Evaluation of a Body-Residue-Based One-Compartment First-Order Kinetic Model for Estimating the Toxicity of Mixtures of Chlorinated Organic Contaminants to Rainbow Trout (*Oncorhynchus mykiss*)

by

Ulysses Klee

A thesis presented to the University of Waterloo
in fulfilment of the thesis requirement for
the degree of Doctor of Philosophy in Biology

Waterloo, Ontario, Canada, 1998

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Acknowledgements

Well, it is hard to believe but I have finally arrived. Who would have thought it possible. "Dr Klee" ..... it sounds a bit pretentious but it beats the heck out of "Almost Dr Klee" or ADK, as I was affectionately referred to by my friends at CanTox..

CanTox Inc. was my first "real" job but it was also the final battle ground for my thesis. For two and a half years my co-workers would ask, "So is it done yet", and for two and a half years I would reply, "It'll be done in a couple more weeks". My friends at CanTox (who are too numerous to name) never let me forget how special this accomplishment was. It wasn't about those three little letters. It wasn't about the money, the fame or the glory. It wasn't about respect (I didn't get any before and I am unlikely to get any now). It was about never having to be called ADK again. I would like to thank everyone at CanTox for their timely words of encouragement and for the cheer when I finally crossed the finish line.

A "finish line" it is, for this, most likely, marks the end of my "distinguished" university career. The memories, however, will last a lifetime for they were the best of times to be sure. What made them special were my friends. I consider myself to be extremely fortunate to have been associated with such a great bunch of people and I would like to thank Allan Arthur, Mark McMaster, Mike van den Heuvel, Niel Karrow, Rachel Gould, Barry Zajdlik, Roman Lanno, Brendon McConkey, Mark Hewitt, Andrea Farwell, Beth Goebel and Lisa Peters. It was great sharing so many laughs with all of you. I would also like to give special thanks to Paul Sibley who introduced me to the joys of cogitating and to the wonderful world of benthic invertebrates; to Janine Clemons who was always there to help and to Mike Dutton who made me realize that flatulants wasn't just a bodily function but something to be proud of too.

Now the Fish Heads, as we were affectionately known, were ruled (and still are) by Dr George Dixon. I don't think that I could have asked for a better supervisor. I can't thank Dr Dixon enough for all the confidence that he showed in me. Thanks for giving me the freedom to think for myself, even if it meant making mistakes, and for the "kicks in the behind" to get me back on the right path.

I would also like to thank Dr. Lynn McCarty for introducing me to 1CFOK modelling and the body-residue approach. Lynn's enthusiasm for science was inspirational and his guidance along the way was invaluable.

I started my PhD in September of 1991, seven and a half years ago, and through it all my family has been by my side. My son Bryce practically grew up in the wet-lab and used to sit beside me at my bench pretending to work on his “fecis” too. There was some discussion as to who would be finished first but Bryce eventually tired of his project and moved on to bigger and better things (Grade 2). Whether I was out collecting fish in a stream, or benthic invertebrates in a swamp or running the GC in the lab, Bryce was often there drawing on the blackboard, doodling on a piece of paper or asking intriguing questions like, “If a mommy horse and a daddy cow were to fight who would win?” ......"What about two mommy horses ?”. Thanks Bryce for just being a kid.

I would also like to thank my mom for making me appreciate and care of what I have, my dad for making me reach further and my sister, Erika, for keeping me humble and preventing me from becoming a smart-ass know-it-all. My brother Marcus deserves a special thanks for he was more than just a brother, he was my best friend. No matter what and no matter when, he was always there to keep me on my feet. We have been and always will be “Brothers in Arms”.

Certainly the person who deserves the greatest acknowledgment for her patience, her endurance and for her undying devotion is my wife Karen. A simple thank-you just does not seem appropriate for I couldn’t have done it without her. In many ways this is as much Karen’s accomplishment as it is mine, for my PhD program was a lot more than just research and experiments. We shared a family together and where ever I fell short Karen was their helping to keep things together. Whether it was helping to look after Bryce, or make dinner, or working ungodly hours at the hospital to make sure the bills were paid, Karen never quit. Thank-you Karen.

Finally, I would like to acknowledge that this research was funded in part by an Ontario Ministry of the Environment Research Grant.

Abstract

A general lack of understanding and inadequate predictive models of toxicant interactions have made it difficult for regulatory agencies to provide water quality guidelines that effectively consider chemical mixtures. Given the amounts and types of compounds found in aquatic ecosystems, research in this area is important in order to ensure that mixtures of chemicals are addressed in a way that provides adequate protection for aquatic organisms. A whole body-residue-based one-compartment first-order kinetic (1CFOK) model was used to examine the combined effects of chlorinated phenols and benzenes on rainbow trout (*Oncorhynchus mykiss*). The use of a kinetic model allows the separation and study of two important aspects involved in the interaction of toxicant mixtures: the influence of one chemical on the kinetics of the other i.e. the effect on uptake and/or depuration, and the influence of one chemical on the resulting toxicity of the other once they have reached their respective sites of toxic action. This approach is relatively well established but only recently have technological advances permitted an extensive analytical evaluation. The overall objective of this research was to assess the ability of the body-residue approach to predict the toxicity of binary mixtures of toxicants to fish.

In order to use a 1CFOK model to predict chemical loading into fish tissue and the potential effects of those residues on organism health, a number of biological and chemical parameters must be determined for each of the compounds to be studied. These parameters include: rate constants for chemical uptake and depuration, bioconcentration factors, and critical body-residues for the effects being studied. These parameters must be derived within the system in which the mixture studies will be done in order to compensate for potential modifying factors. The rate constants for chemical uptake and depuration are derived using kinetic experiments in which the body-residues resulting from waterborne exposure were tracked over time. The critical body-residues and times-to-death are defined using a standard toxicity test format with the addition of quantifying chemical residues in samples of the dead fish.

Based on the results, the 1CFOK model was shown to adequately describe the uptake and depuration of 1,2,4-trichlorobenzene, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol in rainbow trout larvae. Suitable measures of k_1, k_2 and the BCF were derived for each compound which could subsequently be used to predict the chemical concentration in the organism at any time for acute exposure to multiple chemicals. A critical body-residue was used to define the chemical toxicity in terms of the chemical concentration in the fish. The critical body-residues for 1,2,4-trichlorobenzene, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol were shown to be independent of the exposure time and water concentration, and thus could be used, in conjunction with the 1CFOK modelling to accurately predict lethality in an exposed group of organisms.

For binary mixture scenarios, the model was used to track the concentration of each chemical in the whole organism over time. By expressing this predicted value as a ratio of the chemicals respective critical body-residue and summing these ratios, it is possible to predict both if death will occur and when it will occur. It was possible to validate the models predictions by performing the mixture experiments and noting the time to death, the number of mortalities and by quantifying the body-residues in each of the organisms at the time of death. A comparison of the results of the mixture toxicity tests with those predicted from the model demonstrate that the effects on larval rainbow trout of binary mixtures of 1,2,4-trichlorobenzene, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol were accurately modelled, both in terms of exposure and toxicity, using the 1CFOK model and a body-residue-based toxicity endpoint. For the most part, the interaction of the chemicals in the mixture could be described as additive. Deviations from this were noted for the mixture of 2,4,5-trichlorophenol and 2,4,6-trichlorophenol but were marginal. The exact nature of the interaction was not determined but changes in the rates of chemical uptake and depuration by the fish were not responsible. It was concluded that the potential toxicity to fish of mixtures of chlorinated phenols and benzenes should be adequately predicted by assuming addition.

In addition, the research was completely dependent on the ability to quantify chemical residues in the organism. It became rapidly apparent that a new approach to residue analysis was required. Traditional methods of organic extraction using separatory funnels were both inefficient and labour intensive making analysis of the thousands of tissue samples an impossible task. The Cryo-extraction protocol was developed in order to accurately and rapidly accomplish tissue residue analysis. Through the systematic assessment of the extraction efficiency, reproducibility and linearity, the cryo-extraction protocol was shown to meet or exceed the requirements for an acceptable analytical tool for quantifying the chemical body-residues of chlorinated benzenes and phenols in fish. The cryo-extraction protocol provided a faster, more efficient and more reliable approach.

Table of Contents

List of Tables

List of Figures

1.0 GENERAL INTRODUCTION

1.1 Background

1.1.1 Why Study Chemical Mixtures ?

As a result of human activities, thousands of unique chemicals are released into the environment every day. Traditional methods for characterizing the toxicity of these compounds has been on an individual basis. Consequently, an overwhelmingly large proportion of the toxicological studies to date deal with single, pure chemicals. The value of this data and the toxicological foundation it supports can hardly be dismissed. An important function of these studies was to provide information that could be used to protect environmental health. Recently however, considerable scientific and public concern has been raised regarding the ability of these traditional concepts and data to adequately deal with the complexity and uncertainty sometimes associated with chemical mixtures.

"Chemical mixtures" refers to the situation in which an ecosystem is being impacted by two or more potentially toxic substances. The presence of mixtures of chemicals in the environment is the rule as opposed to the exception. Environmental contamination from industrial, urban and agricultural activities, coupled with long-range transport, atmospheric deposition, surface run-off and leaching, has reached global proportions; it is becoming increasingly difficult to find pristine environments. Aquatic ecosystems act as environmental sinks, becoming depositories for a large proportion of what is released within their watershed. Certainly the best examples are from heavily impacted areas. Green Bay and Saginaw Bay within the Great Lakes takes a heavy load of urban and industrial waste and have become contaminated with PCB's, DDE (a derivative of DDT), mercury and chlorinated pesticides (Golden *et al.*, 1993). Inland harbours on the east coast of Canada and the USA near industrial centres such as Halifax and Boston are contaminated with mercury, lead, PAH's. chlorinated pesticides and PCB's (Gearing, *et al.*, 1991; Johnson *et al.*, 1994.). Pulp mill effluent contains thousands of distinct chemical compounds from a variety of classes including resin acids, dioxins, furans, chlorinated benzenes and phenols (Environment Canada, and Health & Welfare Canada, 1991). Most aquatic environments which are considered contaminated contain a variety of chemicals both natural and man-made which have the potential to stress organisms at all trophic levels. As a result, unlike the laboratory, in the real world one rarely has the luxury of assessing the impact of a single chemical on an ecosystem; it is often necessary to deal with numerous contaminants from a variety of chemical classes.

In addition to their prevalence, concerns over chemical mixtures stems from the knowledge of potential chemical interactions; that one chemical may modify the toxicity of another. It has long been known that the potency of certain chemicals can be enhanced or inhibited when in the presence of a second compound as compared to when they are applied singly (Calabrese. 1991). These two properties (enhancement and inhibition) have been used to advantage in medicine, agriculture and pest control (Calabrese, 1991), but lead to some concerns when trying to assess the risk and set acceptable limits for mixtures of chemicals present in the environment. Just as it is difficult to actually measure the toxicity associated with single compounds in every specific situation, it is orders of magnitude more difficult to determine. through experimentation, the toxicity of each chemical mixture scenario. Assessing the risk associated with chemical mixtures is therefore greatly dependent on extrapolation and modelling. Unfortunately, the processes which govern the types of interactions that exists between two or more toxic substances are poorly understood, making accurate modelling and predictions of their combined toxicity difficult. However, if toxicant mixtures are not dealt with properly we may seriously under or over estimate environmental risk.

Decades of chemical misuse and improper waste disposal have left the water, sediment and biota, in many ecosystems, contaminated with an abundance of xenobiotics. Government regulatory policy aimed at reducing emissions, coupled with extensive clean-up and restoration projects, have led to dramatic reductions in contaminant loading and subsequent improvement in environmental quality. Yet, despite these efforts, the health of many ecosystems remains compromised and the steady deterioration of others is observed even though chemical concentrations may be well below their respective "safe" levels based on the assessment of individual chemicals. As scientists probe the environment in an attempt to identify offending chemicals within the plethora of chemicals that exist, two questions often arise:

1) Is the observed environmental impact the product of a single chemical or the result of two or more chemicals acting in concert ?; and
2) Is the toxicity of many chemicals each at a low concentration equivalent to one chemical at a high concentration ?

Addressing these questions, along with the many others associated with chemical mixtures, has been a slow process bogged down by the potential complexity and imposing numbers of combinations presented by even a simple mixture scenario. For example, in order to rigorously examine a group of ten chemicals, one generates just over a thousand mixture experiments. Each would require a range of chemical concentrations as well as the assessment of a variety of important modifying factors. This would be a daunting task and although the required effort is exaggerated in this example, a typical mixture scenario in the environment may include not ten but perhaps twenty distinct compounds.

1.1.2 The Toxic Unit Model

Current approaches for dealing with the combined effects of toxicants on aquatic systems has been the topic of several reviews (USEPA, 1986; de March, 1987a; 1987b; Hermens, 1989). These approaches are based largely on results from the mixture toxicity methodology discussed by Sprague (1970) as the "toxic unit" model. A toxic unit is defined as the ratio of the chemical concentration in water to a toxicological endpoint, also expressed as a water concentration. This endpoint can be either lethal such as the chemical concentration required to kill half the organisms within a specific time (LC50) or non-lethal such as a No Observable Effect Concentration (NOEC) related to growth or reproductive effects. Thus a toxic unit represents the proportion of a given endpoint contributed by the individual chemical. As toxic units are dimensionless ratios, the toxicity of a mixture of compounds can be expressed as the sum total of the toxic units of each of the components in the mixture as:

$$Toxicity=\frac{Cw_1}{LC50_1}+\frac{Cw_2}{LC50_2}+\ldots\frac{Cw_i}{LC50_i}$$

Where Cw_i is the exposure concentration in water and $LC50_i$ is the respective 96 hour LC50 for chemicals 1 to I. Values less than one predict that the combined toxicity of the mixture is insufficient to produce the endpoint defined by the denominator. Values greater than one predict that the toxic endpoint will be observed. This approach is based on two important assumptions: that the concentration of the chemical in the water is an adequate surrogate for the chemical concentration at the site of toxic action and, that the toxicity of each chemical, expressed as a toxic unit, is strictly additive.

The toxic unit approach has several attractive qualities. First, it accounts for all of the chemicals defined in a mixture, giving each an appropriate weight based on concentration and relative toxicity. Second, since the denominator of the toxic unit can be chosen to be any endpoint of biological significance, the model can be used to protect the environment at an individual, population or ecosystem level depending on the availability of information. Third, a considerable amount of the data used to apply the model is readily available. Since LC50s, and NOEC values for a variety of compounds have already been derived, model implementation is quick and inexpensive. Finally, the toxic unit model is simple and, for the most part, effective. The basic premise behind the approach, expressing each chemical in a mixture as an exposure ratio and then summing to predict the total toxicity, is sound. Although it assumes only additivity, not accounting for interactions that may produce greater- or less-than-additive effects, it is still considered a relatively conservative approach. Efforts have been made to increase the sophistication of the method (Placket and Hewlett, 1967, Konemann, 1981, de March, 1987a). Most of this work, however, has been directed towards statistical aspects but still employs the toxic unit approach to deal with multiple toxicity problems.

Despite its apparent advantages, concerns with respect to the adequacy of the toxic unit approach have been raised. The primary concern with the model is actually the way in which the chemical toxicity is expressed, as the chemicals concentration in the water. The traditional bioassay involves measuring the response of an organism exposed to known concentrations of a waterborne toxicant. A primary assumption of the water concentration-response relationship is that the amount of toxicant at the site(s) of toxic action is some function of the amount of chemical to which the organism is exposed (Mancini, 1983). In effect, the exposure concentration is used as a surrogate measure for the dose of chemical an organism actually receives (Connolly, 1985). The relationship between the surrogate exposure-water concentration and the effective dose at the site(s) of toxic action is important to verify but is rarely established (McCarty, 1989). As a result, the use of a water concentration as a surrogate for the concentration of the chemical at the site of toxic action can have serious limitations and these limitations are compounded as a result of the addition model.

The first limitation is that a static model is being applied to a dynamic process and thus ignores the partitioning behaviour of the chemicals in question. Expressions of toxicity such as the LC50 and NOEC are often derived from measurements at fixed times and assume steady-state so that the concentration in the organism is a function of the concentration in the water (Ferguson, 1939). Since the kinetics of accumulation for each of the chemicals in a mixture may vary significantly, the ratio of actual body-residues at a given time may not be proportional to their relative waterborne concentrations. Thus, unless all the chemicals in a mixture have reached a steady-state, it is not valid to assume a proportional internal dose based on the chemical concentration in the water. Relationships, such as interactions between compounds, derived from these expressions of dose can be inaccurate and misleading.

Second, a variety of environmental factors can dramatically influence the relationship between the contaminant water concentration and the concentration in the organism, thus modifying the resulting toxicity (Sprague, 1970; Rand and Petrocelli, 1985). For example, the uptake of phenolic compounds is a function of pH (Spehar *et al.*, 1985). Metal assimilation is also affected by pH, in addition to water hardness and the dissolved organic carbon (DOC) (Rand and Petrocelli, 1985; Wetzel, 1983; Borgmann and Ralph, 1984; Erickson *et al.*, 1996). The bioconcentration of lipophilic compounds is a function of the organisms lipid content and, is therefore influenced by diet (van den Heuvel *et al.*, 1991). All processes, both physical and chemical, are affected by temperature (Rand and Petrocelli, 1985); toxicity is no exception. To summarize, considerable difficulty has been encountered when trying to apply traditional laboratory toxicity data to site-specific environmental situations largely because the concentration in the organism is not accurately reflected by the concentration in the water (Veith *et al.*, 1983; McCarty, 1991).

Finally, for chemicals which are persistent and highly lipophilic, the concentration of the contaminant in an organism can be significantly underestimated by the water concentration. The hydrophobic nature of these compounds causes them to partition out of the aqueous phase leaving only trace amounts in the water column. Since these compounds are both lipophilic and often metabolically recalcitrant, they have a propensity to bioaccumulate (McCarty, 1991). Defining the toxicity of these compounds based on a water-borne concentration can be misleading.

