1.5 x 1.5 cm square glass cover slip was stuck to the horizontal surface of a modified carving bit attached to a Fordom high speed carving tool (Fordom Electric Co., Bethel, CT, USA). Two hundred μl of the VA containing solution was dropped onto the glass cover slip and spread using the tip of a syringe. The Fordom tool was then turned on and quickly ramped up to 5,000 rpm, holding this speed for 10 seconds. Excess solution of VA was spun off leaving an extremely thin film that was completely free of solvent within an hour. Once free of solvent, the cover slips were then placed on a rack in a 30 mL jar containing 5 mL spiked sample. At no time were the cover-slips exposed directly to the sample. All air was displaced with N₂ before capping with a plastic screw-on lid lined with tin foil and sealed with hot wax.

The 30 mL bottles were secured to a turn table inclined at a 20° and spun at 33 rpm to facilitate convection within the sample. Once the exposure time was completed, the jars were taken off the turntable and the cover-slips removed for
analysis. Each glass cover-slip was placed in the bottom of a small glass petri plate and rinsed 4 times with 1 mL of DCM, thereby quantitatively transferring the VA and PCB into a 5 mL glass test tube.

The sample was concentrated to about 1 mL and then 200 µl of 2-octanol was added. The remainder of DCM was evaporated and 2 mL of methanol was added to precipitate out the VA polymer. The sample was then centrifuged at 30,000 rpm for 5 minutes to produce a clear supernatant. The supernatant was transferred to a 2 mL vial and analyzed using GC-ECD.

Reproducibility Experiment

Five 30 mL jars each containing five coated cover slips suspended above 5 mL of HPCB-spiked food sample were equilibrated for 2 weeks. Once removed, the glass cover-slips were analyzed using the technique discussed above and total concentration of absorbed contaminant was compared between slides.

Uptake Kinetics Experiments (thin and thick films)

Five mL of POC spiked food was added to eight 30 mL jars. To all eight jars, three coated cover slips were then added before the jars were purged with N₂, closed and sealed with hot wax. For the 18.5 µg film, sampling times were at 1, 2.5, 4.5, 5.5, 19, 22.5, 35, 43, 96 and 144 hours. For the 126 µg film sampling times were at 2, 9, 19.5, 60 and 132 hours. Film thickness was calculated by determining the coating mass using a sensitive analytical balance, converting.
this number to a volume knowing the density of VA (0.965 g/cm³) and dividing by the surface area of one face of the cover-slip.

Desorption Experiment

A 0.5 mL aliquot sample of 10 ppm chlorobenzenes and PCBs in hexane stock solution was added to 7.5 mL VA stock solution before being mixed. Two hundred mL of this solution was added to each of five glass cover-slips before being spun using the same procedure given above. These impregnated cover-slips (coating thickness: 18.3 ± 0.5 μg) were placed in a fume hood, exposed to passing air and one each was sampled after 7, 15, 35, 187 and 345 minutes.

Thicker coating preparation

The same VA film coating methodology was used as for the thinner coating, but instead a more concentrated VA solution was used. The solution contained 62.0 parts per thousand of VA, 83% 2-octanol and 17% DCM (by mass).

3.4 Data Analysis and Statistics

Sensitivity

The relative sensitivities of the two methods was determined by comparing relative sensitivity values which were calculated as follows.
$$R_S = \frac{\text{contaminant mass injected from analysis of day 45 sample (pg)}}{\text{GC detection limit mass for the same chemical using given technique (pg)}}$$

The detection limit used for head-space analysis and VA analysis was 0.05 pg and 0.5 pg respectively for all chlorobenzenes and PCBs. The detection limits represent masses which yielded peak areas having a signal to noise ratio of about 10 for TCB. The TCB peak was chosen as it had the smallest peak area per unit mass.

**Variability**

Variability was expressed in terms of the coefficient of variation (CV). The CV was calculated by taking the standard deviation of replicates, dividing it by the mean of those replicates and multiplying by 100. The three methodologies tested were compared using average CV values.

3.5 **Results**

**Sensitivity of rod technique**

A true comparison of sensitivity between the head space and VA-dipped rod techniques could not be done as equilibrium was not reasonably achieved for the dipped rod technique, but was for head-space analysis. However a comparison was done at 45 days (Figure 14). At this time, the difference between the two techniques was found to be highly dependent upon the $K_{ow}$ of
POC analyzed. The head-space technique was more sensitive than the dipped rod for TCB and QCB, while the dipped rod was 7x more sensitive for HCB than the head-space technique. Measurement of tetrachlorobiphenyl and hexachlorobiphenyl by the VA-dipped rod technique was considerably more sensitive, 53x and 110x respectively, than the head space technique. Mirex and DPCB were not measured using the head space technique, yet they were easily measurable using the VA-dipped rod.

![Graph showing comparison of sensitivities](image)

**Figure 14:** A Comparison of Sensitivities. Y-axis represents the ratio of dipped rod sensitivity over the sensitivity of a head-space analysis taken from different vials containing the same spiked food sample.
Variability of rod technique

The CV for HPCB was 19.8 for the dipped rod technique compared with 37.6 for head-space analysis. The CVs for the other POC congeners are presented below (Figure 15). In all cases the spun technique exhibited lower variabilities.

![Graph showing variability of different methods](image)

**Figure 15**: A comparison of replicate variabilities for the three methods outlined. The variability is expressed as the coefficient of variation (%). The variability for the thicker VA coating is presented for the spun cover-slip method.
Rod uptake curve

A number of inferences can be drawn for the rod uptake curve data. TCB for instance, appears to have reached equilibrium within the first 10 days of exposure (Figure 16). QCB appears to be assymptoting early in the uptake experiment, but a slight upward trend from day 10 to day 60 suggests that the actual equilibrium may not have been attained during the uptake period. HCB, TPCB, HPCB and Mirex concentrations did not reach equilibrium during the experiment and increased linearly with time suggesting that the time required to achieve equilibrium is much greater than the duration of the experiment.

Variability of cover-slip technique

A 126 ± 5 µg coating of VA on the cover-slip reduced the CV of TPCB and HPCB in relation to the dipped rod technique. However the chlorobenzenes and Mirex still retain a significant variability (Figure 15). Increasing the film thickness had the effect of reducing the CV for all POCs analyzed (Figure 19).

Cover-slip uptake curves

The cover-slip technique using a 18.3 ± 0.5 µg VA coating exhibited a significant reduction in the time-to-equilibrium over the dipped rod technique (Figure 17). Two preliminary uptake experiments used time frames which were too long (2 weeks and 2 days) and consequently equilibrium had already been attained for most analytes before the first data points recorded. The third uptake experiment, presented in Figure 17 revealed that equilibrium was attained for TPCB and
FIGURE 16: UPTAKE CURVES FOR DIPPED ROD TECHNIQUE.
HPCB at about 30 hours and 50 hours, respectively. The variability for the chlorobenzenes as reported earlier is high and interpretation is difficult. However TCB and HCB appear to have reached equilibrium within 4 hours and 24 hours respectively (Figure 17). Analysis of both QCB and Mirex was impossible due to their high variability.

Desorption experiment
Starting with similar impregnated concentrations, all measurable analytes exhibited an inverse relationship between the molecular weight and the depuration rate, shown as slopes in a log(mass/cover slip) versus time plot (Figure 18). The chlorobenzenes dissipated rapidly and except for HCB were not detectable 7 minutes after ambient exposure. HCB was detectable up to 15 minutes and exhibited the greatest fitted depuration slope. TPCB was detectable up to 180 minutes and had the next steepest slope after HCB. Both HPCB and Mirex were measurable for the duration of the experiment and had the shallowest slopes.
Figure 17: Spun cover-slip uptake curve for thinner film (18 mg)
Thicker Coating

A 126 µg coating increased analyte equilibrium times. However, the analysis was easier due to the reduction in variability (Figure 19). All chlorobenzenes appeared to reach equilibrium within 24 hours after which TCB and QCB appear to start losing analyte. The two biphenyls, TPCB and HPCB, did not reach equilibrium during the 140 hours of the uptake experiment, but both were clearly close to their respective equilibrium concentrations. Mirex exhibited a purely linear uptake curve for the duration of the uptake experiment.
Figure 19: Spun cover-slip uptake curve for thicker film (126 mg)
3.6 Discussion

The objective of this study was to develop a method for fugacity determination incorporating VA polymer. The new method was sought in response to the low sensitivity and relatively large variability in head-space fugacity measurements of very hydrophobic organic chemicals. The head-space technique exhibits a CV of 37% for HPCB and has a low sensitivity due to a low Z of air.

Possible explanations for the poor sensitivity and relatively high CV for head-space analysis of PCBs are: 1) contaminated particulate matter from the sample either drawn up inside the syringe or contaminating the tip of the needle could have erroneously increased the head-space analysis. 2) Residual solvent or particulate matter remaining after the needle wash could provide extra surfaces and compartments for the analyzed POC to partition to once drawn inside the syringe and thus be unavailable for analysis. 3) The low variability, could have also been partly due to low sensitivity, as chromatographic noise can interfere with the analyte peak areas when measurements are close to machine detection limits.

By developing a technique incorporating an absorbing film having a high fugacity capacity it was hoped that a better method for fugacity determination could be developed. By concentrating chemical in a small volume of plastic, it was
expected that the signal-to-noise ratio would increase and the effects of transient adsorption of analyte to surfaces would be minimized.

The VA-dipped rod technique yielded improved sensitivity, however equilibrium was not attained for PCBs after two months. The variability, although improved over head-space analysis, still was high. It is suggested that the variability was largely caused by variations of VA surface thickness. Because the rods were hand-dipped, variations in the rate of removal and drying conditions could have significantly affected the rod coating. Due to imperfections, the surface area to mass ratio of the VA coating on the rod was unknown and this could have very significant implication in determining the rate at which POC was absorbed. If equilibrium had been attained during the 2 months of the dipped rod uptake experiment, it is likely that the POC concentration measured in the VA of each rod would have been similar and the 20% variability reduced significantly. At equilibrium the VA concentration is no longer concentration dependent and thus no longer dependent upon variations in the area-to-volume ratio.

The cover-slip spin coating technique was used as a way to avoid problems arising from surface imperfections, while retaining the improvements of sensitivity yielded from the dipped rod technique. As a result of a smaller absorbing mass, the VA spinning technique lost some sensitivity over the dipped rod technique, however variability was notably lower. The VA absorbing mass
was 0.011 mg for the spun cover-slip method as opposed to 0.2 mg of the dipped rod; an eighteen-fold difference.

By spin coating, a greater surface area-to-mass ratio was created and this decreased the equilibrium time of PCBs significantly. The equilibration of TPCB and HPCB are approximately 30 and 50 hours, respectively, for a 18.5 µg film. Chlorobenzene analysis was difficult due to variability between replicates.

The replicate variability seen with the chlorobenzenes during the uptake experiment was thought to be a result of their low concentration in the VA due to the ease by which they desorb from the VA. It was hypothesized that some loss occurred while transferring the cover-slip from the sample vial to the solvent extraction dish. This hypothesis was supported by the desorption experiment results which showed that TCB and QCB were not detectable 15 minutes after the impregnated VA coating was exposed to the ambient environment.

In order to maximize the absorbed concentration and minimize the desorption of chlorobenzenes (CBs), an uptake curve using a thicker VA film was conducted. This had the effect of decreasing the average CB variability. It also had the effect of increasing the sensitivity of all CB and PCB congeners (Figure 17 Figure 19 in results section). It is interesting to note that the improvement in
sensitivity decreases with increasing molecular weight, thus emphasizing the importance of desorptive loss processes.

Before switching to the spun cover-slip method, there was some question regarding which phase was limiting the transfer of POC into the VA film and resulting in an equilibrium time greater than 2 months. There were three distinct compartments in each sample vial: a semi-fluid food sample, $N_2$, and the VA film. As convection was introduced into the $N_2$ and semi-fluid food compartments for both the dipped rod and spun disk methodology, it was thought that diffusion in these compartments would no longer be limiting and mass transfer of analytes would not be dependent upon molecular weight. Thus, if mass transfer through the air or food sample was limiting the uptake curves for each POC analyzed would exhibit identical uptake kinetics. Mass transfer through the semi-solid VA, on the other hand, would be primarily the result of diffusion and consequently, analytes would diffuse at a rate dependent upon the analyte molecular weight. Thus, if mass transfer through the VA was limiting, initial uptake rates would decrease with increasing molecular weight. As discussed previously, Stokes' law states that the diffusion rate should be inversely proportional to the cube root of the molecular weight.