1.1.3 The Body-residue Approach

In order to address the inadequacies of the current toxic unit approach, it has been proposed that the contaminant exposure and the toxic endpoint be expressed as a body-residue instead of the traditional water concentration (McCarty, 1991). The basic framework of the toxic unit approach remains unaltered. Instead of calculating a toxic unit for each chemical as the ratio of the contaminant water concentration and the ILL or NOEC, the toxic unit is derived by dividing the concentration of the chemical present in the whole organism by a critical body-residue. The equation for predicting the toxicity of a mixture becomes:

$$Toxicity = \frac{BR_1}{cBR_1} + \frac{BR_2}{cBR_2} + \ldots \frac{BR_i}{cBR_i}$$

where BR_i is the whole body-residue in question and cBR_i is the respective critical body-residue for chemicals 1 to I. The critical body-residue simply quantifies the chemical residue in the organism at which a specific endpoint is observed. Like its predecessor the LC50, the endpoint for the cBR can be any measurable biological response from the presence of endocrine disruption to mortality. For mortality, the cBR represents the concentration of a chemical in the whole organism necessary to cause death to the "average" organism.

Like the toxic unit approach, this method is quick, simple and all the chemicals are accounted for. In addition, the use of body-residues offer several advantages. Concerns over how and if a chemical will enter the organism are no longer important since the actual tissue concentration in the organism is quantified. The influence of many modifying factors, differential partitioning behaviour and potential for bioaccumulation are accounted for. It is important to note that the body-residue is not equivalent to the toxicant concentration at the site of toxic action. The former should better represent the latter but, like the water concentration, the whole-body toxicant concentration must too be recognized as a surrogate measure.

1.1.4 One-Compartment First-Order Kinetic Modelling

An important co-requisite to the body-residue approach is the use of kinetic modelling. For many organic chemicals, it is possible to describe their movement between an organism and its surroundings by using one-compartment first-order kinetics (1CFOK) (Landrum *et al.*, 1992). The model reduces an organism to a single compartment within the aqueous environment. The diffusion of a chemical between the water and the tissue is characterized by rate constants for both uptake and depuration. The mathematical relationship is described by the following equation:

$$C_f = C_w \frac{k_1}{k_2} (1 - e^{-k_2 \cdot t})$$

where C_f is the toxicant concentration in the organism at time t; C_w is a constant and represents the concentration of the toxicant in the water; k_1 and k_2 are the rate constants for uptake and depuration, respectively (Spacie and Hamelink, 1982; Van Hoogen and Opperhuizen, 1988).

Although currently impractical for application in a "real" ecosystem, kinetic modelling is a valuable design tool for studying chemical mixtures within a controlled setting such as a research laboratory. Specifically, the kinetic model allows one to more accurately view the assessment of toxicant mixtures as a time-dependent phenomenon where the concentration of each toxicant in the organism can be evaluated independently of the others over a continuous time interval and related to each chemical's respective critical body-residue. In addition, the model breaks the problem down into its toxicokinetic and toxicodynamic elements, which potentially offer a more direct means of extrapolating between laboratory and field situations (Mackay *et al.*, 1992).

1.1.5 Chlorinated Benzenes and Phenols

Chlorinated benzenes and phenols, specifically 1,2,4-trichlorobenzene, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol, and pentachlorophenol, were chosen to study the application of a body-residue based 1CFOK model based approach to mixture toxicity. These compounds are relatively easy to handle with respect to aquatic toxicity studies. They are reasonably soluble in water and can be easily quantified in both water and tissue samples using standard analytical techniques. In addition, 1,2,4-trichlorobenzene, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol, and pentachlorophenol have been the focus of a number of studies and thus their potential toxicity to aquatic organisms and their mode of toxic action have been determined. This latter point was of particular interest because of the potential for chemical interactions. Mixtures of chemicals which share a common mode of toxic action are expected to be strictly additive whereas compounds whose mode of toxicity differ may deviate from this (Hermens *et al.*, 1984 a&b; Broderius, 1991). The 1,2,4-trichlorobenzene is a non-polar narcotic while 2,4,5-trichlorophenol and 2,4,6-trichlorophenol are polar narcotics. Pentachlorophenol has been demonstrated to be an uncoupler of oxidative phosphorylation (Brown *et al.*, 1987; McKim *et al.*, 1987). Chlorinated benzenes and phenols are still of great interest and further study particularly with respect to mixtures is warranted

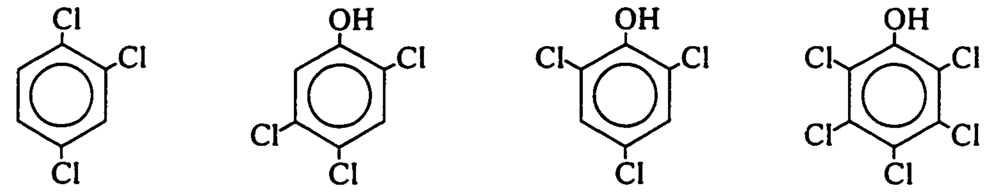
Chlorinated organics have gained considerable notoriety in recent years and their continued production by industry is the subject of heated debate. Environmental advocacy groups are calling for a complete ban on chlorine as a feedstock in industrial chemistry. The growing concern stems from the fact that in comparison to their nonchlorinated analogues, chlorinated compounds tend to be more lipophilic and less biodegradable. They are therefore more bioaccumulative and potentially more toxic to both humans and the environment. Most of the publicity has focussed on compounds such as DDT, PCB's, dioxins and furans but due to their ubiquitous nature chlorinated benzenes and phenols also share some of the spot-light and are represented in the Priority Pollutants List under the Canadian Environmental

Protection Agency (Canada Gazette).

For most of the chlorinated benzenes and phenols there are no known natural sources; the abundance of these compounds in the environment is the result of human activity. They are present at detectable concentrations but with varying composition in the effluent of pulp and paper mills, iron and steel mills, organic and inorganic chemical plants, textile mills, petroleum refineries, and activated sludge waste water treatment plants (Jones, 1981). In addition, a few are produced commercially for use as solvents, deodorizers, wood preservatives and fungicides (Jones, 1981). The estimated total release of chlorinated benzenes and phenols into the Canadian environment is difficult to quantify but is in the range of thousands of tonnes per year (Jones, 1981).

Chlorinated benzenes and phenols are a chemically and biologically diverse group of compounds which share a single substituted benzene ring (See Figure 1.1). The extent of ring chlorination ranges from a single chlorine atom to complete substitution. Certain structures however are much more common then others in the environment partially due to human use but also resulting from differences in chemical stability (NRCC, 1982.). As a general rule, compounds with low chlorine substitution tend to degrade more quickly than those which are highly chlorinated. The latter also display an increase in lipophilic character resulting in compounds with a tendency to bioconcentrate (higher BCFs) (Eisler, 1989; WHO, 1989). The pattern of chlorine substitutions also influences a compounds susceptibility to biotransformation, and thus isomers do not necessarily have equivalent bioconcentration potential (Eisler, 1989). For the most part, except for hexachlorobenzene, chlorinated benzenes and phenols are degraded and excreted rapidly enough so that their potential to bioaccumulate is limited (McKim *et al.*, 1986).

Figure 1.1 Chemical structures of chlorinated benzenes and phenols.



1,2,4-trichlorobenzene 2,4,5-trichlorophenol 2,4,6-trichlorophenol pentachlorophenol

1.2 Statement of Problem

Current methods for assessing the impact of chemical mixtures in the environment based on water-borne chemical concentrations are inadequate. Although they are useful for many toxic compounds, they fail to address those chemicals which are lipophilic and persistent i.e. those for which the water concentration is not a good surrogate for the concentration at the site of toxic action. This is a significant problem since contaminants with these properties, such as chlorinated organics, are of greatest concern in the aquatic environment. The body-residue approach coupled with one-compartment first-order kinetic modelling provides a potential solution but there is only limited scientific data to support this hypothesis. It is thus necessary to assess the utility of this approach under controlled laboratory conditions.

1.3 Research Outline and Objectives

The overall objective of this research is to assess the ability of the body-residue approach to predict the toxicity of binary mixtures of chlorinated phenols and benzenes in an aquatic system. The following compounds: 1,2,4-trichlorobenzene, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol, were chosen because they have been well characterized (physically, chemically and biologically), they are easily quantified and are designated as priority pollutants.

Since body-residues are a function of chemical uptake and depuration, a 1CFOK model can be applied not only to predict body-residues for a given water concentration but to express the increase in body-residues over time. This latter ability makes the experimental approach more amenable to the dynamics of an ecosystem.

This approach should provide a more comprehensive and detailed analysis of the mixture toxicity tests than could be obtained by looking simply at the water concentration. For any mixture scenario, it will be possible to track the concentration of each chemical in the whole organism over time. By expressing this predicted value as a ratio of the chemical's respective critical body-residue and summing these ratios, we can not only predict if death will occur but when, during the exposure time, it will occur. Finally, it is possible to validate the models predictions by performing the mixture toxicity experiment and noting the time to death, the number of mortalities and by quantifying the body-residues in each of the organisms at the time of death.

In order to run the models, a number of biological and chemical parameters must be defined for each of the compounds used in the mixture toxicity tests. These parameters include: rate constants for chemical uptake and depuration, and critical body-residues. Although much of this information is available in the literature, these parameters have been shown to be sensitive to modifying factors such as temperature, pH, hardness, species, and lipid content just to name a few. As a result, reported values from the literature show considerable variability (summarized in Mackay et al, 1992b; Mackay et al, 1995). These parameters must therefore be derived within the system in which the mixture experiments will be done. This will make comparison between the predicted and the observed experimental values more accurate and valid.

The rate constants for chemical uptake and depuration are derived via kinetic experimentation. Fish are exposed to a fixed, sublethal concentration of the chemical for a period sufficient to achieve steady-state between the chemical in the organism and the chemical in the water. At specific intervals within that time period, a subsample of fish is taken and the chemical concentration in the whole organism is quantified. The remaining fish are then transferred into clean water and the sampling regime and body-residue analysis continued until chemical depuration is complete. By plotting the measured body-residues against the time at which the fish were sampled, a curve is generated that represents chemical uptake (during the exposure phase) and another is generated that represents chemical depuration (the clean water phase). Values for k_1 and k_2 are calculated from these curves by using non-linear curve fitting.

The critical body-residues and times-to-death are defined using a toxicity test. This test is equivalent to the classic toxicity test in which fish are exposed to a geometric series of chemical concentrations and mortality recorded. In addition, the time at which each fish dies will be accurately recorded and the chemical concentration in the dead fish will be quantified.

The research is completely dependent on the ability to quantify chemical residues in the organism. Although not part of the original research proposal, it became rapidly apparent that a new approach to residue analysis was required. Traditional methods of organic extraction using separatory funnels were both inefficient and labour intensive making analysis of the thousands of tissue samples an impossible task. The Cryo-extraction protocol was developed in order to accurately and rapidly accomplish tissue residue analysis.

The success of the 1CFOK model and the body-residue approach is based on a number of assumptions. The first assumption is that a whole-body-residue can be used as a surrogate for the toxicant concentration at the site of toxic action thus allowing for one to define a critical body-residue. The cBR can define one and only one endpoint and itself must be unique. That is there is a body-residue which when achieved by a fish, for example, will result in mortality. The second assumption is that the toxic units expressed as a ratio of the body-residue and the critical body-residue can be simply added to predict the toxicity of the chemical mixture. The final assumption is that the movement of the chemical in and out of the organism can be accurately described with the 1CFOK model regardless of chemical concentration or mixture composition. Thus, in addition to testing the body-residue based model, the above mentioned assumptions will also be verified so that they can be used with some measure of assurance.

2.0 QUANTIFYING CHLORINATED BENZENE AND PHENOL RESIDUES IN WATER AND TISSUE SAMPLES USING THE CRYO-EXTRACTION METHOD

2.1 Introduction

The ability to accurately quantify the concentration of a contaminant in an aquatic organism can be extremely useful in toxicology. Highly lipophilic chemicals, such as chlorinated benzenes and phenols, tend to partition out of an aqueous phase and into an organic phase leaving only trace concentrations in the water but significant concentrations in the exposed biota (Veith *et al.*, 1983; McCarty, 1991). Assessing the toxic potential of these compounds based on water concentrations can thus be misleading and may result in a serious underestimation of the compound's toxic potential (McCarty, 1991). It has been proposed that body-residue concentrations may be a more accurate and meaningful way of expressing the exposure dose for these chemicals to aquatic organisms (McCarty, 1991). The body-residue approach is gaining acceptance and it is conceivable that, in addition to water concentrations, tissue concentrations in organisms from various trophic levels will be incorporated into the current regulatory framework.

This chapter describes a quick, simple and accurate method for extracting chlorinated organics from both water and tissue samples for subsequent analysis and quantification. Although it specifically addresses chlorinated benzenes and phenols, it can be applied to any compound with a similar affinity for organic solvents.

Although a number of extraction techniques already exist, the development of the cryo-extraction method, presented here, arose from the need to quantify the concentration of contaminants in the large number of water and tissue samples necessary to examine the relationship between chemical body-residues, chemical kinetics and toxicity in fish. The three R's of statistics and experimental design (replication, replication, replication) ensure a rigorous and scientifically sound approach which will provide meaningful results. Unfortunately this comes at a cost; the need to generate extensive data sets. For this situation, the standard methods of extraction were found to be inadequate. The conventional "separatory funnel" organic extraction method is labour intensive and uses large solvent volumes, leading to waste in terms of time and materials. When confronted with several hundred samples requiring residue analysis, being equipped with even as many as twenty separatory funnels still represents a significant level of effort. The old method requires a constant level of activity; a great deal of time is spent cleaning, drying and rinsing glassware. Significant variability in extraction efficiency can be introduced by subtle changes in day to day routines, lowering the overall precision of the process. For small numbers of samples, the above may not be an issue, but for larger numbers the problems can be overwhelming.

In comparison, the cryo-extraction protocol is quick and simple. The method owes its speed and efficiency to the fact that the entire digestion and extraction process can be carried out in a test-tube. This is accomplished by taking advantage of the differential freezing points between the organic solvent and the aqueous sample. Freezing of the lower aqueous phase physically stabilizes it thus accommodating removal of the upper liquid organic phase by decanting. An important restriction of the method, however, is that the organic solvent must have a very low freezing points (below -70°C); a density less than one (i.e. less than water) and be immiscible in water. Solvents that meet these three criterion include hexane and diethylether.

The cryo-extraction method is also a micro-method ie. developed for small samples. It is best suited for water samples of less than 3 to 4 ml and tissue samples in the range of 10 to 1000 mg wet weight. The method has been stream-lined and as a result is fast with good accuracy and precision. Once proficient, it should be possible to process 40 samples plus standards and blanks in an 8 hr day.

2.2 Materials and Methods

All reagents were GC/HPLC grade. This included the organic solvent (hexane), the concentrated hydrochloric and sulfuric acid, silica gel, and deionized water. The 1,2,4-trichlorobenzene, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol were listed as 99% pure and purchased from Alderich Chemical Company (Milwaukee, USA).

Other materials included a dry-ice bucket, a Vortex mixer, two 16 mm x 150 mm glass test tubes per sample and a centrifuge capable of spinning the glass test tubes at 1200 x g. A drying rack was set up to deliver a gentle stream of nitrogen over the surface of the solvent in the test tube as it was warmed using a hot-plate and heating block.

2.2.1 Sample Preparation

Water samples were carefully measured and put into the 16 mm x 150 mm test tubes. The sample volume was based on the nominal concentration of the chemical but typically ranged from 1 to 3 mls. An acid extraction was used to optimize the recovery of the chlorinated phenols therefore the pH was adjusted to below pH 2.0 with an appropriate addition of concentrated hydrochloric acid (roughly 1 ml). An overlay of 6 ml of hexane solvent was added. Water samples were immediately processed further (See Water Extraction, Section 2.2.2, below). The acid was added to all water samples even when only chlorinated benzenes were being extracted. The acid prevented the formation of large ice crystals. These crystals caused cracking of the test tube when attempts were made to freeze the "pure" water.

Tissue samples were processed "wet" to facilitate digestion. Samples typically ranged between 20 and 400 mg wet weight. Once weighed, the tissue was placed in a 16mm x 150 mm test tube and digested with a minimum (enough to completely cover the tissue , 1-2 ml) of concentrated H_2SO_4 for 8 to 10 hours at room temperature. Alternatively, digestion was accomplished quickly by heating the sample to 75 °C for 30 minutes followed by a 1 hour cooling. Sulfuric acid was used instead of hydrochloric because it oxidized the lipid in addition to solubilizing the tissue. This lipid digest did not partition into the solvent phase during subsequent extraction thus providing a cleaner extract. A 6 ml solvent overlay was applied prior to digestion in order to capture any volatile compounds being released during digestion. An aluminum foil or rubber cap was used in order to reduce both solvent evaporation and contamination.

2.2.2 Water Extraction

The two phases (hexane and water) were thoroughly mixed by Vortexing for 10 seconds and then allowed to settle. The settling time was reduced by centrifuging at 1200 x g for 1-2 minutes. Once restabilised, the test tube was placed in dry ice or in a -70° C freezer until the lower aqueous layer was frozen solid. The tube was removed and the solvent carefully decanted into the second test tube. A second 5 ml aliquot of hexane was added to the thawing aqueous layer and the procedure was repeated. The second decant was pooled with the first. If the chemical concentrations in the solvent were expected to be small, the final 10 ml of solvent was reduced to a 1 ml volume under a gentle stream of nitrogen and mild heating (40° C) prior to analysis. This concentrated the chemical making it easier to quantify during the subsequent GC analysis. The final volume of the sample was determined by adding a known amount of dibromobenzene to act as an internal standard. The final volume was very important and was determined accurately by quantifying the dibromobenzene peak from the GC tracing.