The results from the spun cover-slip uptake experiment showed that the initial uptake rates decidedly decreased with increasing molecular weight and,
therefore, it can be concluded that diffusive transfer through the VA is limiting the uptake of chemical.

A simpler way of coming to the same conclusion is to compare the uptake kinetics of both the dipped rod technique and the spun cover-slip technique. By the very fact that the time-to-equilibrium for chlorobenzenes and PCBs was decreased greatly by using a thinner film indicates that the VA film was the limiting compartment.

**Improved uptake rate explained: dipped rod vs. spun cover-slip:**

Results of the cover slip spinning method showed that an equilibrium for HPCB can be achieved within two days. A simple calculation reveals why equilibrium was attained so quickly. Knowing the mass of coating, density of VA and approximate surface area of both the dip and spin methods, film thickness were calculated for each (Figure 20).

The dipped rod method film was estimated as having a film 1.68 μm thick and the spin method 2.36 x 10^2 μm thick - the latter being almost 71-times thinner. Einstein's relationship (Chang 1981) states that diffusion rate is proportional to the distance squared, thus it is easy to understand why PCB uptake showed no sign of reaching equilibrium for the dip method even after two months.
Calculation of film thickness

For rod:

assumption made: coating is very thin and can be approximated as being a sheet with an area:

2π r h and a thickness d.

Then:

thickness d = V/A = volume of ethylene vinyl acetate / area of ethylene vinyl acetate coating.

A=2π r h = 1.23 cm²
where:

r = 0.15 cm = radius of metal rod
h = 1.3 cm = depth to which rod was dipped in VA solution

V = m/ρ = 2.1 x 10⁻⁴ cm³
where:

m = 0.2 mg = mass of coating (measured on an analytical balance)
ρ = 965 mg/ml = density of VA (taken for Dow chemical product information)

Then:

d = V/A = 1.68 x 10⁻⁴ cm = 1.68 μm

For Cover slip:

A = L² = 4.84 cm²
Where:

L = 2.2 cm = the length and height of the cover slip

V = m/ρ = 1.14 x 10⁻⁵ cm³
where:

m = 1.10 x 10⁻² mg = mass of coating (measured on an analytical balance)
ρ = 965 mg/ml = density of VA (taken for Dow chemical product information)

Then:

d = V/A = 2.36 x 10⁻⁶ cm = 2.36 x 10⁻² μm

In Comparison:
The cover slip methodology produces a film which is 71-times thinner.

Figure 20: Calculation of VA film thickness.
**Fugacity Capacity of VA**

The uptake curve using the spun cover slip methodology revealed that equilibrium is achieved for HPCBs within 50 h and the equilibrium concentration of HPCB within the VA was 2.60 mg/g or 6.95 mol/m³. Knowing the VA concentration it is possible to calculate the fugacity capacity of VA for 2,2',4,4',6,6' HPCB as long as the fugacity of the sample can be determined. In the trout dosing experiment (section 2), it was revealed that fugacity can be easily calculated if the head space concentration of a sample at equilibrium is known. In this section, the same HPCB-spiked food was used and the head space concentration was known to be 3.71 pg/mL (value taken from section 2 - stomach content fugacity), which corresponds to a fugacity of $2.55 \times 10^5$ Pa. By dividing the VA concentration by the sample fugacity, the HPCB fugacity capacity was calculated to be $2.73 \times 10^5$ mol/m³Pa (Figure 21). It is interesting to note that the calculated fugacity capacity for lipid using a Henry's law constant of $16.7$ Pa·m³/mol is $4.98 \times 10^5$ mol/m³Pa, which is only a factor of 1.82-times larger. This suggests that VA may be a good surrogate for lipid in chemical partitioning studies (Figure 22), at least for HPCB. Further studies with other POCs are needed.
Calculation of HPCB concentration in ethylene vinyl acetate (C\textsubscript{VA}): 

\[ C\textsubscript{VA} = \frac{M_{\text{HPCB}}}{M_{\text{VA}}} = 2.60 \times 10^{-3} \text{ g/gVA} \]

\[ M_{\text{HPCB}} = 2.86 \times 10^{-2} \text{ ug (determined from gas chromatograph)} \]

\[ M_{\text{VA}} = 11.0 \text{ ug (measured on balance)} \]

Knowing the ethylene vinyl acetate density and the molecular weight of HPCB, the concentration can be expressed in terms of mols/m\textsuperscript{3}:

Now \[ C\textsubscript{VA} = 6.95 \text{ mols/m}^3 \]

Density\textsubscript{VA} = 965 Kg/m\textsuperscript{3} (from Dow chemical product information)

MW\textsubscript{HPCB} = 360.88 g/mol

Calculation of sample fugacity \( f \):

\[ f = P_{\text{HPCB}} = nRT/V = 8.10 \times 10^{-5} \text{ Pa} \]

Where:

\[ n/V = 1.028 \times 10^{-8} \text{ mol/m}^3 = \text{head-space concentration of HCB spiked food used (derived in the trout experiment)} \]

\[ R = 8.314 \text{ (Pa m}^3)/\text{ (mol K)} = \text{Avigadro's constant} \]

\[ T = 273 \text{ K = temperature in degrees Kelvin} \]

Calculation of ethylene vinyl acetate fugacity capacity \( Z_{\text{VA}} \):

\[ Z = \frac{C\textsubscript{VA}}{f} = 2.73 \times 10^5 \text{ mol/(m}^3\text{. Pa)} \]

Where \( C\textsubscript{VA} \) and \( f \) are calculated above.

\textbf{Figure 21: Calculating The Fugacity Capacity of VA}
Usefulness of VA as a fugacity measuring tool

i. Sensitivity Issues

The biological samples used for testing VA as a fugacity measuring tool were all spiked with a large concentration of several POCs. HPCB, for example, was used at a concentration of 900 ppm. A ten-fold reduction in concentration could still be easily measured using the spun cover-slip method with the thicker VA coating. However, neither VA-film technique would be suitable for measuring the much lower concentrations often found in the environment. Because both VA methodologies require that the analyte be extracted from the VA using solvents,

Calculation of fugacity capacity for other matrices

Lipid: \( Z_L = K_{OW} \cdot \frac{D}{H_{HPCB}} = 4.98 \times 10^5 \text{ mol/(Pa m}^3\text{)} \)

Air: \( Z_A = \frac{1}{RT} = 4.04 \times 10^{-4} \text{ mol/(Pa m}^3\text{)} \)

Water: \( Z_W = \frac{1}{H_{HPCB}} = 9.90 \times 10^{-3} \text{ mol/(Pa m}^3\text{)} \)

Where:

\( K_{OW} \) = the octanol-water partition coefficient for HPCB = \( 10^{6.92} \) (Hawker 1988)

\( D \) = the density of lipid = 0.9

\( H_{HPCB} \) = Henry's constant for HPCB = 16.7 Pa m\(^3\)/mol (Dunnivant 1992)

\( R \) = Avigadro's constant = 8.314 (Pa m\(^3\))/(mol K)

\( T \) = temperature in degrees Kelvin = 273 K

**Figure 22:** Determination of HPCB fugacity capacity for several different matrices.
a considerable dilution occurs. Of the final 2 mL collected, only a 1 ul sample was injected. This, as discussed earlier, represents a 2,000-fold sample dilution. If the total extract could be reduced or more injected, sensitivity could be improved, thus making VA coatings more usable.

The use of a GC-injection port which thermally atomizes VA may be the ultimate solution. This type of injection port would enable the introduction of VA film and absorbed PCB directly to the GC eliminating the need for a extraction and concentration step. The VA film would be atomized, allowing the full sample to be passed through a gas chromatographic column with out fear of precipitation. This approach would, for reasons stated above, effectively increase sensitivity approximately 2,000-fold over the approach presented earlier in this thesis.

ii. Uncertainty issues.

As the VA methods stand, they cannot be used to measure actual fugacities in units of Pa and hence will only be useful where a researcher wants to examine the difference (in terms of a ratio) between two matrices. This is fine for experiments like in chapter 2, where the difference between the food and intestinal chyme fugacities was highlighted.
To determine the actual fugacities, the $Z$ of VA for each POC analyzed for must be known. In this chapter, $Z$ was determined for HPCB. However, the calculation was dependent upon determining the actual fugacity, which was done by performing a head-space analysis on the sample. By relying on head-space analysis the 37% CV associated with head-space analysis of HPCB fugacity is incorporated into the determination of $Z$. $Z$ must also express any error inherent in the measurement of POC concentration.

Having to include error due to head-space analysis into a fugacity determination using VA, defeats the purpose of using VA as an alternative fugacity measuring technique. One of the main reasons for switching from head-space analysis was to avoid the large inherent errors.

3.7 SUMMARY

VA was found to be an effective absorbing medium for POC fugacity analysis. Compared to head-space analysis, the VA techniques had smaller replicate variabilities and better sensitivity for the POCs having larger molecular weights. For HPCB the variabilities were found to be 37.6% for head-space, 19.8% for VA coated rod and 12.2% for VA (126 μg) coated cover-slip. Sensitivities using VA dipped rod were 7.0x, 52.9x and 110x better than head-space analysis for HCB, TPCB and HPCB respectively. Mirex was not detectable using head-space
analysis. Head-space analysis had a greater sensitivity for measuring TCB and QCB, being 4.3x and 1.3x better, respectively.

The time to equilibrium for the VA-dipped rod was impractical, as the slopes of the uptake curves for the PCBs and Mirex were still linear even up to 2 months of VA exposure. The VA spun cover-slip method was a vast improvement over the dipped rod method as equilibrium was attainable within 30 and 50 hrs for TPCB and HPCB respectively using a 18.5 µg coating. However, the decrease in equilibrium time was at the expense of replicate reproducibility.

The three methods exhibited sensitivities which would make them useful where only high concentrations of analyte are used, such as in the laboratory. As an example, the suggested concentration of 2,2',4,4',6,6' -hexachlorobiphenyl (HPCB) in biological samples is greater than 900 ppm. Concentrating of VA extracts down from 2 mL would be an easy and effective approach to improving the sensitivity.

The VA methods used in this thesis can not be used practically to determine actual fugacities. Z values of VA for the POCs used in this thesis must first be known. One sample calculation was performed using HPCB, however the approach used makes switching to VA redundant as it requires the use of head-space analysis results (and their inherent uncertainty). The uncertainty
associated with head-space analysis was one of the primary reasons for developing the VA methods.

Presently the VA methods can be used in applications where the difference in fugacity (expressed as a ratio of POC concentrations in VA) must be known.
4. Appendix 1: Factors which may determine the extent of biomagnification.

There are several theories which strive to explain how biomagnification can occur, two of which explain how uptake can occur against a fugacity gradient. One theory suggests that chemical is taken up via some energy dependent active transport process and the other suggests that modification of food during digestion reduces the fugacity capacity, $Z_d$, thus creating a fugacity gradient favorable to absorption via passive diffusion (Gobas et al. 1993, Vetter et al. 1985). The latter theory suggests that a drop in diet fugacity capacity would result in a BMF greater than 1 once the fish had reached equilibrium with its intestinal content, i.e., once $f_F = f_d$. Where $f_F$ is the contaminant fugacity in the intestinal chyme and $f_d$ is the contaminant fugacity in the diet. Therefore, to understand biomagnification, there is a need to understand how food is altered during digestion and the factors which determine the degree of this alteration.

4.1 Digestion/Absorption of Lipids By the GI Tract

Although there is some disagreement regarding how the presence of dietary lipid impacts upon biomagnification, many researchers believe that dietary lipid influences the extent of dietary uptake of contaminants (Gobas 1988, Connolly
1988). To grasp the importance of digestion in determining the uptake efficiency of xenobiotics, a basic understanding of gastro-intestinal physiology and lipid digestive processes must first be attained.

The following section will also present the reader with some factors which have the potential to determine the extent to which lipids are digested and absorbed; factors which may have an impact on the extent of POC absorption. Triglycerides will be focused upon as, in most animals, they make up the bulk of total lipid in the diet. In humans, for instance, they provide 90% of the total calories supplied by fat (Borgstrom 1972, Carey 1983, Northfield 1975). Lipid digestion has been divided into several sub-categories below. As most work has been done with mammals, the following section will discuss lipid digestion and absorption from a mammalian perspective. Differences between mammals and fishes will follow in section 4.1.8.