2.2.3 Tissue Extraction

Once the tissue was completely solubilized, the two phases (hexane and aqueous) were thoroughly mixed with the Vortexer for 10 sec. After centrifuging the test tube for 5 minutes at 1200 x g in order to reestablish the two discrete phases, 1 ml of deionized water was carefully added. Being less dense than the digest it formed a layer between the sample and the solvent. The test tube was then placed in dry ice or in the -70° C freezer until the intermediate, deionized water layer was frozen solid. This ice cap over the solvent layer prevented the underlying sample from mixing with the solvent when the latter was subsequently decanted into the second test tube. A second 5 ml aliquot of hexane was added to the thawing aqueous layer and the procedure was repeated. The second decant was pooled with the first. If the chemical concentrations in the solvent were expected to be small, the final 10 ml of solvent was reduced to a 1 ml volume under a gentle stream of nitrogen and mild heating (40° C) prior to analysis. This concentrated the chemical, making it easier to quantify during the subsequent GC analysis. The final volume of the sample was determined by adding a known amount of dibromobenzene to act as an internal standard.

2.2.4 Gas Chromatography Analysis

The GC analysis was performed using a Varian 3400 GC equipped with an 8100 Autosampler and Nickel^{63} EC detector. The hexane sample (1 ul) was injected at 250°C onto a DB-5 Mega-Bore capillary column (30 m length, 0.53 mm ID, 0.25 um film thickness). The initial column temperature was set at 50°C for 2 minutes then ramped at 25°C/minute to 210°C where it was held for 12 minutes. The total run time was 20 minutes. The detector temperature was set at 250°C. The peak areas for the compound of interest were recorded and compared to a standard curve.

2.2.5 Cryo-Extraction Validation

In order to assess the validity of the cryo-extraction method, water and tissue samples were prepared which were spiked with trichlorobenzene, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol. By comparing the chemical concentration of these samples quantified via cryo-extraction with either a theoretical concentration or a level derived via a standard extraction process, it was possible to define the recovery, reproducibility and linearity of the new method.

Rainbow trout (*Oncorhynchus mykiss*) were used to prepare the tissue samples. Rainbow trout were purchased as "eyed-up" eggs from the Rainbow Springs Trout Farm in Thamesford, Ontario. Once hatched, they were reared in the wet-lab until the fry had completely absorbed their yolk-sack (5-6 weeks post hatch). At this point, the fish were placed in exposure tanks (40 fish per tank) containing known concentrations of trichlorobenzene, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol, where they remained for 48 hours. The exposure system was a 12 hour static-renewal with the water temperature maintained at 15°C. Moderate aeration of the water was supplied but the fish were not fed for the duration of the exposure. After the prescribed time, the fish were removed, quickly rinsed, sacrificed and stored at -70 °C until body-residue analysis was performed. Residues were determined using the cryo-extraction method, previously described, and the standard separatory funnel extraction method (Helrich, 1990).

In order to separate the variability associated with the extraction protocol from that which is naturally inherent to the tissue residues, chemical concentrations in both individual fish and aliquotes of a fish homogenate were subject to the cryo-extraction method.

The exposure tanks were set-up to contain four separate and distinct chemical doses for 1,2,4-trichlorobenzene, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol plus a blank control. In order to facilitate chemical addition to the exposure water, stock solutions of the chlorinated organics were prepared. The chlorinated phenols were dissolved in 0.1N NaOH while the chlorinated benzene was mixed with a minimum amount of DMSO as a carrier. An appropriate amount of these stock solutions was added to the water in the exposure tanks in order to achieve the required exposure concentration. The final pH of the exposure water was checked in order to assure that NaOH addition did not significantly alter the hydrogen ion concentration. The pH of the water was typically 7.8 and the maximum pH shift was 0.2 units. Where DMSO was used, the concentration of the carrier never exceeded 0.2 % v/v. The theoretical water concentrations for each of the four contaminants are given in Table 2.1. Water samples (in triplicate) were obtained directly from the exposure tanks at the beginning of each study and were quantified immediately in order to verify that the actual exposure concentration was not significantly different from the nominal concentration.

Table 2.1 Nominal chemical concentrations used in the exposure tanks in order to provide water and tissue samples for method validation.

Compound	Nominal Water Concentrations (ug/L)			
1,2,4-trichlorobenzene	96	187	370	722
2,4,5-trichlorophenol	103	218	315	410
2,4,6-trichlorophenol	111	210	412	821
pentachlorophenol	26	62	113	218

2.2.6 Statistical Analysis

In order to assess chemical recovery and reproducibility, comparisons between the results of the methods used to quantify the chemical residues in both the water and fish samples, was accomplished using the paired t-test or the one-way analysis of variance (ANOVA). The MGLH module of SYSTAT 5.1 (Wilkinson, 1988) was used to conduct the statistical analysis. The linearity of the method was assessed using linear regression, also conducted with the MGLH module of SYSTAT 5.1 (Wilkinson, 1988).

2.3 Results and Discussion

Although the cryo-extraction protocol employs the same fundamental strategy for the transfer of organic contaminants from the tissue to the organic solvent as the standard separatory funnel method, a detailed assessment of its overall performance is important in order to verify its utility. The method validation involves addressing the following criterion:

-1- chemical recovery;
-2- reproducibility; and

-3- linearity

2.3.1 *Chemical Recovery*

The recovery represents the amount of the compound that is extracted from the biological matrix relative to the actual amount that was originally present. The recovery is usually expressed as a percentage of this actual value. The assessment thus requires an accurate estimate of the original chemical concentration in the sample. For water samples, this is relatively straight forward since a measured amount of compound is added to a known volume of water. In the absence of volatilization, incomplete solubility or adsorption to the sampling vessel, the concentration of water samples can be accurately predicted.

The recovery of 1,2,4-trichlorobenzene, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol from spiked water samples ranged from 74% to 105% (see Figure 2.1). The recovery was independent of dose but appeared to be chemical specific. Interestingly, the percent recovery demonstrated a degree of correlation to the compounds solubility in water. The solubility affects the accuracy of predicting the expected water concentration as well as influencing the partitioning behaviour between the aqueous and organic phases. Both 2,4,5-trichlorophenol and pentachlorophenol dissolved readily in water using a 0.1 N NaOH carrier. The recovery of both these compounds was consistently above 90%. The recovery of 2,4,6-trichlorophenol was slightly lower, ranging between 86% and 94%. The poorest recoveries were obtained with 1,2,4-trichlorobenzene and ranged from 74% to 85%. The solubility of this latter compound in water is relatively low. DMSO was used as a carrier but, even at concentrations well below the listed saturation limit of 34.6 mg/L (Chiou, 1985), a clearly visible sheen was observed on the surface of the water. This suggested that a proportion of the 1,2,4-trichlorobenzene that was added had not gone into solution. Thus, the expected calculated concentration is likely overestimating the actual concentration. The percent recoveries of 1,2,4-trichlorobenzene, if corrected for this overestimation may have been closer to that which was obtained for 2,4,5-trichlorophenol and pentachlorophenol.

Water is a simple matrix compared to tissue and the extraction of chlorinated organics from it using hexane should be an efficient process. Thus, the values obtained were not unexpected. For an extraction process to be useful as a tool for quantification, an extraction efficiency of greater than 80% is preferred. Extraction efficiencies of greater than 100%, although theoretically impossible, are the result of the variability associated with the injector and detector of the gas chromatograph (GC). The quantification of a chemical in the GC sample is determined by taking a peak area provided by the GC and converting it to a concentration by using the sample injection volume and a previously generated standard curve. Slight fluctuations in the injection volume or the detector sensitivity can influence the relationship between the peak area and the sample concentration and this can be in either a positive and negative direction.

The extraction of tissue samples is somewhat more complicated than extraction from water. One of the primary impediments to tissue extraction is tissue solubilization and subsequent removal of the appropriate chemical from the fragmented and degraded biological matrix. A simple spike, as was done for the water samples, does not adequately mimic the interaction between the tissue and the contaminant, making the measured recovery, from samples prepared in this way, unrealistic. It is thus necessary to use tissue which was "naturally" contaminated with a chemical, i.e. exposing live fish to the contaminant of interest.. Samples of this nature were generated by exposing rainbow trout to 1,2,4-trichlorobenzene, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol for a period of time necessary for the compounds to reach steady state. Contaminant residue concentrations were quantified with the cryo-extraction protocol and the values compared to results of duplicate tissue samples extracted and quantified using an accepted (separatory-funnel) method. An important assumption is that the accepted method attains close to 100% recovery. In this case, even this assumption is not critical since the cryo-extraction process was developed to replace the older separatory funnel method. As long as the new method operates as efficiently and consistently as the older one, then it will be useful.

The recovery of the four compounds from fish tissue is displayed in Figure 2.2. Statistical analysis (paired t-test) showed there to be no significant difference between the mean of the tissue residues concentrations determined via the separatory funnel method compared to the cryo-extraction method. Although the graph seems to suggest that the cryo-extraction method tended to provide a poorer recovery with the chlorinated phenolics, the p-values from the t-test were all greater than 0.05. However, the lack of significant differences between the two methods was not a good measure of the efficiency of the cryo-extraction method because it was primarily a function of the high variability rather than the similarity of the values. Visual inspection of Figure 2.2 suggests that recovery of the compounds from the tissue using the cryo-extraction method was acceptable . Pentachlorophenol and 1,2,4-trichlorobenzene ranked the highest with the average percent recovery being consistently over 90%. The recovery of 1,2,4-trichlorobenzene was actually better with the cryo-extraction than the separatory funnel. The volatility of 1,2,4-trichlorobenzene is relatively high and the separatory funnel method requires several lengthy evaporation steps in order to reduce the large volumes of solvent that are generated during extraction. Loss of the chlorinated benzene is expected during these steps. In contrast, the cryo-extraction protocol uses much smaller volumes of solvent, thus requiring only a single short evaporation step and is therefore not prone to the same loss in recovery. The samples and solvents are also maintained at low temperatures during cryo-extraction which further reduces volatolization of the chlorinated benzene. The recovery of 2,4,5- and 2,4,6-trichlorophenol was also quite respectable averaging above 80% for both.

For the comparison of chemical recoveries from tissue samples, a quantitative determination of the chemical concentration in the exposure water was not critical since this value was not used to derive a theoretical body-residue. The recovery of the cryo-extraction method is being evaluated by comparing it directly to the recovery of the separatory-funnel method. As a result, the caveat noted with the water samples, with respect to relating the low water solubility of the trichlorobenzene to an exaggerated water concentration, is not a factor here.

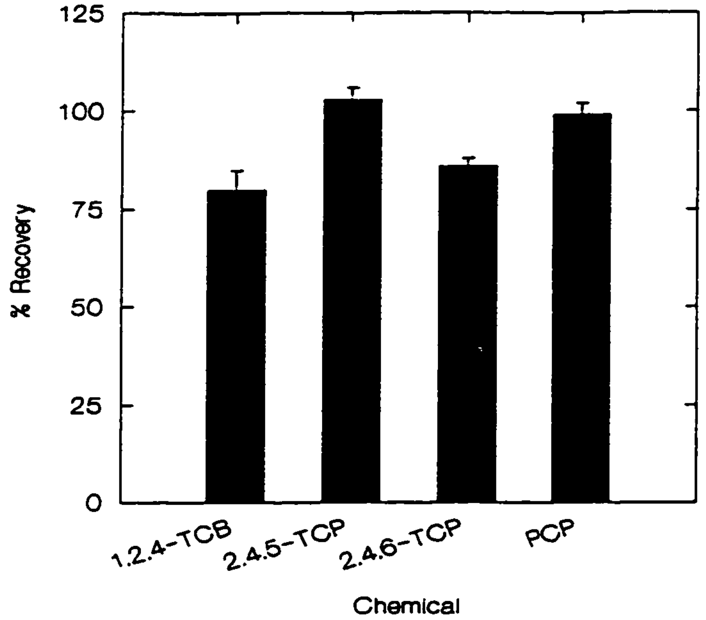


Figure 2.1 The recovery of chlorinated benzene and phenols from spiked water samples (n = 20) using the Cryo-extraction method. Error bars represent 95% confidence limits.

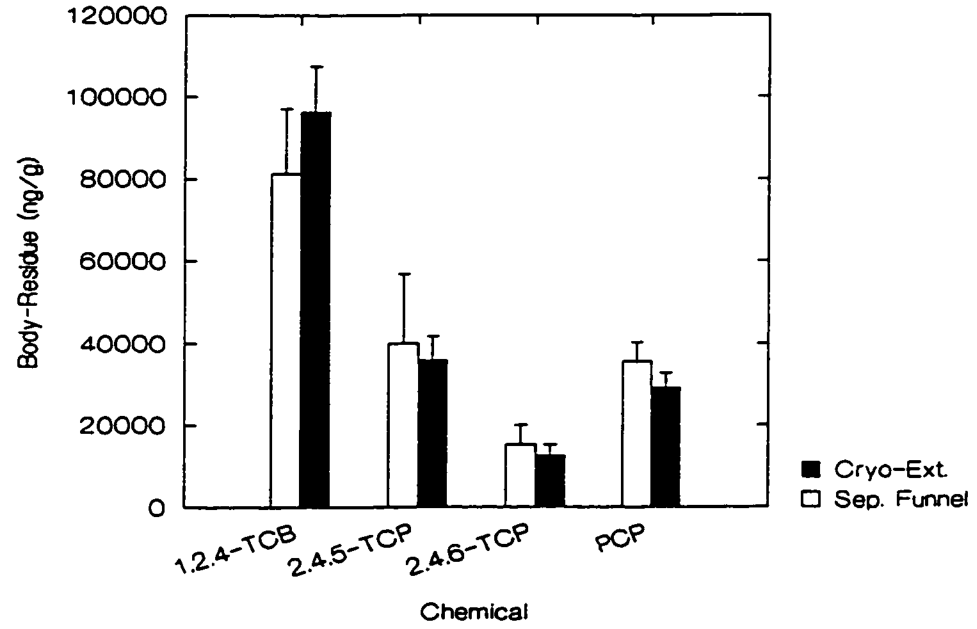


Figure 2.2. A comparison of the recovery of chlorinated benzene and phenols from fish tissue (n = 20) between the Cryo-extraction procedure and the traditional method using a separatory funnel. T-tests conducted for each compound indicated no significant differences between the two methods. P-values were 0.15, 0.65, 0.37 and 0.05 for 1,2,4-TCB, 2,4,5-TCP, 2,4,6-TCP and PCP, respectively.

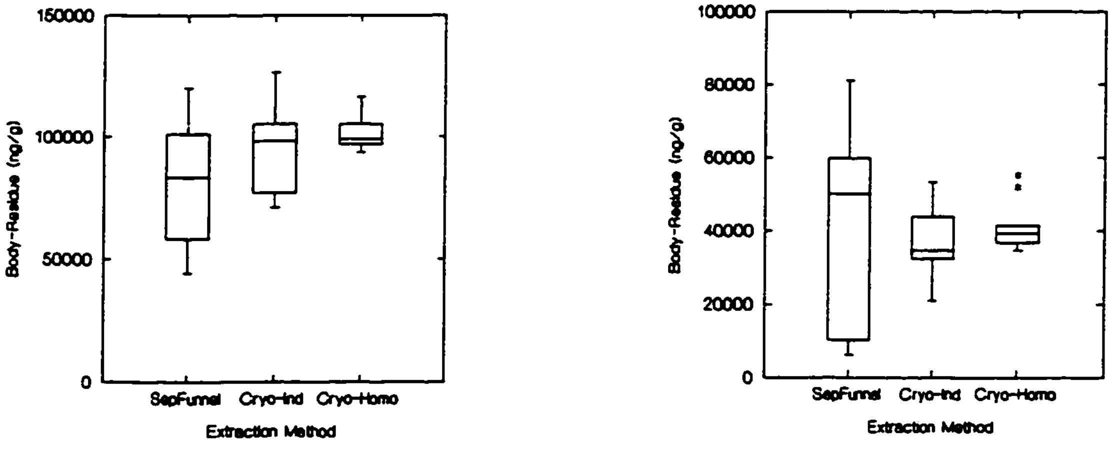
2.3.2 Reproducibility

The reproducibility or precision represents the ability of the method to generate equivalent results for equivalent samples and is defined by the observed variability between replicate samples. Variability arising from measurement error is introduced within the method at every step. Some sources are relatively insignificant, others are not and some cancel each other out. Common sources of variability include errors in volume measurement, inconsistent decanting, incomplete digestion, sample loss during heating or evaporation and the performance of gas chromatography. Despite care during the procedure, these factors can rapidly compound to produce excessive variability in the final quantitative values, greatly reducing the precision of the results. For a simple matrix such as water, the chemical concentrations determined for replicate samples should not differ by more than 10%of the mean. With a more complex matrix such as tissue, this level of variability is often hard to achieve and the acceptability of the data spread is determined by the goal of the study. The body-residues of chemicals quantified in the fish will be used to define rates of chemical uptake and depuration, and the tissue-residues at the time-of-death. A significant amount of uncertainty is added due to natural variability associated with living organisms and therefore a maximum uncertainty range of 2-fold would be considered acceptable within replicate tissue samples.

The reproducibility of the cryo-extraction method was assessed for both water and tissue samples by analysing sample replicates and quantifying the distribution. The evaluation relied heavily on an ability to generate replicates. For water samples, this was achieved by carefully subaliquoting a single stock solution which had been prepared by adding a known amount of chemical to a known volume of water. As indicated by the size of the error bars in Figure 2.1, the variability within the water samples was very low, with the 95% confidence intervals ranging from 5 to 10 % of the mean. The reproducibility was independent of dose and although minor differences existed between compounds, they were not considered significant. Again, the simplicity of the matrix and the simplicity of the refined protocol are very conducive to high performance. The reproducibility of the Cryo-extraction process quantifying chlorinated benzenes and phenols in water samples is considered good.

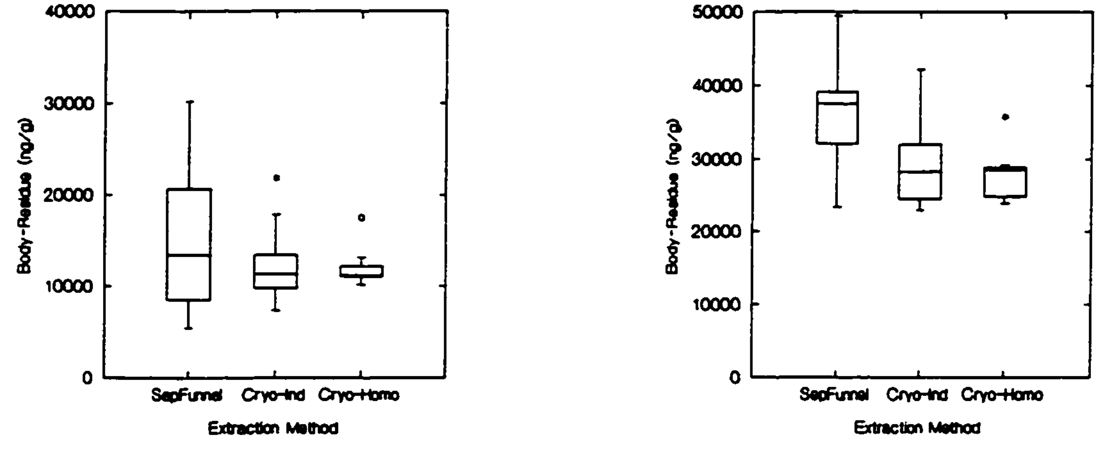
For tissue samples, the reproducibility of the Cryo-extraction method was evaluated by comparing its variability to that of the Separatory funnel method. Again, this comparative approach was necessary since contaminated tissue had to be generated by exposing rainbow trout to the contaminants and thus the actual chemical residues in the fish could not be accurately predicted. In addition, even though fish were exposed to the same concentration of chemical for the same time, natural variability in rates of uptake and depuration will result in slightly different concentrations between individual fish. If this is not taken into account, a significant overestimation of the method variability will result. In order to evaluate this natural variability, an homogenate of several contaminated fish was produced and subaliquotes of this preparation were analysed. Thus the variability in the residues from the subaliquoted homogenate represents the actual method variability, while the variability in the residues from the individual fish residues is the total variability including both the method variability and the natural variability. The difference between the two provides an estimate of the natural variability.

As depicted by the box plots in Figure 2.3, the two methods displayed significant differences in variability. The extraction results from the separatory-funnel method showed excessive variability with tissue residues within replicate samples varying by more than two-fold. Only pentachlorophenol was extracted with a reasonable degree of reproducibility. Previous experience with this method has given similar results. The high variability displayed in these studies significantly impacted on the power of the experiments and was a primary driving force for the development of the cryo-extraction protocol. In contrast, the variability of the cryo-extraction method, as represented by the 95% confidence intervals ranged from 15 to 35% of the mean. Although this variability is also relatively high, it is much more respectable and is well below the two-fold cut-off. A comparison of the variability between individual fish replicates and the homogenate reveal that a good proportion of this variability



Chemical: 1,2,4-trichlorobenzene

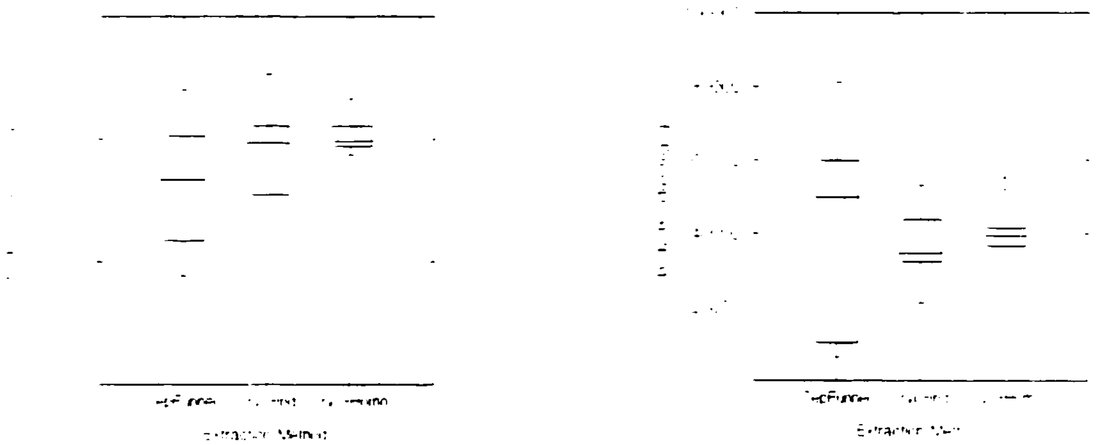
Chemical: 2,4,5-trichlorophenol



Chemical: 2,4,6-trichlorophenol

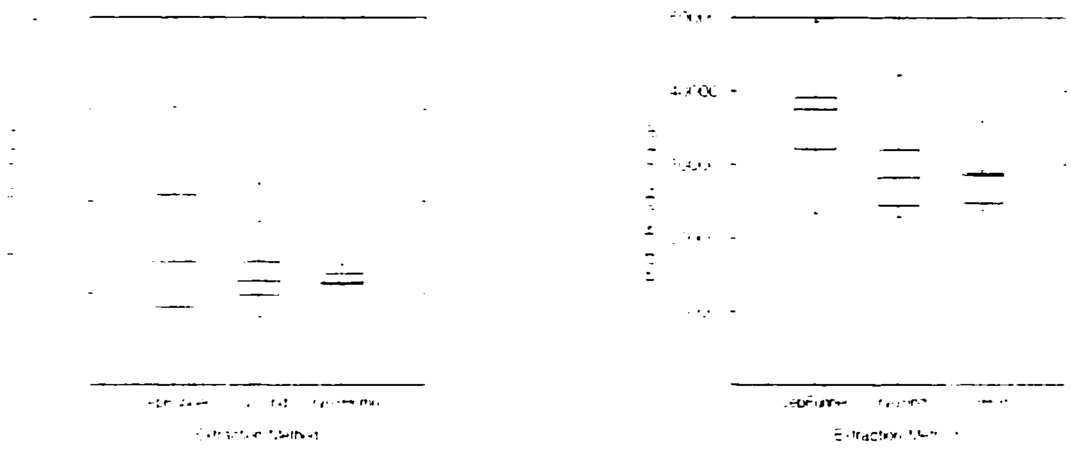
Chemical: pentachlorophenol

Figure 2.3 Box and whisker plots showing the variability associated with chemical body-residues quantified using either the Separatory funnel approach on individual fish (n = 10), the cryo-extraction method on individual fish (n = 10) and the Cryo-extraction method on a fish homogenate (n = 10).



Chemical: 1,2,4-trichlorobenzene

Chemical: 2,4,5-trichlorophenol



Chemical: 2,4,6-trichlorophenol

Chemical: pentachlorophenol

Figure 2.3 Box and whisker plots showing the variability associated with chemical body-residues quantified using either the Separatory funnel approach on individual fish (n = 10), the cryo-extraction method on individual fish (n = 10) and the Cryo-extraction method on a fish homogenate (n = 10).

is natural. The extracted chemical values from the homogenate replicates had a relatively low variability with deviations ranging from 15 to 20%. This variability represents the actual reproducibility of the cryo-extraction method. These values are higher than what was observed for the water samples. However, the increased number of steps and complexity accounts for this and the results are still quite acceptable. The difference in the variability (10 to 15%) represents the natural variability introduced by the biology of the experiment. Typically, this is much higher but the small and very uniform sizes of the fish may have minimized this influence.

For lipophilic chemicals, normalizing chemical body-residues for lipid content has the potential to account for some of the variability seen in body-residue data. A number of papers describe the effect of lipid content on the kinetics of chemical uptake and depuration in fish. Typically, a higher fat content is associated with higher bioconcentration factors while the leaner fish display lower rates of chemical accumulation. Since body-residues of the chemicals are dependent on lipid content it is prudent to express the chemical concentrations in terms of the total lipid instead of the total body weight.

The application of lipid normalization to the rainbow trout tissue quantified in the reproducibility experiments did not significantly reduce the variability between replicate fish samples. When comparisons are made between fish species, this normalization is important in order for the comparisons to be meaningful. However, for fish of the same size , of the same age and raised in the same hatchery from the same batch of eggs, the deviations in lipid content were minor and in such small fish had little consequence on the final chemical tissue concentrations. Lipid levels in the fish ranged from 0.9 to 2.7%.

As a result of their size, smaller fish and invertebrates more closely approximate a single compartment where chemicals are simply taken up and depurated. The partitioning of the chemical within and between tissues is relatively rapid and distribution within the body reaches a steady-state quickly. Smaller fish, unlike the larger ones, are less prone to the complications imposed by a multicompartment system where chemical kinetics vary from gill, to blood, to muscle, to fat and so on.

2.3.3 Linearity

The linearity of the method describes the relationship between the concentration in the sample and that determined by the extraction, but unlike the recovery, it describes this relationship over a wide range of sample concentrations. This relationship includes both the extraction process and the analytical analysis. Theoretically, a linear relationship should hold true for an infinite range of values where an incremental increase in the body-residue is reflected by an equivalent increase in the body-residue measurement. However, in practice there is a saturation point above which the method's efficiency drops significantly. In addition, there is a detection limit below which the quantified value is not significantly different from background.

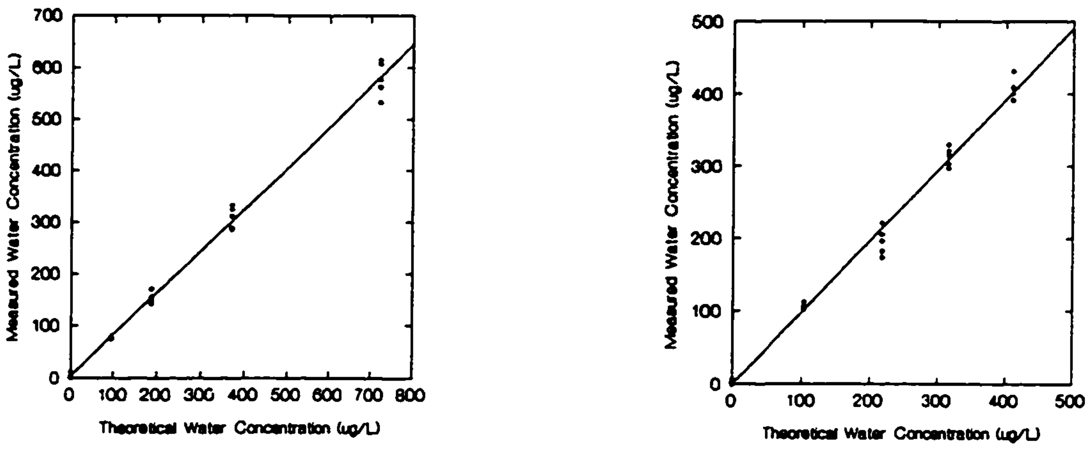
A linear relationship between the actual concentration and the measured signal from the analytical instrument is important because it provides a simple yet statistically rigorous approach for interpolation. Standard curves that are linear functions are easy to apply and are usually more powerful than non-linear functions.

The linearity of the cryo-extraction method was assessed for both the water and tissue samples. For both sample types, a change in the expected chemical concentration was matched by a proportional change in the measured chemical concentration as shown in Figures 2.4 and 2.5, respectively. Within the range of chemical concentrations available both in the water and the fish tissue, no significant deviations from linearity were observed. Simple linear regression analysis was performed in order to define the linear model and to statistically evaluate the goodness-of-fit between the data and the linear model. For the water samples, where the quantified chemical concentrations at each dose could be compared directly to the nominal water concentration, the plot of one against the other should produce a slope of 1. The regression analysis showed that the slopes were consistently lower than 1 suggesting that the recovery was not 100%. However they ranged from 0.802 to 0.982. The correlation coefficients (r-squared) for the water data were also very good being consistently above 0.95, indicating that the data fit the linear model very well. A similar trend was observed with the tissue residues (Figure 2.5). At this point, the slope of these graphs are somewhat arbitrary but again, the correlation coefficients demonstrate a very good fit of the linear model. The r^2-values ranged from 0.92 to 0.99. A value above 0.8 is considered a good fit.

There are practical limitations to the linear model, more so as the chemical concentration to be quantified gets smaller and smaller. Where dilution can be used to accommodate very high chemical concentrations there is a limit of detection below which the response is no longer linear. For both the water and the tissue samples the detection limit was chemical specific and was related to the sensitivity of the EC Detector on the GC. For water samples the detection limit of 1,2,4-trichlorobenzene was 30 ug/L, of 2,4,5-trichlorophenol was 20 ug/L, of 2,4,6-trichlorophenol was 50 ug/L and of pentachlorophenol was 10 ug/L. For tissue samples the detection limit of 1,2,4-trichlorobenzene was 60 ug/g, of 2,4,5-trichlorophenol was 20 ug/g, of 2,4,6-trichlorophenol was 5 ug/g and of pentachlorophenol was 10 ug/g.

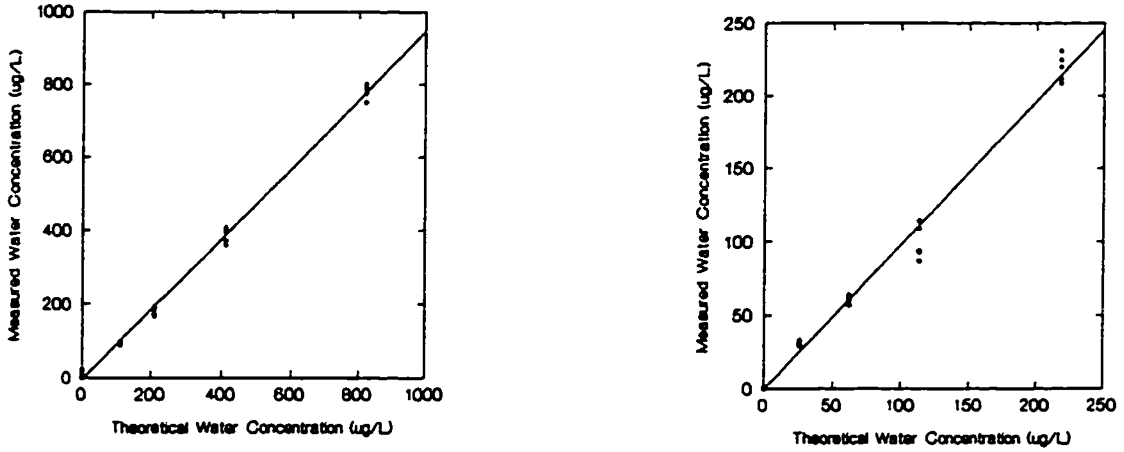
2.3.4 Sample Contamination

The impurity of greatest concern was fish lipid derived from the extracted tissue. If in high enough quantities, this lipid severely impacts on the GC baseline making peak resolution and integration difficult thus requiring a lengthy column burn-out which wastes considerable time and greatly reduces the life-expectancy of the column. A great deal of effort was centred around reducing the level of the lipid impurity. Initially, solubilization employed conc. HCl. This is still the acid of choice providing complete solubilization at room temperature after only 6 to 8 hours. However, the HCl does not degrade the lipid and thus a significant portion of these molecules co-extract with the chemicals and end up contaminating the organic



Chemical: 1,2,4-trichlorobenzene

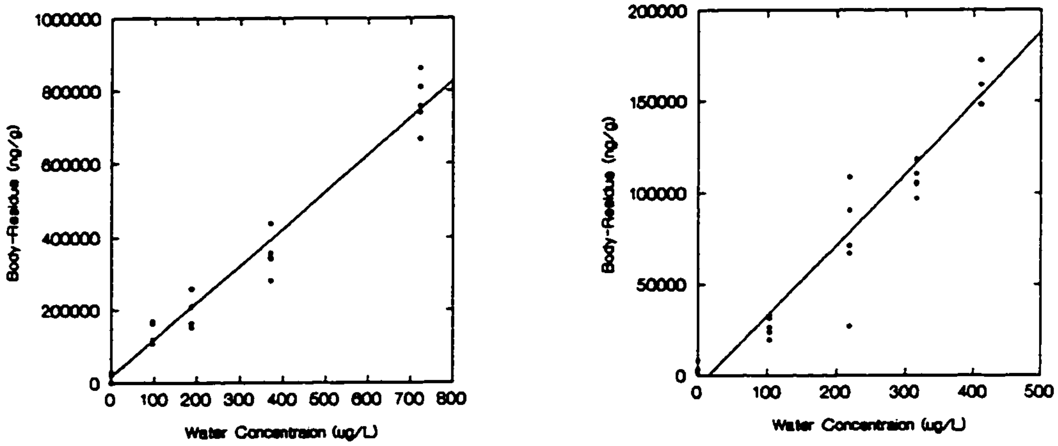
Chemical: 2,4,5-trichlorophenol



Chemical: 2,4,6-trichlorophenol

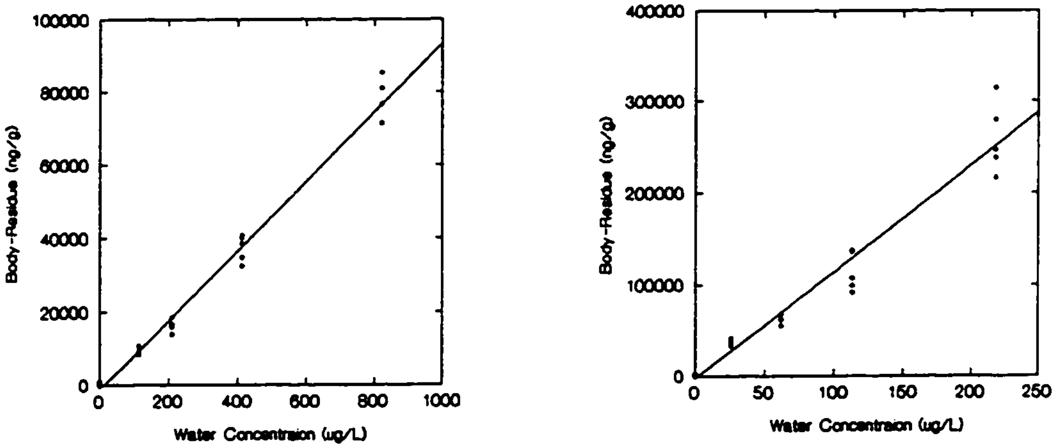
Chemical: pentachlorophenol

Figure 2.4 The relationship between the expected water concentration and the concentration determined using the cryo-extraction method. A straight line was fitted to the data to demonstrate linearity between the observed and the expected values.