4.1.1 GASTRIC LIPID PARTITIONING

Due to the erect posture of humans there is some evidence to suggest that fats will float to the top of the stomach and not be released until all the aqueous soluble gastric contents of been emptied (Sanford 1992). Although the implications of this are unclear, it would be logical to predict that an improvement in lipid absorption could occur because they are digested somewhat independently of the aqueous food components (Granger 1985,
Sunvold 1995). Animals living in the prone position are unlikely to enjoy a similar separation of food components based on component density.

4.1.2 EMULSIFICATION / HYDROLYSIS

Before reaching the intestine, food is exposed to two types of lipase, lingual lipase and gastric lipase. Both are called acid lipases in reference to having their highest activity in the acidic medium of stomach (Field 1983, Roberts 1984). These enzymes cleave one fatty acid off triglycerides, forming diacyl glycerol and a fatty acid (Stafford 1981, Abrams 1988, Hamosh 1981, Cohen 1971). Triglycerides that have short fatty acid chain lengths are cleaved faster than those with long fatty acid chain lengths (Liao 1984); neonates feeding on milk may require this faster metabolism as milk fatty acids are fairly short and the pancreatic function (i.e. emulsification and hydrolysis) is not fully functional (Grand 1976).

In the lower regions of the stomach, the chyme is repeatedly squirted, ground and retropelled, resulting in the emulsification of free fatty acids, diacyl glycerol and triglycerides. (Tso 1994)

4.1.3 MICELLE FORMATION

The addition of bile acids in the anterior intestine creates micelles, which solubilize the products of lipolysis, allowing a further reduction in lipid particle
size (Hofmann 1964). Addition of lipid metabolites beyond the micelle saturation point results in the formation of unimellar vesicles (liposomes) (Carey 1983, Stafford 1981). Although liposomes are found in the gastrointestinal tracts of healthy people, they are thought to play a greater role in those people who have low intraluminal bile salt concentrations (Carey 1983, Porter 1971). The relative roles of both micelles and liposomes are presently not well understood.

The reduction of size of lipid particles increases the effective surface area of dietary lipid, thus increasing the enzymatic efficiency of pancreatic lipase which cleaves fatty acids off diacyl glycerol (Mattson 1964 and 1968).

In addition to increasing the surface area, micelles have the added benefit of increasing the aqueous solubility of ingested lipids. The unstirred aqueous layer within the glycocalyx can be an effective barrier to lipophilic macromolecules, but transported within a micelle, these molecules diffuse through the unstirred layer at a rate about 100- to 1000- times faster (Dietschy 1971, Westergaard 1976, Wilson 1971). Thus, lipolytic products and lipid soluble chemicals are more available to be passively absorbed by enterocytes. Any factors that decrease the formation of micelles, could also decrease the diffusion of lipolytic products to the absorptive surface.

Both the emulsification of lipid and the formation of micelles enhance the surface area of ingested lipids, allowing a greater interaction of lipase which is only
active at the lipid-aqueous interface (Mattson 1964 and 1968). If either the extent of fat emulsion is decreased or the ability to form micelles is retarded, it is known that the efficiency of fatty acid production and hence, absorption can be diminished (Tso 1994).

4.1.4 Absorption

Fat absorption occurs primarily in the proximal intestine (Honknen 1985). The prevailing theory today is that absorption of lipid metabolites (monoacylglycerol, glycerol and fatty acid) by the brush border is a passive process (Thomson 1981). In the past, it was believed that this process was unregulated diffusion. However, new evidence suggests that large fatty acids may be mediated by protein carriers (Stremmel 1988, Ockner 1974, Fleischner 1977). Research has shown that during food digestion cytosolic and membrane F.A. binding proteins are vastly outnumbered (100x) by fatty acids and it has been suggested that fatty acids may be transported through the cytosol as small aggregates or micelles (Vetter, 1985). Vetter appears to be suggesting that pinocytosis is involved in fatty acid absorption and omits the possibility of free diffusion through the brush border membrane. Others believe that fatty acid in excess of that which can be handled by protein binding is taken up via passive diffusion (Chow 1979). Recent histological studies have shown that pinocytotic vesicles
in the proximal regions of the intestine are rare, thus adding support to the diffusion theory (Garrido, 1993).

There are several factors which can determine the rate of diffusional uptake of lipid by-products; exposure duration, resistance to passive diffusion and size of lipid by-product potential gradient. Diffusional resistance is primarily a function of two parameters; molecular weight and polarity of diffusing molecule (Berne & Levy). In order for a molecule to diffuse through the phospholipid bilayer of a cell membrane it must first be able to completely dissolve in the non-polar region, thus the rate of membrane diffusion tends to increase with decreasing degree of polarity (Berne & Levy). Molecular weight is important as it is indicative of the size of a diffusing molecule. The larger a molecule the slower it's diffusion rate as it must displace the membrane phospholipids to a greater degree. Some molecules are so large that the large threshold energy required for diffusion makes membrane diffusion unlikely (Berne & Levy). This may be why triglycerides are not absorbed intact. The threshold energy of diffusion may also be the reason why larger fatty acids have specific membrane binding proteins (1).

Exposure duration is primarily a function of intestinal transit time. As diffusion does not occur instantaneously, a shorter residence time of food within the intestine may preclude efficient absorption of lipid by-products. Increasing the meal size, for instance, results in an increase in the amount of food digested per
unit of time and a decrease in time that food is in contact with the intestine (Jobling 1977). As the absorptive surface of the intestine is typically in excess to what is required for normal lipid absorption, small fluctuations of intestinal motility is not expected to make a significant difference in the absorption efficiency of lipid. However if lipid is included in a food containing a high percentage of indigestible roughage, the decreased surface exposure of the dietary lipid combined with the increased motility can affect lipid absorption efficiency (Meienberger 1993, Granger 1985).

The chemical potential gradient of lipid by-products determines the magnitude of diffusion in the same manner as it does for chemical contaminants. The larger the chemical potential in lumenal lipid by-products and the smaller the chemical potential of lipid by-products in the enterocytes, the greater the chemical potential gradient and the greater the net flux of lipid byproducts into the enterocytes. Determining the size of chemical potential on either side of the cell membrane is the amount of freely dissolved lipid byproducts whose levels are dependent upon the degree of micelle diffusion through the unstirred layer, de-esterification in the intestinal lumen and re-esterification within the enterocyte cytosol. Increasing de-esterification by lipases, increases the concentration of lipid byproducts on the outside of enterocytes and re-esterification decreases the concentration of lipid byproducts on the inside of enterocytes, both of which result in a chemical potential favoring the uptake of lipid byproducts. As both
lipases and T.G. synthetases can efficiently catalyze their respective reactions in reverse, it would be logical to assume that cytosolic triglyceride removal is also crucial to the maintenance of a lipid chemical potential favoring the efficient absorption of dietary triglycerides.

4.1.5 **Cytosolic Transport of Lipid Metabolites and TG Resynthesis**

Once inside the enterocytes of the brush border, the lipid metabolites either freely diffuse or are transported by specific protein carriers to the area of triglyceride synthesis, which is the smooth endoplasmic reticulum (Ockner 1974). Several binding proteins have been isolated each with varying degrees of specificity for fatty acids (Marquardt 1986).

Triglycerides are re-synthesized via the monoacylglycerol pathway and packaged predominantly within pre-chylomicrons, although a small percentage of fatty acids, those derived from the hydrolysis of lesolecithin, are re-synthesized using the $\alpha$-glycerophosphate pathway and packaged into pre-very low-density-lipoproteins (VLDL's) (Tso 1984).

Fatty acids having less than 12 carbons are less likely to be made into triglycerides and instead pass unchanged to the portal blood stream (Mansbach 1991, Hyun 1967). The path taken by these molecules appears to depend on
diffusion into and through cells rather than traveling through intracellular spaces, as chylomicrons do.

4.1.6 Chylomicron/LDLP SYNTHESIS AND TRANSPORT

Once synthesized, chylomicrons and VLDLs are transported to the basal-lateral membrane of the enterocyte by cytosolic microtubules, where they are released into the intercellular space via phagocytosis (Sanford 1992). Chemicals known to impede microtubule action have been found to reduce lipid absorption when administered within the diet (Glickman 1976).

Phospholipid levels have also been found to have an effect on the absorption of triglycerides, and it is now thought that phospholipids are an important component of pre-chylomicrons and pre VLDLs (Bennet-Clark 1978, Tso 1981). If levels of phospholipids are too low, there will be a triglyceride build up within the enterocyte and re-esterification of lipid byproducts will be retarded (Bennett-Clark 1991). Most of the phospholipid found in the lumen is biliary in origin. Thus conditions which inhibit the production and excretion of bile, combined with dietary shortages of phospholipids, may result in poor absorption of triglycerides from the diet.
Once pumped out of the enterocyte cells, the volume of chylomicrons in the basolateral, intercellular space begins to increase until the basement membrane separating the enterocytes form the lamella propria breaks (Tso 1973, 1994). The chylomicrons and absorbed fluid then diffuse through the intercellular spaces in the lamella propria towards the central lacteal where the chylomicrons join up with the lymphatic system (Shepherd 1959, Barona 1975). The endothelium of the central lacteal contains gaps through which intact chylomicrons can flow. Vesicular transport through the endothelial cells is also known to occur (Dobbins 1970). The extent to which each of these mechanisms operate is presently unknown (Tso 1994).

The absorption of fluid is thought to be crucial in the transport of chylomicrons, as the convective flow through the lamella propria vastly decreases the time which it take for chylomicrons to reach the lymph (Tso 1985). Lymph flow appears to be stimulated by amino acid and fatty acid absorption (Turner 1977, 1978), and may also be facilitated by piston-like contractions and relaxations of the intestinal villi. (Sanford 1992).

In humans, the appearance of chylomicrons increases up to a flow of about 40 µl/min for each central lacteal, beyond this flow rate, the appearance of chylomicrons remains constant indicating that flow rate is no longer the limiting step (Tso 1985). It has been hypothesized that re-esterification, chylomicron
formation and subsequent discharge into the lamella propia together become limiting to the uptake of lipid (Tso 1994). The potential exists that if very low fluid flows coincide with lipid digestion/absorption, lipid products will accumulate in the enterocyte and will impede diffusive uptake of fatty acids. Low fluid flows may occur in instances where a dry diet is being consumed.

4.1.7 Bile Acid Reabsorption and Enterohepatic Circulation

After lipid metabolites have been absorbed within the duodenum and proximal jejunum, bile acids (BA) continue traveling through the intestine where they are absorbed both actively and passively (Sanford 1992). Passive uptake occurs in all regions of the small intestine, while active transport only occurs within the posterior ileum (Granger 1985). De-conjugation and dehydroxylation of bile acids by bacteria within the posterior ileum and colon increase their lipid solubility and hence enhance passive uptake through the brush border (Sanford 1992). Approximately 50% of BA uptake is via passive diffusion, while 7 to 20% are lost via the feces (Sanford 1992). This loss is made up from synthesis of bile acids within the organism (Granger 1985).

Once absorbed, BAs enter the hepatic-portal vein. Around 80% of BA are absorbed during the first pass through the liver where the deconjugated BA's are
re-conjugated before being released to the total bile pool. This enterohepatic recirculation must occur 2- to 5- times per average meal (Granger 1985).

Efficient bile acid absorption is important, because without recycling, the levels of bile acids available for effective intestinal micelle formation becomes compromised (Sanford 1992). As discussed earlier, micelles increase the surface area available to lipase action and assist in the absorption of fatty acids, glycerol and monoglycerides.

4.1.8 A PHYSIOLOGICAL COMPARISON OF FISH AND MAMMALS RELATING TO LIPID ABSORPTION

Although the GITs of fish and mammals share many similarities in both structure and function, there are a number of notable differences. Like mammals, the structure of fish gastro-intestinal tracts (GITs) vary considerably. Trout, and many other fish have caeca, finger-like projections radiating from the proximal intestine. The true function of the caeca is unknown although there is some evidence which suggests that it may play an important role in lipid absorption due to it's large surface area (Buddington and Diamond 1986, Bauermeister 1979). Cyprinid or carp-like fishes, may be missing defined stomachs and have very long alimentary tracts with few folds (De Silva and Anderson 1988). Diets of this group of fish are usually low in lipid and it is probable that if fed a high fat diet lipids would be absorbed poorly. Compared
with mammals, the intestine of fish is relatively undifferentiated and have been found to adjust morphologically to changes in feed nutrient proportions over generations. (De Silva 1984).