Chemical: 1,2,4-trichlorobenzene

Chemical: 2,4,5-trichlorophenol



Chemical: 2,4,6-trichlorophenol

Chemical: pentachlorophenol

Figure 2.5 The relationship between the water concentration in the exposure tanks and the whole body-residue in the exposed rainbow trout determined using the Cryo-extraction method. A straight line was fitted to the data to demonstrate linearity.

solvent. Once in the solvent, further clean-up is required before the sample can be injected into the GC. This step required the preparation of an acid-silica column. The sulfuric acid treated Davisil silica gel oxidized the lipid as the sample passed through the column. This oxidized lipid came out of the hexane and was deposited in the column leaving the column eluent free of lipid. Unfortunately, the entire process consumed large volumes of wash solvent (much of which had to be evaporated off to reduce the sample volume), was very labour intensive and was prone to cross-contamination and unexpected changes in column efficiency. In order to bypass this step, tissue solubilization was carried out with conc. H_2SO_4. Although solubilization with the sulfuric acid was a bit slower at room temperature, mild heating for 1 hour greatly enhanced the performance and both tissue solubilization and lipid oxidation was accomplished in one step. Subsequently, during the extraction, the lipid residues remained in the aqueous phase leaving the organic phase relatively uncontaminated. No further sample clean-up was required.

2.4 Conclusions

Through the systematic assessment of the extraction efficiency, reproducibility and linearity, the cryo-extraction protocol was shown to meet or exceed the requirements for an acceptable analytical tool for quantifying the chemical body-residues of chlorinated benzenes and phenols in fish. As a substitute for the conventional separatory funnel methods, the cryo-extraction protocol provides a faster, more efficient and more reliable approach. The speed and efficiency positively impact on both the number of samples that can be processed per day and on the amount of labour intensive effort required.

3.0 DERIVATION OF UPTAKE/DEPURATION RATE CONSTANTS AND CRITICAL BODY-RESIDUES FOR SELECTED CHLORINATED BENZENES AND PHENOLS USING RAINBOW TROUT

3.1 Introduction

An important co-requisite for applying the body-residue approach for predicting the toxicity of chemical mixtures to aquatic organisms is the use of toxicokinetic modelling. For many organic chemicals, it is possible to accurately describe the movement of the compound between an organism and its surroundings by using a one-compartment first-order kinetic (1CFOK) model (Landrum *et al.*, 1992). Thus, with the appropriate chemical specific information, one can predict the concentration of chemical residues in an aquatic organism for a given chemical exposure at any given time. The same model used for individual compounds should be applicable to a mixture of compounds making it possible to predict multiple residues within an organism. The ability to model body-residues for a mixture of chemicals becomes a valuable design and prediction tool when combined with a measure of the critical body-residue and an assumption of strict addition.

Although somewhat impractical for application in a "real" ecosystem, as a result of the numerous factors which could potentially modify the partitioning behaviour of a compound (Sprague, 1985), 1CFOK modelling is ideal within a controlled setting such as a research laboratory. However, even with a highly regulated system, it is important to have site specific data. Models are only as good as the information one uses to run them. The purpose of this section was to derive the necessary kinetic and toxicological parameters so that body-residues and toxicity for mixtures of 1,2,4-trichlorobenzene, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol could be accurately modelled and studied.

3.1.1 The 1CFOK Model

Models, in general, can be defined as mathematical representations of complex biological systems designed to simplify, clarify and predict responses in those biological systems based on experimentally derived parameters (McCarty *et al*, 1992). Models have also been described as "simplified nonfunctional replicas of the real thing" (Dixon, pers. comm). Both of these definitions are equally valid and in fact serve to accent the expectations and limitations associated with any modelling exercise. Models provide a fast and efficient means for addressing complex dynamic issues and, although they tend to oversimplify a system, their limitations are primarily a function of our understanding of the mechanisms and the data used to derive them. A good understanding of a model is imperative in order to ensure that its application is appropriate, and that the results are properly interpreted.

The term "kinetic model" implies a change over time. In this case, the concentration of a chemical within the organism changes over a given exposure time. This change is the product of the interaction between the organism and its environment. When a substance enters the organism it is termed "uptake"; when it leaves it is termed "depuration". A difference in the rates of these two processes results in a change in the amount of chemical in the organism. For chemicals that are lipophilic, the chemical has a stronger affinity for the organism relative to the water resulting in partitioning from the water into the organism. The concentration of a chemical in an organism is referred to as a body-residue or body-burden and has units of ug of compound per kg of tissue. A schematic representation of the 1CFOK model, as it relates to a fish, is presented in Figure 3.1.

A one-compartment model actually simplifies the biological system into two compartments, the environment and the organism. The organism is reduced to a single compartment and is not divided up into anatomically or physiologically related units which are expected to contain similar concentrations of the contaminant. It is a black-box. Contaminants go in and contaminants come out. This is obviously inaccurate but it represents the simplest case and is thus an appropriate starting point. Increasing the number of compartments to provide a more realistic model results in an exponential increase in complexity, making the process more complicated than it may need be.

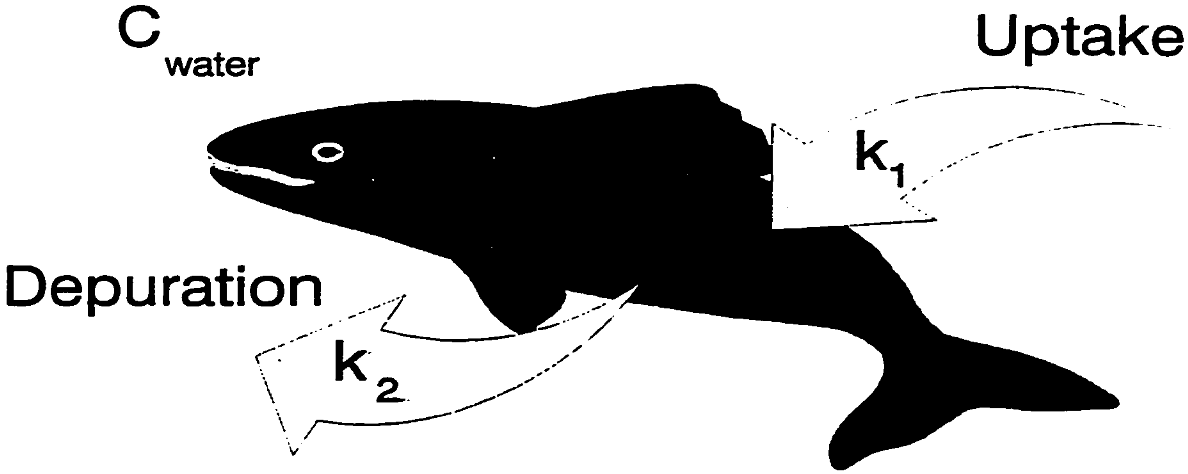


Figure 3.1 A schematic representation of the one-compartment first-order kinetic model as it describes the uptake and depuration of organic chemicals by fish. C_{fish} and C_{water} designate the chemical concentrations in the organism and the water, respectively. The parameters k_1 and k_2 represent rates of chemical uptake and depuration, respectively.

The "order" of the model describes the relationship between the rate of chemical movement (i.e. uptake and depuration) and the chemicals concentration in a particular compartment. For a first-order model, the rate is dependent on the concentration of the chemical in both the exposure medium and the organism itself. That is, the rate of uptake is a function of the concentration of the chemical in the environment (the water) while the rate of depuration is a function of the chemical concentration in the organism. The rates of uptake and depuration are related to the concentrations by the rate constants k_1 and k_2, respectively.

The mathematical form of the 1CFOK model, which provides the relationship between the water-concentration , the body-residue, k_1, k_2, and time for both uptake (Eqn. 1) and depuration (Eqn. 2), is presented by the following differential equations:

$$dCf/dt=(Cw*k_1)-(Cf*k_2) \tag{1}$$

$$dCf/dt=Cf*(-k_2) \tag{2}$$

Once integrated, the respective equations take the more common form:

$$Cf=Cw*\frac{k_1}{k_2}*(1-e^{-k_2t}) \tag{3}$$

$$Cf=Cf_o*e^{-k_2*t} \tag{4}$$

Where:

Cf - toxicant concentration in the fish at a given time (ug/kg)
Cw - a fixed toxicant concentration in the water (ug/l)
Cf_o - toxicant concentration in the fish at the start of the depuration phase (ug/kg)
k_1 - uptake rate constant (hours^{-1})
k_2 - depuration rate constant (hours^{-1})
t - time (hours)

When the body-residue (Cf) is plotted against time for a fixed exposure concentration, standard uptake and depuration curves are obtained (see Figure 3.2).

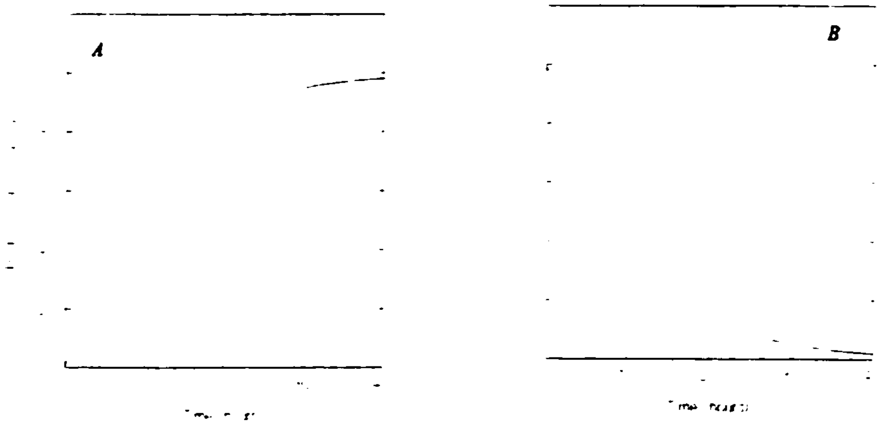


Figure 3.2. Standard uptake (A) and depuration (B) curves as predicted by a 1CFOK model.

Figure 3.2 illustrates a number of characteristics of the 1CFOK model. At time zero the chemical concentration in the organism is also zero and logically there is no depuration. As a result, the net flux into the organism is at a maximum as indicated by the steepness of the curve at this point. In theory, depuration counters this influx the second that the chemical is present in the organism, although in practice there is certainly a lag-time. As the concentration of the chemical in the fish increases so does the rate of depuration. At some point in time (barring death of the organism) the rate of depuration equals the rate of uptake and equilibrium is achieved as indicated by the levelling-off of the curve. Theoretically a steady-state is never achieved since the curve asymptotically approaches this maximum. However, in practice, the organism is considered to have reached steady-state when the body-residue is between 90 and 99% of the theoretical maximum, which is equivalent to an exposure time of four half-lives. The body-residue, at this point, when divided by the water concentration of the chemical, defines the bioconcentration factor (BCF). The BCF is also expressed as k_1/k_2. This follows from Equation 1, since at equilibrium the rate of uptake equals the rate of depuration. Thus k_1 x Cw = k_2 x Cf and k_1 / k_2 = Cf / Cw (by cross-multiplication).

Although depuration is constantly taking place, the depuration phase of the curve results when exposure stops (i.e. the fish is transferred to water where the chemical concentration is zero). The body-residue in the organism during depuration is a function of the initial residue concentration, time and k_2. The rate constant, k_1, is not a factor since the concentration of the chemical in the water is always assumed to be zero. As with uptake, the rate of depuration is maximal during the initial stages and minimizes as the curve approaches zero asymptotically.

3.1.2 Assumptions for Model Application

In order to apply simple models to predict the behavior of complex systems, assumptions are usually necessary. An important assumption of the 1CFOK model is that the values of the rate constants, k_1 and k_2, are independent of the exposure concentration; as their designation implies, they remain constant (Landrum, 1992, McKim and Heath, 1983). Theoretically, a linear relationship exists between the water concentration or dose and the resulting body-residue at any given time (See Figure 3-3).

It has been suggested that the 1CFOK model reduces the fish to "a chunk of fat suspended by a piece of string" (Mayfield, pers. comm.) and that the movement of the toxicant in and out of the organism is modelled as a completely passive process dictated simply by the physical and chemical properties of the environment, the organism and the chemical. This analogy clearly illustrates the apparent oversimplification of the "real" system by the model. Fish are active, dynamic organisms and toxicant uptake and depuration result from a number of active processes (McKim and Heath, 1983). A major route of uptake for chlorinated organic chemicals in the water column is via the gill (Schmieder and Weber, 1992; McKim *et al.*, 1985) and thus ventilation rates and blood flow should play an important role in uptake rates. The depuration of these chemicals can involve biotransformation of the parent compound prior to excretion via the biliary-fecal route (de Wolfe *et al.*, 1993; James, 1986; Andersson and Koivusaari, 1986; Oikari and Anas,

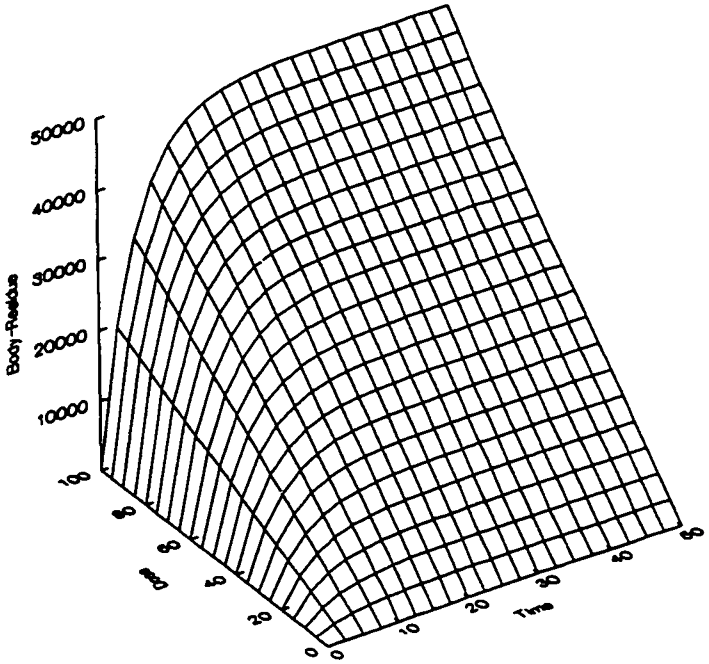


Figure 3.3 A 3-dimensional graph describing the theoretical relationship between the chemical dose or water concentration and chemical uptake (body-residue vs time) for a fish given the assumption that rate constants k_1 and k_2 are independent of dose.

1985). Biotransformation often requires energy input and can be under the regulation of enzymatic processes. Thus, by definition, this is not typically passive. Perhaps the most compelling reason for skepticism arises when a fish is observed lying nearly motionless, save a faint gill movement, at the bottom of an exposure tank for twelve hours. It becomes difficult to expect the rates of chemical uptake and depuration in this fish are equivalent to that of a healthy active fish and are unaffected by the state of the organism.

The assumption that k_1 and k_2 are independent of exposure concentrations and fish health is critical to the application of the 1CFOK model. If the rate constants are a function of dose, or worse, the body-residue, then the model will not accurately predict the concentration of a chemical in the organism at high chemical concentrations and long exposure times. To avoid problems, it would seem logical to simply derive the rate constants at a dose that closely approximates the acute exposure of interest. There are however, practical limitations to this approach: acute exposures result in mortality and rates of uptake and depuration are not typically measured on dead fish. Therefore, it is necessary to apply k_1 and k_2 derived from chronic exposures to acute situations with the assumption that the higher exposure concentrations will not affect rates of uptake and depuration. Curiously enough, although 1CFOK models are frequently used in the literature, rarely is a test of this underlying assumption presented.

A second assumption, important for model application, is that the response, in this case mortality, occurs when and only when a critical body-residue (cBR) is reached within the exposed organism. Like the rate constants, ideally, the cBR must also be independent of the exposure concentration and, in addition, it must be independent of the time-to-death. Ideally, if a fish is exposed to a toxic compound at a high enough water concentration, then a cBR will be achieved at some time defined by 1CFOK's. The time at which this occurs is dependent on both the exposure concentration and the kinetics of uptake and depuration. On the other hand, if the exposure concentration is insufficient, then regardless of the time allowed, the cBR will not be reached and no mortality is expected for the average fish. The application of a cBR based predictor for an acute endpoint must be verified for 1,2,4-trichlorobenzene, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol.

Whole body-residues have been shown to be a suitable surrogate for the chemical dose for some compounds. For neutral narcotics, lethality in fish occurs at a body-residues between 2 and 8 mmoles/kg (Van Hoogen and Opperhuizen, 1988; McCarty, 1989; Carlson and Kosian, 1987). As an example, Van Hoogen and Opperhuizen (1988) have shown that for three chlorobenzenes, lethality occurred at a body-residue of 2.0-2.5 mmol/kg fish. This lethal body-residue was shown to be independent of exposure concentration and duration and allowed them to predict lethality using a simple first order bioconcentration model. Mackay and Hughes (1984) reported lethal body-residues of 4-5 mmol/kg for a series of alkyl esters of p-aminobenzoic acid. The lethal body-residue of pentachlorophenol, a metabolic uncoupler, ranges from 0.28 mmol/kg for fathead minnows (Spehar *et al.*, 1985) to 0.34 mmol/kg for goldfish (Kobayashi and Kishino, 1980). Body burdens associated with thresholds of chronic toxicity have been estimated using QSAR models (McCarty, 1987). Examples of sublethal dose-response relationships may also be drawn from the pulse exposure studies by Seim *et al.* (1984) with copper and Curtis *et al.* (1985) with fenvalerate. Carlson *et al.* (1987) reported mean tissue concentrations associated with chronic toxicity endpoints for four chlorinated benzenes. The geometric mean of tissue concentrations associated with the NOEC and LOEC were 140 ug/g, 85 ug/g, 270 ug/g, 820 ug/g for 1,3 dichlorobenzene, 1,4-dichlorobenzene, 1,2,4-trichlorobenzene and 1,2,3,4-tetrachlorobenzene respectively.

3.1.3 Purpose

Although the initial purpose of this work was to simply derive kinetic and toxicological parameters for use in subsequent modelling of chemical mixtures, it became apparent that validation of the aforementioned assumptions was also required in order to avoid potential complications with data interpretation during the mixture studies. Thus, in addition to generating values for k_1, k_2, BCF, and cBR's for 1,2,4-trichlorobenzene, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol the following issues were also addressed:

- the effect of exposure concentration and organism health on the rate constants k_1 and k_2 (dose-dependent kinetics)
- the relationship between time, dose and the cBR (time-dependent kinetics)

3.2 Materials and Methods

The chemicals used as toxicants in the kinetic studies and the toxicity studies, including 1,2,4-trichlorobenzene, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol, were purchased from the Sigma Chemical Company and assayed to be 99% pure by GC-MS. In order to facilitate chemical addition to the exposure water, stock solutions of the chlorinated organics were prepared. The chlorinated phenols were dissolved in 0.1N NaOH while the chlorinated benzene was mixed with a minimum amount of DMSO as a carrier. An appropriate amount of these stock solutions was added to the water in the exposure tanks or fed into the diluter in order to provide the required dose. The final pH of the exposure water was checked in order to assure that NaOH addition did not significantly alter the in-tank pH. Where DMSO was used, the concentration of the carrier never exceeded 0.2 % v/v.

All studies were carried out with 4 to 6 week old rainbow trout larvae (*Oncorhynchus mykiss*). The fish were purchased as "eyed-up" eggs from the Rainbow Springs Trout Farm, Thamesford, Ontario. The eggs were incubated in the lab and once hatched, were reared until the fry had completely absorbed their yolk-sack (2-3 weeks post hatch). At this stage they were transferred to an artificial stream system where water was continually fed into the troughs at one end and syphoned off at the other. This system was found to maintain the trout in excellent health while making them readily accessible (thus reducing stress during capture and transfer) for subsequent experimentation. Fish were fed twice a day *ad libitum* with a mix of 0.5 and 1-point starter chow purchased from Richies Seed and Feed, Elmira, Ontario. Fish weights at the time of chemical exposure ranged from 80 to 250 mg (wet weight).

3.2.1 Kinetic Studies

Kinetic studies were conducted in order to determine the rate constants for chemical uptake and depuration for 1,2,4-trichlorobenzene, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol. Although rate constants can be adequately derived at a single chemical concentration , the fish were exposed to a geometric range of sublethal doses in order to test the assumption that the rate constants k_1 and k_2 were independent of the exposure concentration. The rate of chemical uptake was quantified by exposing fish to a fixed concentration of each compound for 48 hours in a 12-hour static renewal system. Fish were sacrificed at 8 hour intervals and stored for whole-body-residue analysis. The depuration phase was initiated by transferring the remaining fish to tanks containing clean water with a flow-through system in order to maintain water quality. A similar sampling regime was followed for another 48 hours. The temperature of the exposure water was maintained at 15°C and only ambient lighting was used. The exposure water was aerated for the duration of the study. For each chemical of interest, 4 concentrations were chosen and 3 replicates were performed for each. Approximately 80 fish were used per replicate with three to four being sampled at each time point. All fish tissue was stored at -80 °C pending whole-body-residue analysis.

In order to determine the significance of fish activity on chemical kinetics, additional uptake and depuration experiments were performed using dead fish. Experiments were carried out as described above with several modifications. The fish exposure was limited to 48 hours; the dead fish were showing noticeable signs of decomposition beyond this time. Therefore the uptake phase, where dead fish are exposed to the chemicals, were run independently from the depuration phase. The fish were sacrificed immediately before being placed in the exposure tanks. Live fish were exposed at the same time for a direct comparison of the body-residue accumulation over time. In order to determine the depuration rates for dead fish, a subsample of the live fish exposed previously were sacrificed and then placed in clean water. This guaranteed that a measurable quantity of chemical was present in the organism so that its loss could be accurately quantified. As with the uptake phase, live fish which were previously exposed, accompanied the dead fish. Fish were sacrificed by saturating the water with nitrogen; it was felt that freezing or a "blow to the head" would potentially rupture cells and compromise the integrity of the fish. The kinetic studies comparing live and dead fish were conducted only with 2,4,5-trichlorophenol and pentachlorophenol.

3.2.2 Toxicity Tests

Toxicity tests were conducted with acutely lethal chemical concentrations of each of the compounds in order to determine both the time to death and the whole-body-residue at the time of death for the rainbow trout larvae. A continuous-flow nine-concentration diluter was used to expose fish to a geometric range of 1,2,4-trichlorobenzene, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol concentrations. The system was set-up to provide a 20-fold difference between the highest and lowest concentration plus a control. The range of concentrations was chosen to produce greater than 50% mortality in half the exposure tanks thus providing ample fish for which critical body-residues could be derived, while still illustrating a dose response relationship.

Twenty fish were randomly placed in each exposure tank and the tanks were monitored for fish behaviour and mortalities every 4 hours for 7 days. Mortality was assessed based on gill movement. Dead fish were removed immediately and stored at -80°C until their critical body-residues could be quantified. Both the number of mortalities and the "time to death" were recorded for each exposure tank. The temperature of the exposure water was maintained at 15°C and only ambient lighting was used. All tanks were aerated for the duration of the exposure time. More than 10% mortality in the control tank resulted in the termination of the toxicity test; it was subsequently repeated. This occurred only twice during the course of the experiments both a result of inadvertent contaminant exposure due to an overhead leak.

3.2.3 Tissue and Water Analysis

All tissue samples were weighed and the chemical residues quantified using the Cryo-extraction protocol outlined in Section 2.0. In order to improve peak resolution, samples containing pentachlorophenol were methylated as described by Levitt (1973) prior to GC-ECD analysis. All results were reported in terms of measured chemical concentrations.

Water samples from the exposure tanks were routinely taken (once per day) during the course of the kinetic and toxicity studies. Chemical quantification was done on these samples immediately in order to verify the expected exposure concentration. At no time did the exposure concentration deviate by more than 10% during the course of an experiment. All analysis was accomplished using the Cryo-extraction protocol outlined in Section 2.1 followed by GC-ECD analysis.

3.2.4 Statistics

Kinetic Studies

The body-residues quantified for the fish sampled at each time point during both the uptake and depuration phases of the kinetic experiments were used to generate plots of body-residue vs time for 1,2,4-trichlorobenzene, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol. A 1CFOK model was fitted separately to the uptake and depuration phases of the curve using the non-linear curve fitting provided by SYSTAT 5.0. The value of k_2 was derived first using the depuration curve and then this value was substituted into the uptake function in order to model the uptake curve to derive k_1. In this way the estimation procedure only solved for one unknown at a time.

An ANOVA procedure was used to evaluate the relationship between the chemical concentration in the water and the values of either k_1 or k_2. The rate constants were derived at four chemical concentrations and each exposure concentration was repeated in triplicate. The null hypothesis for the ANOVA was that the rate constants were constant and were not influenced by the exposure concentration. Graphical representation of the data (rate constants vs the exposure concentration) also aided in identifying trends that might otherwise be masked by the sample variability.

Toxicity Studies

From the exposure tanks in which greater than 50% mortality was observed, the body-residues were quantified for a random selection of samples. A total of 40 fish were chosen. The body-residue information for these fish were plotted against the time of death and the linearity and slope of the resulting scatter plot was tested. The null hypothesis was that the data fell along a straight line with a slope of zero. Linear regression was used to derive the slope and check deviations from linearity.

3.3 Results

3.3.1 Kinetic Studies

During exposure to sublethal concentrations of 1,2,4-trichlorobenzene, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol, the juvenile rainbow trout displayed no signs of acute toxicity in response to the presence of the toxicants. The majority of the fish showed no behavioural effects such as loss of equilibrium, hyper- or hyposensitivity. Those fish that did show signs of stress recovered rapidly and completely when returned to uncontaminated water.

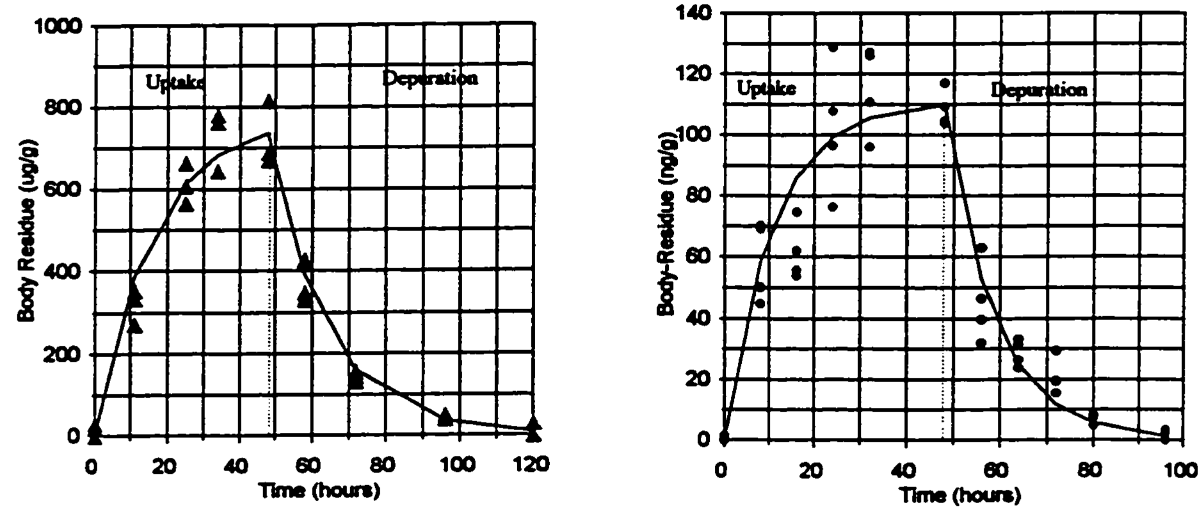
Graphical representation of body-residues versus time, for each of the chemicals being studied, produced uptake and depuration curves typical of what would be expected if the movement of the chemical between the water and the fish followed 1CFOK's (see Figure 3.4). Although significant differences in kinetic activity existed between the compounds, all of the curves depicting uptake and depuration, regardless of chemical type or concentration, followed one-compartment first-order kinetics. Non-linear curve fitting conducted with SYSTAT 5.0 confirmed the goodness of fit with the 1CFOK model. Correlation coefficients between the data and the predicted values, based on the 1CFOK model, ranged from 0.7 to 0.98. The examples shown in Figure 3.4 had correlation coefficients greater than 0.87.

The rate constants, k_1 and k_2, derived from the uptake/depuration curves are summarized in Table 3.1. Statistical regression analysis of the relationship between the exposure concentration and the magnitude of the rate constants k_1 and k_2 indicated no significant difference between the rate constants obtained at each of the chemical doses; the slopes were not significantly different from zero. This supports the assumption that the values of k_1 and k_2 are indeed constants, independent of the chemical exposure concentration.

Since the rate constants were shown to be independent of dose, all of the empirical values derived for k_1 and k_2, for each chemical, were pooled in order to provide a single representative value to be used for modelling. This and other relevant information obtained from the uptake/depuration curves is summarized in Table 3.2. The values of k_1 ranged from 21 hours^{-1} for 2,4,6-trichlorophenol to 110 hours^{-1} for pentachlorophenol. The values of k_2 ranged from 0.063 h^{-1} for 1,2,4-trichlorobenzene to 0.193 h^{-1} for 2,4,6-trichlorophenol. In more practical terms, the time it took for the compounds in the water to reach 90% of its maximum in the body of the fish (T_{90}) ranged from 36.5 hours for 1,2,4-trichlorobenzene down to 11.9 hours for 2,4,6-trichlorophenol. The same trend was seen for the measure of depuration rate with the biological half-life ($T_{1/2}$) of the compounds in the fish ranging from 3.6 to 11 hours.

1,2,4-trichlorobenzene (at 728 ug/L)

2,4,5-trichlorophenol (at 313 ug/L)



2,4,6-trichlorophenol (at 220 ug/L)

pentachlorophenol (at 201 ug/L)

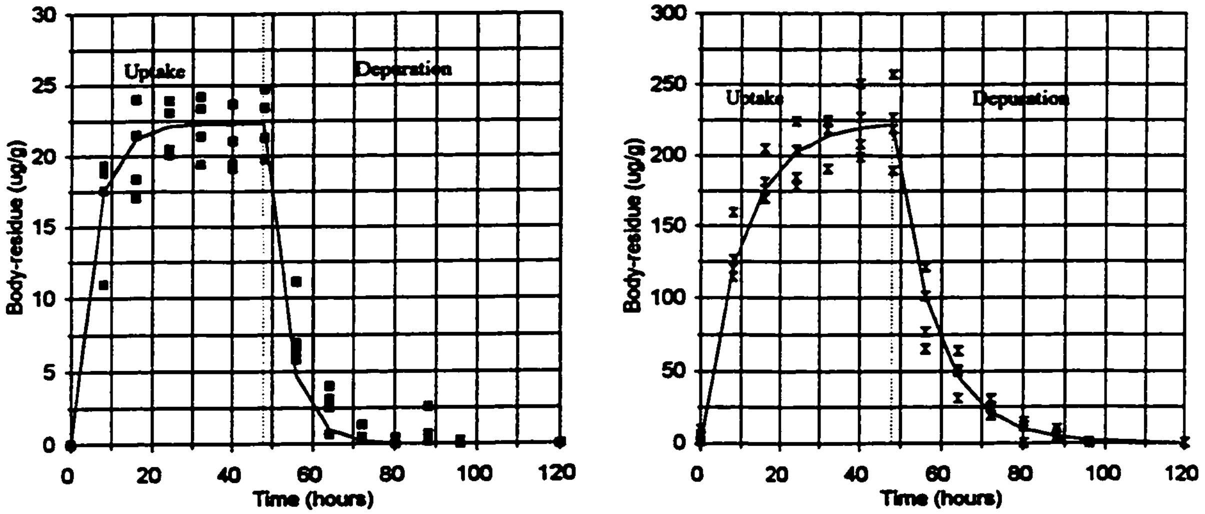


Figure 3.4 Typical uptake and depuration curves for rainbow trout larvae exposed to sublethal concentrations of 1,2,4-trichlorobenzene (▲), 2,4,5-trichlorophenol (●), 2,4,6-trichlorophenol (■) and pentachlorophenol (X). Data points represent quantified body-residues in replicate fish sampled at the designated times. The 1CFOK curve was fitted to the data points using the NONLIN module in SYSTAT 5.0.

Table 3.1. 1CFOK rate constants (k_1 and k_2) derived for 1,2,4-trichlorobenzene. 2,4,5-trichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol using a range of sublethal exposure concentrations. The error term represents the 95%CI of the three replicates.

Chemical	Dose (ug/L)	k_1 (hours^{-1})	k_2 (hours^{-1})
1,2,4-trichlorobenzene	89	72.8 ±2.1	0.069 ±0.006
	182	60.5 ±11.8	0.062 ±0.015
	363	70.0 ±6.6	0.067 ±0.002
	724	61.5 ±7.4	0.054 ±0.007
2,4,5-trichlorophenol	104	35.5 ±2.8	0.093 ±0.007
	209	31.4 ±3.5	0.087 ±0.010
	311	31.2 ±7.1	0.088 ±0.009
	416	33.9 ±4.4	0.102 ±0.008
2,4,6-trichlorophenol	103	17.5 ±3.2	0.163 ±0.016
	210	22.0 ±3.2	0.204 ±0.013
	413	24.8 ±3.7	0.215 ±0.009
	811	19.9 ±1.3	0.192 ±0.009
pentachlorophenol	25	126 ±21	0.104 ±0.016
	53	78 ±28	0.076 ±0.011
	100	107 ±12	0.096 ±0.017
	208	127 ±7	0.111 ±0.017

The units used to designate the cBR in Table 3.2 have been converted to nmoles of chemical per gram of fish versus the more standard mg of chemical per gram of fish. The conversion was done to put the values of the cBRs, for each chemical, in perspective. The response of an organism to a chemical insult is the product of single contaminant molecules interacting at a target sites. The number of molecules of a chemical in the organism defines its activity but is not immediately apparent when the concentration is expressed based on the chemical weight. The use of nmoles instead of mg is more appropriate because it provides this information directly.

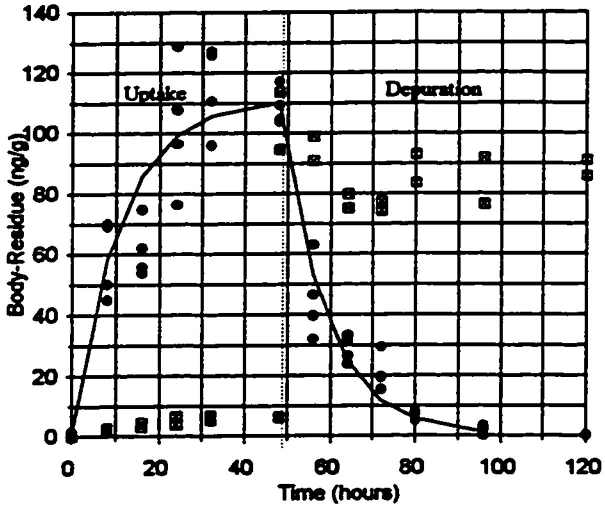
Table 3.2 Summary of modelling parameters for 1,2,4-trichlorobenzene, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol.