The absorption and distribution of lipids in fish is highly dependent upon the number of carbons in the free fatty acids. Fatty acids shorter than about 10 carbons will diffuse easily and directly to the blood vessels lining the epithelial cells (Sheridan 1987). Free fatty acids longer than 10 carbons are re-esterified to triglycerides in the endothelial cytosol and packaged into vesicles resembling chylomicrons. These vesicles are transported to the lamella propria where they build up for reasons not entirely understood. Robinson and Mead (1973), citing histological work done by Greene (1913 as cited in Robinson and Mead 1973), postulated that the build up of vesicles was due to a lack of pores in the walls of circulatory vessels permeating the lamella propria.

Fish are also known to be lacking a defined lymphatic system. Chylomicrons which manage to leave the intestine are thought to be carried either by blood vessels or by a lymphatic-like system called the secondary circulatory system (Steffensen 1992). Unlike a true lymphatic system, the secondary circulatory system of fish is continuous; originating from arteries, branching out into capillaries and merging again before meeting up with veins (Steffensen 1992). Most of the capillary beds of the secondary circulatory system have been found
on the surfaces of tissues suggesting that the secondary circulatory system plays primarily an immune system role (Steffensen 1992). An exception to this is the secondary circulation of the gut.

The triglyceride content of fish blood and liver is consistently low, prompting some researchers to suggest that lipids are transported primarily as free fatty acids (Robinson and Mead 1973). However new evidence suggests that lipids are transported as chylomicrons (Sheridan 1987), low density lipoproteins (LDLs) and high density lipoproteins (HDLs). The low concentrations of triglyceride makes sense if the diffusion of vesicles from the lamella propia is limiting and may also explain why fish are known to be slower at absorbing lipid than mammals.

The main lipid storage sites for fish and mammals are also different. Mammals use predominantly adipose tissue while fish rely on their liver and skeletal muscle. Sluggish bottom fish predominantly use their livers while active fish predominantly use their muscle (Sheridan 1987). Although to a smaller degree than mammals, excess fat can also be deposited around the GI tract.

The function of chylomicrons in TG absorption has been discussed in chapter one. However, the role of chylomicrons in the thermodynamic model of biomagnification requires special mention here. Chylomicrons perform two
functions which are crucial to the maintenance of a fugacity gradient across the brush boarder and thus biomagnification; (a) they increase the fugacity of POC in the intestinal lumen, and (b) they maintain the fugacity of POC in the enterocyte at a low level.

Without chylomicrons, resynthesized triglycerides in the cytosol of enterocytes would build up and impede the synthesis of new triglycerides. Lipolytic products such as fatty acids, glycerol and monoglycerides in the cytosol would diffuse to the lymphatic system and arterioles, but at such a slow rate that their higher cytosolic levels would thermodynamically discourage the diffusional absorption of luminal lipolytic byproducts, hence poor triglyceride absorption. As previously discussed, with out the efficient absorption of lipid, the fugacity of POC would not increase in the lumen and diffusional uptake of POCs by the enterocytes would be poor.

By-actively removing triglycerides with partitioned POCs from the enterocyte cytosol, chylomicrons are able to maintain a low cytosolic POC fugacity and thus create a fugacity gradient favoring the diffusional absorption of POC from the lumen. If chylomicrons were not actively removed, the fugacity of POC associated with cytosolic lipid would soon become comparable to luminal POC fugacity and all intestinal absorption of POC would halt.
4.2 SOME FURTHER IMPLICATIONS OF DIGESTION AND DIET TYPE ON POC

ABSORPTION:

4.2.1 LIPID DIGESTION AS A PARAMETER OF THE FUGACITY GRADIENT

The results of chapter 2 present evidence that digestion of dietary lipid is primarily responsible for the increase in luminal fugacity, referred to in literature as gastrointestinal magnification (Gobas 1993). It is the increase in luminal fugacity which creates a fugacity gradient which essentially drives the diffusional uptake of POC across the brush border of the intestine and ultimately determines the extent of biomagnification. Logically it would follow that any factor affecting the digestion of dietary lipid will also have a profound affect on the contaminant fugacity gradient and thus the extent of biomagnification. The uptake efficiency of POC would also be a factor of lipid digestion, but would likely exhibit a poorer correlation to lipid absorption efficiency than biomagnification because POC uptake efficiency is dependent upon another variable, time. POC uptake efficiency, therefore, would be affected by factors such as ingestion rate, perfusion rate and diffusion rate, which have no effect on the ultimate biomagnification.

As both uptake efficiency and biomagnification may be profoundly affected by changes in lipid digestion, it follows that an understanding of the fugacity capacity of lipids and factors which determine the extent of lipid absorption could
be important in predicting the magnitude of POC dietary uptake efficiency and biomagnification. Previous paragraphs have discussed the mechanism of lipid absorption and gave examples of impairments which could significantly affect normal lipid absorption and thus POC gastrointestinal magnification.

Examples of crucial steps were: formation of micelles, formation and transport of chylomicrons, absorption of fluids, and re-esterification of triglycerides.

Following are a number of non-mechanistic variables which would likely have profound effects on the extent of gastro-intestinal magnification. In a nutshell, these are: physiological differences in the GI tract, fatty acid chain length, food consistency and variation in fugacity capacities for different lipid types.

4.2.2 LIPID DIGESTIBILITY / ABSORPTION

4.2.2.1 Physiological Differences Between Fish Species

Differences in physiology are known to result in differences in lipid absorption rates of different fish species (Takeuchi 1979). Differences in neiche often dictate the function and structure of the digestive tract. For instance, planktivores, such as the Milkfish, have long narrow GITs with no defined stomach, while most carnivores, such as the trout, have defined stomachs and much shorter intestinal tracts (De Silva 1995). Research has shown that rainbow
trout (a carnivore) exhibits a greater lipid absorption efficiency than carp (an omnivore, emphasizing plant sources of food) when fed a diet containing fish oil (Takeuchi 1979). The greater lipid absorptive efficiency suggests that carnivores, such as trout would also exhibit greater fat soluble xenobiotic BMF.

4.2.2.2 Source of Lipid

Fatty acid absorption is known to be a function of animal type and the chain length and degree of saturation of fatty acid. Trout, for instance, tend to be poor at absorbing long chain saturated fatty acids (Austreng 1980). This tendency would have an impact on the fugacity capacity achieved within the GIT.