Modelling Parameters	**Chemicals**			
	1,2,4-TCB	**2,4,5-TCP**	**2,4,6-TCP**	**PCP**
k_1 (hours^{-1})	66.4 (±12.0)	33.0 (±2.1)	21.0 (±2.1)	110.0 (±36.2)
k_2 (hours^{-1})	0.063 (±0.014)	0.093 (±0.007)	0.193 (±0.022)	0.097 (±0.024)
BCF	1054 (±152)	355 (±20)	109 (±5)	1134 (±116)
cBR (nmoles/g)	6779 (±696)	1942 (±256)	1695 (±160)	989 (±138)
T_{90} (hours)	36.5	24.8	11.0	23.7
$T_{1/2}$ (hours)	11.0	7.4	3.6	7.1
ILL (ug/L)	1157	1074	3099	220

Values designated by brackets represent the standard deviation calculated for the sample mean.

When dead fish were exposed to chemical concentrations in the uptake /depuration studies, a significant difference was noted, in comparison to live fish, between the rates of both uptake and depuration. The live fish accumulated the compound as predicted from the previous kinetic studies while the dead fish showed a decrease in chemical accumulation by 10 to 20-fold. Thus, the resulting body-residues at equilibrium were significantly lower (see Figure 3.5). A similar result was seen during chemical depuration. When previously exposed fish were placed in clean water, the live ones rapidly depurated the chemical and the body-residues asymptotically approached zero. Fish that had been sacrificed just prior to transfer into the tanks with uncontaminated water, depurated less than 10 % of their body-burden within the same time frame. Kinetic modelling of the data in order to obtain values for k1 and k2 was not possible since the data was not accurately modelled by 1CFOK's. The data indicates that at the time of death, chemical partitioning between the water and the fish is significantly restricted.

2,4,5-trichlorophenol (at 313 ug/L)



pentachlorophenol (at 201 ug/L)

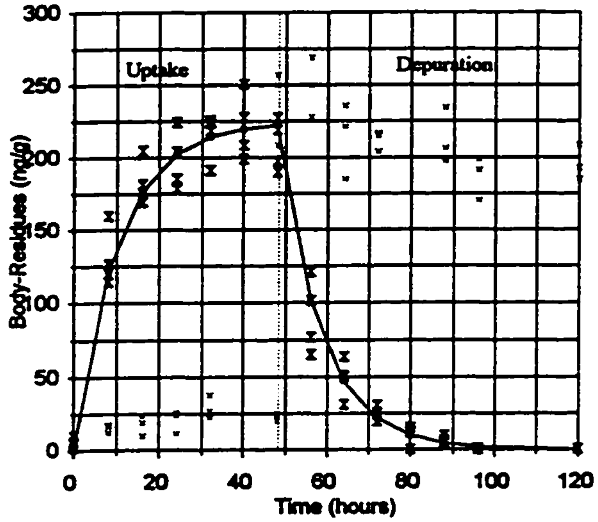


Figure 3.5 Uptake and depuration curves for rainbow trout larvae exposed to sublethal concentrations of 2,4,5-trichlorophenol (●) and pentachlorophenol (⧗). The data points represent body-residues quantified in both live fish (●,⧗) and in fish sacrificed just prior to being introduced into either the uptake or depuration tanks (⊠,X). A 1CFOK curve was fitted to the body-residues of the live fish using the NONLIN module in SYSTAT 5.0. Fitting a curve to the body-residue data from the sacrificed fish was not possible due to the significant deviations from the 1CFOK model.

3.3.2 Toxicity Tests

The juvenile rainbow trout exhibited good dose-response relationships during toxicity tests with 1,2,4-trichlorobenzene, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol The response of the fish was proportional to the chemical exposure concentration, with those exposed to the highest chemical concentrations dying first. With decreasing exposure, the rate of mortality decreased while the time to death increased. With all four compounds, where the exposure concentration was low enough, the lethal response was limited to less than 50%; the majority of the fish survived for the entire exposure period and showed full recovery within a short time of being returned to clean water. The maximum exposure concentration at which less than 50% of the fish died was considered a reasonable approximation of the incipient lethal level (ILL). The ILL is defined as the concentration below which 50% of the organism are expected to survive for an indefinite period of time. This value is useful as a time-independent measure of lethal toxicity and marks a boundary concentration between the potential for lethal effects and sublethal effects.

Chemical residues quantified in the bodies of the fish which died as a result of chemical exposure showed good agreement with each other in that the concentration of the chemical at the time of death was relatively constant, regardless of exposure dose and duration (Figures 3.6 and 3.7). Statistical analysis of the data using an ANOVA showed no correlation between the cBR and either dose or time-to-death. The slopes of the lines fit to the data in these plots were not significantly different from zero (p-values <0.05).

Predicted body-residues based on the chemical concentrations in the water, time-to death and the kinetic parameters derived above agreed for the most part with the actual values. Some discrepancy was noted for the higher chemical concentrations where mortality occurred within 8 hours. In these cases, the predicted values tended to overestimate the expected residue concentration in the organism. This was most noticeable with 2,4,6-TCP and PCP.

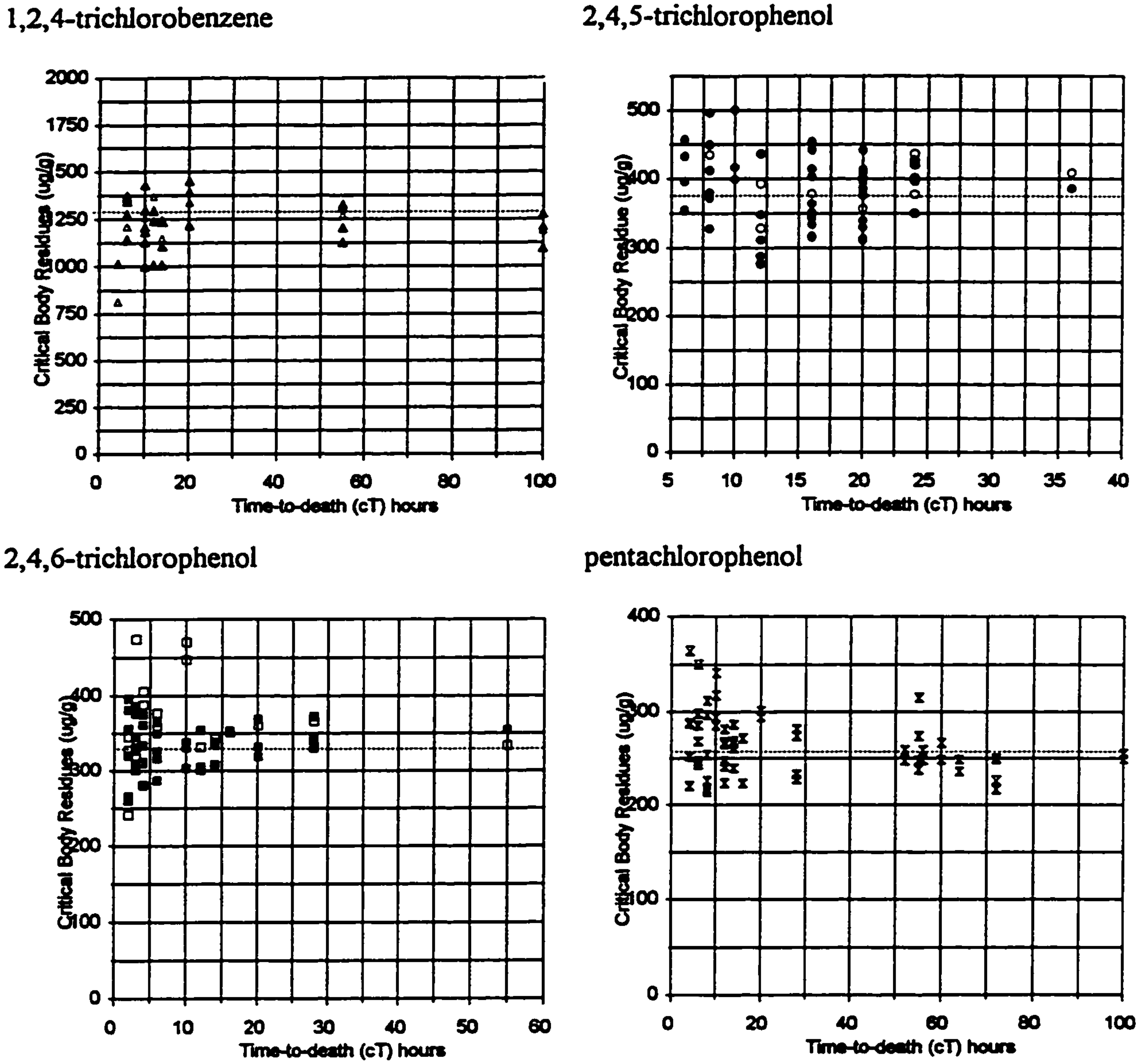
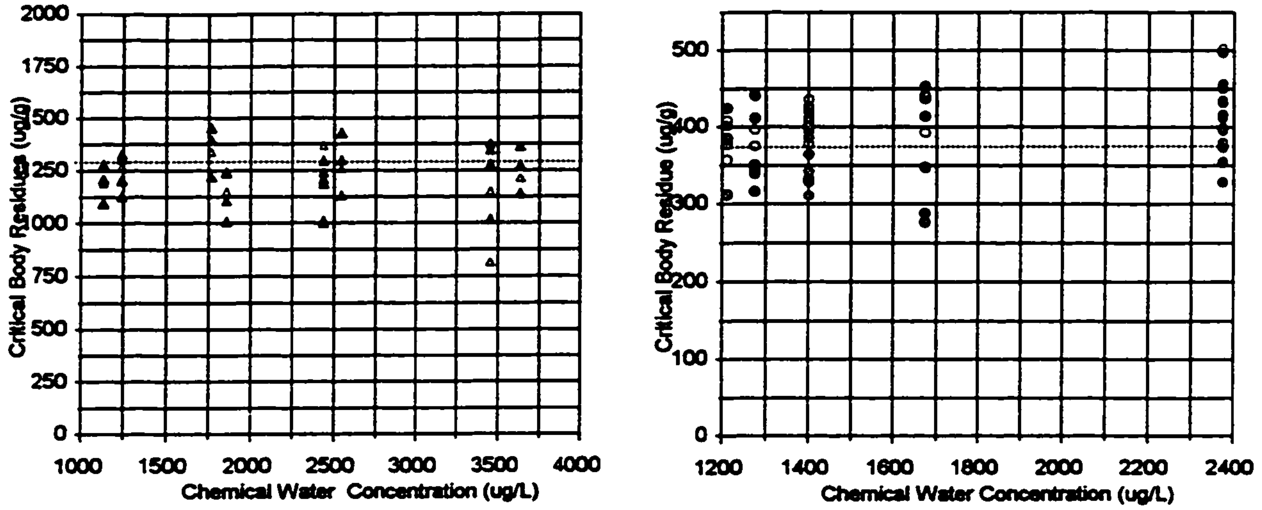


Figure 3.6 Scatter plots showing the dependence of the critical body-residue on the time-to-death for rainbow trout larvae exposed to lethal concentrations of 1,2,4-trichlorobenzene (▲), 2,4,5-trichlorophenol (●), 2,4,6-trichlorophenol (■) and pentachlorophenol (I). The shaded symbols represent quantified body-residues from replicate fish. Open symbols represent predicted body-residues using the 1CFOK model and based on the exposure level and the time-to-death. The dashed line intercepts the cBR-axis at the mean cBR, and with a slope of zero, designates the trend if the cBR was independent of the time-to-death.

1,2,4-trichlorobenzene 2,4,5-trichlorophenol



2,4,6-trichlorophenol pentachlorophenol

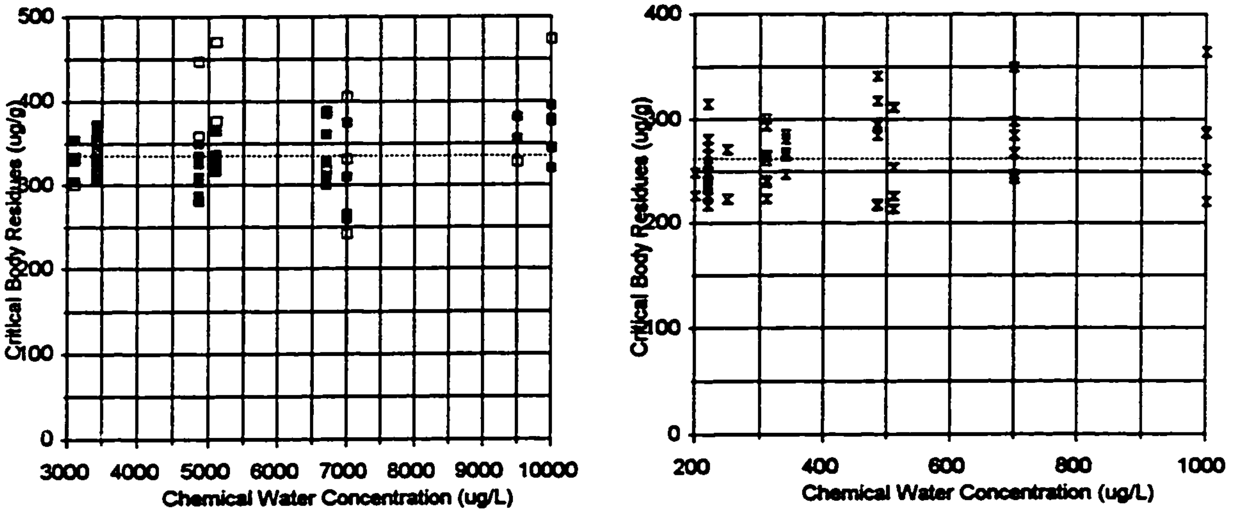


Figure 3.7 Scatter plots showing the dependence of the critical body-residue on the exposure concentration for rainbow trout larvae exposed to lethal concentrations of 1,2,4-trichlorobenzene (▲), 2,4,5-trichlorophenol (●), 2,4,6-trichlorophenol (■) and pentachlorophenol (I). The shaded symbols represent quantified body-residues from replicate fish. Open symbols represent predicted body-residues using the 1CFOK model and based on the exposure concentration and the time-to-death. The dashed line intercepts the cBR-axis at the mean cBR, and with a slope of zero, designates the trend if the cBR was independent of the exposure concentration.

In general, variability in both the measured and predicted values was greatest for the higher chemical concentrations where the fish died quickly . This variability decreased for those fish which died at more moderate exposures and at later times (i.e. after 12 hours). The cBR for each chemical, derived from the mean (± the s.d.) of all the data points, are summarized in Table 3.2.

3.4 Discussion

3.4.1 Uptake/Depuration Kinetics

A number of researchers have demonstrated the ability of the 1CFOK model to accurately describe the uptake and depuration of organic compounds in aquatic organisms. (Makela *et al.*, 1991; Makela and Oikari, 1990; Smith *et al.*, 1990; Van Hoogen and Opperhuizen, 1988; Galassi *et al.*, 1988). The results presented here support the utility of the model. For 1,2,4-trichlorobenzene, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol, and pentachlorophenol. uptake and depuration by the fish were accurately described using one-compartment first-order kinetics. As discussed further below, the predicted relationship between the body-residue and time showed a good fit with the measured data, the kinetic parameters for each compound correlated well with their expected behaviour, and the importance of generating "site-specific" modelling information was reinforced.

The correlation between the predicted uptake/depuration curves and the uptake/depuration data was very good, with correlation coefficients ranging from 0.70 to 0.98. Although more complex multi-compartment modelling is possible (Leung, 1991; Wijnand, 1987; Gingerich and Rach, 1985), the 1CFOK model was expected to adequately describe the partitioning and associated kinetics of chlorinated organics between the fish and the water. The lipophilicity of these compounds naturally drives them from an aqueous phase into an organic phase and the movement of the molecules from the water into the fish tissue is considered completely passive (Hunn and Allen, 1974). There are no direct active mechanisms of transport across membranes and the kinetics are controlled primarily by diffusion. When passive diffusion is the driving mechanism, first-order kinetics should be observed.

The use of small fish in the studies also helped to ensure the goodness-of-fit between the uptake/depuration data and the 1CFOK model. Physiologically, rainbow trout larvae are clearly not single compartments but were still well represented by a one-compartment model. As with larger fish, the larvae still assimilate the chlorinated benzenes and phenols through the gill, where it is transferred to the circulating blood and on to the liver, muscle and fat stores (Barron, 1995). During depuration, some of the chemical is excreted to the water back through the gill but the majority requires biotransformation subsequent to its release via the bilary/fecal route (McKim and Heath, 1983). All of these discrete areas within the fishes body represent separate compartments and the ideal multi-compartment model would consider the chemical kinetics between each. However, in smaller fish, the interaction between the numerous compartments seems to be quick enough so that the different tissues in the fish rapidly attain steady-state with each other. Therefore, they behave as a single compartment even though they are not. In larger fish, as a result of size and increased volumes, the rates of chemical partitioning within the fish are slower (Barron, 1995). As a result, these become a factor in the overall uptake/depuration process and may cause significant deviations from the one-compartment model.

The compounds 1,2,4-trichlorobenzene, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol have similar physical and chemical properties but even within this closely related group of compounds, marked differences in uptake/depuration kinetics and chemical partitioning were observed (see Table 3.2). Differences in chemical structure lead to differences in molecular weight, solubility and lipophilicity which in turn are reflected in the partitioning behaviour and kinetic parameters. For 1,2,4-trichlorobenzene, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol, the BCF values (or the k_1/k_2) correlated well with the compound's lipophilicity as described by the water solubility and the octanol/water partition coefficient (log Kow). Pentachlorophenol and 1,2,4-trichlorobenzene, which have the lowest solubility and the higher log Kow, had the higher values for the BCF. The two trichlorophenols, which are much more water soluble and have lower log Kow's, had lower values for the BCF. The BCF is a function of a compounds lipophilicity and the correlation between the two should be good. The lipophilicity or log Kow indicates the partitioning behaviour between an aqueous phase and an organic phase represented by octanol. The BCF also indicates the partitioning behaviour, but between an aqueous phase and the organism.