4.2.2.3 Consistency of Food

Digestibility studies comparing natural and formulated fish foods have found that natural foods tend to be digested to a much greater degree (Jobling et al. 1986). The argument presented by Jobling et al. was that formulated foods which typically break down in to small particles quickly are released from the stomach at a larger rate and end up overloading the digestive and absorptive capacity of the intestine. Just by mincing the natural prey (whole fish), Jobling observed a 14% reduction in the absorption of energy yielding components of the food. If the energy yielding components of the food in this case are predominantly lipid, the reduction in lipid absorption may minimize the reduction in chyme Z and thus
decrease the fugacity gradient achieved between the intestine and the rest of the fish.

4.2.3 Fugacity Capacity of Lipid Types

In most bioaccumulation studies, lipids are assumed to have homogeneous qualities and, hence, are considered to have similar POC absorbing capacities. Differences in fugacity capacity between lipid types could have an impact on the fugacity gradient created within the GIT. For instance, if one type of lipid having a high $Z$ is absorbed to a lesser degree than lipids with relatively lower $Z$ values, the fugacity gradient produced would be smaller than that predicted by the total lipid mass. As will be discussed next, evidence does exist that different lipid types may have different $Z$ values.

Animals having long-term exposure to POCs often have a tissue distribution of fat soluble xenobiotic which can not be solely explained by the concentration of fat in each tissue. One study found muscle having concentrations 30-times that of the brain when concentration is expressed on a lipid weight basis (Philips 1981). A possible explanation of this discrepancy was that the lipid types found in each of these tissues differ significantly and that the solubility of xenobiotic in (or fugacity capacity of ) different lipid types varies widely (Philips 1981).
Opposing evidence that different lipids have distinct fugacity capacities for POCs is the research done by Dobbs et al. (1983, 1984). Their studies showed that the type of fat is not a critical parameter in determining the solubility of lipophilic xenobiotics. (Dobbs, 1983, 1984). Also, other investigations of body POC distribution suggest that chemicals are evenly distributed when tissue concentrations are compared on a lipid weight basis (Noguchi & Heffelburg 1991, Guarino et al 1978). This would then indicate that steady state may not have occurred within the organism for Holden and Marsden's experiment, even if steady state appears to have been reached between an organism as a whole and its surroundings. It is interesting to note that those tissues with the highest lipid weight concentrations of contaminant metabolize lipid for energy. The brain, on the other hand, only utilizes glucose (Berne & Levy 1988). By traveling in association with lipids, POCs are more soluble and will likely realize a greater flux into those cells utilizing fat as an energy source, thus explaining the tissue concentration discrepancy.

4.2.4 Resistance to Contaminant Uptake

It is noteworthy that despite intestinal uptake of contaminant, fugacities of the anterior intestinal content consistently remained much higher than the below-detection-limit fugacities of fish tissue. The continual difference indicates that resistance to absorption must be considered in addition to the size of fugacity gradient when trying to model contaminant uptake efficiencies. This resistance is
a function of the solubility of the organic contaminant within a given matrix as well as the matrix's viscosity.

Factors which may affect resistance to GIT absorption are: ambient temperature, number of micelles, solubility within micelles, solubility in enterocytes cytoplasm, ability to diffuse through membranes.

4.3 OTHER FACTORS:

4.3.1 DEPENDENCE OF BMF ON BODY LIPID COMPOSITION

Although temperature has been found to have little or no affect on the dietary POC absorption efficiency (Santulli, 1993), it could have an affect on more long term biomagnification studies. Temperature has been found to be a parameter in determining the lipid composition of fish. One study yielded up to a 10% increase in body lipid composition when the ambient water temperature decreased from 22 to 15 °C (Alliot, 1983). The change in lipid composition would increase the total body fugacity capacity of the fish and the fish body concentration of POC achieved at steady state would be greater. As the biomagnification factor (BMF) is the POC concentration in a fish divided by the
POC concentration in its diet, the BMF would be higher for a fish in cooler water (refer to formula 1-2).

4.3.2 Ectotherms vs. Endotherms

When an endotherm ingests food at environmental temperatures and consequently warms it to the internal temperature of the ingesting organism, the fugacity of the ingested food should also increase. The increase in fugacity suggests that uptake efficiency of endotherms should be greater than ectotherms ingesting the same food if all other factors are the same. A quick calculation using the ideal gas law reveals that an organism with a 37.5°C blood temperature will have an intestinal fugacity 1.1 x (10 percent) higher than that for an organism having a blood temperature of 10°C, regardless of the original temperature of the food.
Table 1: Numerical results of the gastro-intestinal experiment - Chyme data only.
Table 2: A comparison of replicate variabilities for the three methods outlined. The variability is expressed as the coefficient of variation (%). The variability for the thicker VA coating is presented for the spun cover-slip method.

<table>
<thead>
<tr>
<th>Method</th>
<th>Coefficient of Variation (%)</th>
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<tbody>
<tr>
<td>VA spun</td>
<td>9.0</td>
</tr>
<tr>
<td>Dip rod</td>
<td>9.6</td>
</tr>
<tr>
<td>Spin (126ug)</td>
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</tbody>
</table>

Table 3: A Comparison of Sensitivities. The mass injected column represents POC mass injected into the GC from head space analysis and using the VA dipped rod method - both taken from separate but identical samples allowed to equilibrate for 45 days. The relative sensitivity column represents the mass injected divided by the detection limit for each chemical. Note that the detection limit was similar for each chemical and therefore, a single detection limit was applied to all. The final column compares the relative sensitivities of the two methods by dividing the dipped rod relative sensitivity by the head-space relative sensitivity.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Mass injected (pg)</th>
<th>Relative Sensitivity</th>
<th>VA dipped/Head-space</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCB</td>
<td>1234</td>
<td>9.3</td>
<td>22</td>
</tr>
<tr>
<td>QCB</td>
<td>2.1</td>
<td>0.29</td>
<td>16</td>
</tr>
<tr>
<td>HCB</td>
<td>0.29</td>
<td>0.14</td>
<td>20</td>
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<tr>
<td>TPCB</td>
<td>2255</td>
<td>0.14</td>
<td>75</td>
</tr>
<tr>
<td>HPCB</td>
<td>224466</td>
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<td>168</td>
</tr>
<tr>
<td>Mirex</td>
<td></td>
<td></td>
<td>46</td>
</tr>
<tr>
<td>Detection limit</td>
<td>0.05</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

VA dipped rod: 22, 16, 20, 75, 168, 46
6. Literature Cited


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