In contrast to the BCF, the rates at which 1,2,4-trichlorobenzene, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol were taken-up and depurated, as indicated by the T_{90}'s and the $T_{1/2}$'s, respectively, did not correlate well with either the water solubility, log Kow or the molecular weight of the compounds. The rainbow trout assimilated 2,4,6-TCP at the greatest rate; only 11 hours elapsed between the initiation of exposure and the fish reaching 90% saturation (t_{90}). The times required to achieve the same relative level were 40.1 hours, 24.8 hours and 23.7 hours with 1,2,4-trichlorobenzene, 2,4,5-trichlorophenol and pentachlorophenol, respectively.

The apparent discrepancy between partitioning behavior and kinetics of the chlorinated organics is due to the fact that, while the BCF is influenced by both the k_1 and the k_2 rate constants, the kinetics are driven only by k_2. The uptake rate constant (k_1), contrary to its name, does not affect the rate at which the chemical is assimilated. Due to its position in the 1CFOK equation, k_1 is a simple scalar. It affects the uptake curve in only one dimension, along the body-residue axis. Since the time axis is unaffected, the height of the curve changes with k_1 but the time necessary to reach any given level remains unchanged. Since the height of the curve at equilibrium determines the BCF, k_1 influences the partitioning behaviour but not the kinetics. The k_2, on the other hand, is a complex scalar that affects both the BCF and the rates of chemical uptake and depuration. From its position in the 1CFOK equation, k_2 influences the uptake and depuration curve along both the body-residue and time axes. As the denominator in the expression $C_w{*}k_1/k_2$, it influences the height of the curve or the BCF. This is an inverse relationship with the final body-residue at equilibrium decreasing as the value of k_2 increases. More importantly, the k_2 is also part of the $e^{-k2{*}T}$ expression and therefore has an affect on the time required to reach equilibrium during both the uptake and depuration phase.

The independence of k_1 and k_2 and their individual and collective affects on the overall kinetics and partitioning of a compound between water and fish is well illustrated by a comparison of the behaviour of 1,2,4-trichlorobenzene and 2,4,6-trichlorophenol. Despite a lower molecular weight, 1,2,4-trichlorobenzene was assimilated and depurated more than 3-fold more slowly by the rainbow trout larvae than 2,4,6-trichlorophenol. As discussed above, this is the result of their respective k_2 values. The k_2 for 2,4,6-trichlorophenol was more than 3-fold greater than that of 1,2,4-trichlorobenzene. The k_1 actually showed the opposite trend, with the value for 1,2,4-trichlorobenzene being more than 3-fold greater then that for 2,4,6-trichlorophenol. The respective k_1's and k_2's combined to produce a BCF for the chlorinated benzene that was almost 10-fold greater then that for the chlorinated phenol. Thus. both the k_1 and k_2 affect the BCF but the k_2 alone drives the kinetics or rate at which the chemical enters or leaves the fish.

The relationships between the values of k_1, k_2, and the BCF, displayed by the rainbow trout larvae, along with chemical specific characteristics such as molecular weight, water solubility and the log K_{ow}, support an important conclusion: the regulation and control of chemical partitioning and kinetics comes from the ability of the fish to eliminate the chemical from its body. Thus, the mechanisms within the fish by which chemical metabolism and depuration take place are very important for understanding the kinetics of uptake and depuration. Fish have only limited control over the uptake of lipophilic organic compounds from the water. The lipophilicity of the chemical drives the movement of the compound from the water into the fish (Gobas and Mackay, 1987; Opperhuizen, 1986). The gill, which is designed to provide an efficient surface for gas exchange, offers no barrier or control over the uptake of these chemicals.

As indicated above, the value of k_2 and the subsequent rate at which the compound is assimilated and depurated will be dependent on the mechanism by which the compound is eliminated from the organism. The excretion of chlorinated organics from fish is facilitated by the biotransformation of the compound from its lipophilic form to a more hydrophilic one (Rand and Petrocelli, 1985). This is accomplished within the fish by hydroxylation, where the chlorines are substituted for hydroxyl groups and then conjugated with sugars (glucuronidation) (Andersson and Koivusaari, 1986; Oikari and Anas, 1985). The whole process is enzyme mediated and thus requires the enzyme and the chlorinated organic to interact. The efficiency of this interaction is related to the molecular configuration of the chlorinated organic. It is possible that a difference in rates of biotransformation are responsible for the observation that 2,4,5-trichlorophenol was depurated significantly more slowly than 2,4,6-trichlorophenol. For 2,4,5-TCP, the chlorines are positioned side by side thus leading to steric hindrance and inhibition of the reaction. The chlorines on 2,4,6-TCP are much less crowded and thus more easily accessible which facilitates biotransformation and possibly the subsequent elimination from the organism (James, 1986; Shaw and Connell, 1980).

Despite an abundance of information in the literature describing the kinetics of 1,2,4-trichlorobenzene, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol, it was felt necessary to derive parameters that reflected the conditions present in the wet laboratory in which the mixture experiments were going to be conducted. Numerous factors can potentially modify the kinetics of chemical uptake and depuration in aquatic organisms including pH, temperature, water hardness and the type of organism, its health, age and lipid content. By generating kinetic parameters within the system which we want to model later, we ensure the parameters used for subsequent modelling will be applicable and relevant. Models are often criticized for being too inaccurate and either significantly under or over estimating the actual response. The fact that a model is only as good as the information used to define and validate it is often overlooked.

A review of the kinetic parameters available from the literature (see Table 3.3) underlines the need to assess these parameters on a site and organism specific basis if accurate modelling of chemical uptake and depuration is to be successful. Considerable variability exists indicating how sensitive the k_1's, k_2's and BCF's are to modifying factors. Between flagfish, guppies and various types of trout, values of k_1, k_2 and the BCF vary by as much as 10-fold (see Table 3.3). Although the kinetic parameters generated here for the rainbow trout larvae are in agreement with those presented in the literature, there is enough difference to cause potentially significant discrepancies during modelling. Accurate modelling of a system requires system-specific parameters.

In addition to generating information regarding the kinetics of chemical uptake and depuration of 1,2,4-trichlorobenzene, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol, the kinetic studies were designed to test the assumption that the rate constants for chemical uptake and depuration remain constant. Specifically, they must be independent of both the exposure concentration of the chemical in the water and the subsequent tissue concentration in the organism. The results of the kinetic studies, in which the values of k_1 and k_2 were derived for a range of sublethal exposure concentrations, clearly show that the rate constants for 1,2,4-trichlorobenzene, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol were not significantly affected by the concentration of chemical exposure. The slopes of the lines relating the k_1 and k_2 to the exposure concentrations was not significantly different from zero.

Although the above information suggests that the rate constants are independent of dose, a significant limitation of using the kinetic studies is that the rate constants were derived from sublethal chemical concentrations but are to be applied to model lethal exposures. The exposure concentrations in lethal studies are much higher and the response of the fish is very

Table 3.3 Comparison of experimentally derived kinetic parameters with those obtained from the literature.

Chemical	Fish Species	k1 (hours-1)	k2 (hours-1)	BCF	Reference
1,2,4-trichlorobenzene	rainbow trout	66.4 ±12	0.063 ±0.014	1054 ±152	*
	flagfish	48.3 ±3.5	0.024 ±0.0004	2026 ±154	Smith et al., 1990
	guppy	345	0.018	19166	Koneman and Van Leeuwen, 1980
	rainbow trout			123 -1288	Geyer et al., 1985
	rainbow trout			1288 -3235	Oliver and Niimi, 1983
2,4,5-trichlorophenol	rainbow trout	33.0 ±2.1	0.093 ±0.007	355 ±34	*
	fathead minnow			1905	Veith et al., 1979
	fathead minnow			62	Van Gestel and Ma, 1988
2,4,6-trichlorophenol	rainbow trout	21.1 ±3.1	0.192 ±0.022	110 ±5	*
	flagfish	17.5 ±3.7	0.199 ±0.024	88 ±21	Smith et al., 1990
	rainbow trout			30 - 120	Oikari et al., 1987
	fish			309	Klein et al., 1984
pentachlorophenol	rainbow trout	110.0 ±23	0.097 ±0.015	1123 ±84	*
	flagfish	9.3 ±1.08	0.043 ±0.002	216 ±29	Smith et al., 1990
	rainbow trout	50.6 ±3.4	0.040 ±0.002	1261 ±104	Smith et al., 1990
	brook trout	58.4 ±3.9	0.043 ±0.002	1356 ±113	Smith et al., 1990
	fathead minnow			776	Veith et al., 1979
	trout			100	Hattala et al., 1981

* Kinetic parameters derived by author

different. In the kinetic studies it is advantageous to maintain good fish health throughout the study whereas in the toxicity tests the fish health can be significantly compromised resulting in death. Observations taken during the acute toxicity tests showed that exposure of rainbow trout larvae to 1,2,4-trichlorobenzene, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol resulted in a premortality response that is typical of fish exposed to narcotic organic chemicals. This included a short period of hyperactivity, followed by loss of equilibrium. The fish then lay nearly motionless on the bottom of the tank until death, which occurred within a few hours to a few days. Given the sensitivity of the kinetic parameters to other modifying factors, it was reasonable to question the assumption that the chemical kinetics derived from relatively healthy fish also applied to these fish.

In order to evaluate the potential impact of fish health on the kinetics of chemical uptake and depuration, kinetic studies were conducted which compared the chemical uptake and depuration between live and dead fish. The results of these experiments clearly demonstrated that fish health can potentially affect chemical kinetics between the fish and the water thus supporting the scepticism outlined above. In comparison to live fish, dead fish showed significantly reduced rates of chemical uptake and depuration as indicated by the body-residues in the fish after 24 hours. This suggests that the mechanisms of uptake and depuration require gill movement , blood flow and metabolic activity within the fish. When they were absent, chemical uptake and depuration decreased by 12 and 14-fold respectively. The results also demonstrate that rate constants derived with relatively healthy fish will not apply to fish which are subject to chemical concentrations high enough to cause changes in fish activity and mortality.

A final test of the assumption was to model the uptake of 1,2,4-trichlorobenzene, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol for the chemical exposures in the toxicity tests and compare the predicted body-residues to those quantified in the fish at the time of death. The results show good agreement between predicted body-residues and measured body-residues. This indicates that the rate constants derived from sub-lethal exposures do apply to acute doses and that any influence organism health and activity has on these constants is minor. Despite the fact that accumulation of organic chemicals in the tissue of the fish is not simply a passive process and that activity on the part of the fish is required, the system is efficient enough such that even extreme incapacitation of the organism does not significantly impact on the rates of chemical uptake and depuration and the subsequent concentration of chemical residue in these small fish.

3.4.2 Critical Body-Residues

The use of whole-body-residues as a measure of exposure relies on there being a strong relationship between the chemical concentration in the organism and the organism's response. More specifically, fish mortality must be associated with a given whole-body-residue, such that the fish dies when this residue is exceeded but lives when it is not.

Toxicity tests were conducted using 1,2,4-trichlorobenzene, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol in order to quantify the body-residues in the rainbow trout larvae at the time of death. The results indicated that, for each chemical, the concentration in the dead organisms was relatively constant and a representative critical body-residue (cBR) was determined for each. The cBR was shown to be independent of both the time-to-death and the water exposure concentration. Thus fish exposed to high chemical concentrations died quickly but had the same body-residues as fish that were exposed to lower concentrations and took longer to die. For each compound there was an exposure concentration below which no significant mortality was observed regardless of the exposure time-length.

The cBR's exhibited a reasonable degree of variability but were normally distributed (based on a probability plot of 40 cBR data points) about the mean with the most sensitive and most resistant fish being represented at either extreme. In addition to the natural variation in fish response, further variability would have been introduced by the analytical method for quantifying the chemical residues but this is not considered significant and the measured cBRs were expected to accurately reflect the chemical concentrations in the fish at the time of death. There was some concern that the sampling procedure during the toxicity tests would affect the results. Considerable effort was expended to remove dead fish from the tanks as soon as possible after death. It was felt that if chemical transfer from the water into the tissue was maintained post mortem, then the body-residue would reflect the sampling time and not the time of death. This would represent a source of considerable bias since fish are never removed prior to death; the error is always in one direction. Toxicity tests conducted with worms showed higher cBR's at the higher exposures and it was suggested that post-mortem accumulation was responsible (Fitzgerald *et al.*, 1995). Rates of chemical uptake and depuration derived for dead fish indicated that chemical movement in and out of the fish essentially stops upon death and the body-residue in the dead organism is preserved. Therefore, removing the fish immediately after death was not critical in order to obtain an accurate measure of the critical body-residue and thus the sampling time, as long as it was reasonable, did not represent a significant source of error in the measured body-residue.

It has been proposed that chemicals with similar structure and a common mode of toxic action will also display similar cBR's. Investigations by McCarty (1990) indicated that there was an inverse relationship between the log Kow and the LC_{50} but that the theoretical cBR's were independent of both and relatively constant. Research by Van Hoogen and Opperhuizen (1988) indicated that fish exposed to hydrophobic test chemicals, exhibited little variation in internal toxicant concentrations at death, despite considerable variations in their exposure concentrations. A comparison of the cBR's measured for the chlorinated phenols showed close agreement with each other. They ranged from 989 nmoles/g for pentachlorophenol to 1942 nmoles/g for 2,4,5-trichlorophenol, which is just under a 2-fold difference. In contrast, the ILL's for these compounds ranged from 0.8260 umoles/L for pentachlorophenol to 15.695 umoles/L for 2,4,6-trichlorophenol (a 19-fold difference). The cBR for the chlorinated benzene, 1,2,4-trichlorobenzene was significantly higher then those for the chlorinated phenols

The relationship between the cBR, the time-to-death and the chemical concentration in the water is in keeping with the kinetics and partitioning behaviour of these compounds. If it is assumed that the mortality of the average fish, when exposed to an organic compound, occurs at a known and constant cBR, then a relationship between the incipient lethal level (ILL), time-to-death, and the cBR can be derived by modifying the integrated form of the 1CFOK model equation describing chemical uptake. The ILL represents the concentration of the toxicant in water below which the fish will live indefinitely. When the Cw term in the 1CFOK equation is replaced with the ILL, the Cf term becomes the cBR as the time (t) approaches infinity. The equation is then rearranged in terms of the ILL.

$$1/ILL = 1/cBR * k_1/k_2 * (1 - e^{-k_2 t})$$

The equation indicates that there is a direct relationship between the ILL and the cBR based directly on the kinetics and partitioning behaviour of the compound. This relationship allows one to move from a static measure of toxicity to a more dynamic one where a lethal response can be predicted based on the exposure concentration and the exposure time.

Furthermore, if the assumption is made that similar compounds with similar modes of toxic action share a common cBR, then the equation suggests that differences in the ILL values for these compounds are a product of the chemical specific uptake and depuration rates and not the potency of the compound itself. Thus, compounds with lower ILL's are not more active or deleterious at the site of toxic action, they simply get there faster or more efficiently and a lower water concentration is required in order to achieve the same response. An examination of the behaviour of 2,4,5-trichlorophenol and 2,4,6-trichlorophenol illustrates this point. The 2,4,5-trichlorophenol has an ILL that is 3-fold lower than the 2,4,6-trichlorophenol which would suggest that it is more toxic. This sort of inference is made routinely in the literature. A comparison of the respective cBR's, however, indicates that their potency in the fish is not significantly different which is consistent with the structural similarity of these two compounds. The difference in the ILL's is a function of the kinetics. The 2,4,6-trichlorophenol is biotransformed at a faster rate thus leading to a higher elimination rate constant (James, 1986; Shaw and Connell, 1980). The 2-fold higher k_2 in addition to a slightly lower k_1 results in a BCF for 2,4,6-trichlorophenol that is 3-fold lower than 2,4,5-trichlorophenol. Therefore, neither phenol is more toxic than the other, it is just that the kinetics are such that 3-times more of the 2,4,6-trichlorophenol is required in the water to achieve the same internal concentration in the fish.

The use of the 1CFOK model in conjunction with cBR's to predict the response of an organism to chemical exposure relies on the assumption that the body-residue and the chemical concentration at the site of toxic action are in steady state. Thus the chemical activity is similar in all phases and the whole body-residue represents a reliable surrogate for the effective dose (McCarty, 1990). For high exposure concentrations, where mortality is induced very quickly, this may not be the case. Chemical concentrations at the site of toxic action will lag behind the total residue concentrations in the whole organism. This will lead to an overestimation of the actual cBR. At lower exposure concentrations, this lag is not as pronounced because, as time goes on and the rate of uptake slows, the two concentrations rapidly converge. This trend was not observed in our studies, although the variability within the cBR was higher for the shorter times than for the longer ones, based on exposure and time-to-death. There was generally good agreement between the predicted body-residues and the measured body-residues.

3.5 Summary

In summary, the 1CFOK model was shown to adequately describe the uptake and depuration of 1,2,4-trichlorobenzene, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol in rainbow trout larvae. Kinetic parameters including the k_1, the k_2 and the BCF were derived for each compound and can be used to predict the chemical concentration in the organism at any time for both chronic and acute exposure concentrations. In order to define the toxicity of the compounds in terms of the chemical concentration in the fish, a cBR was also determined for 1,2,4-trichlorobenzene, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol. The cBR's were shown to be independent of the exposure time and water concentration, and thus can be used, in conjunction with the 1CFOK modelling to predict lethality in an exposed group of organisms.

4.0 USING A BODY-RESIDUE BASED MODEL TO PREDICT THE TOXICITY OF BINARY MIXTURES OF CHLORINATED BENZENES AND PHENOLS TO FISH

4.1 Introduction

Both the prevalence of multiple chemical exposures in the environment and the potential interactions between the individual compounds has raised some interest in how mixtures of chemicals are addressed with respect to protecting the health of aquatic ecosystems. Currently, methods for assessing the effects of multiple chemicals on aquatic ecosystems are based on the "toxic unit" model (Sprague, 1970). The ability of this model to accurately predict the joint toxicity of complex mixtures is limited by its use of water concentrations to define chemical exposure. A majority of the chemicals of concern have a propensity to bioaccumulate in the environment being both lipophilic and persistent. For these compounds, water concentrations are an adequate surrogate when assessing a single contaminant. With mixtures of chemicals, differences in chemical behaviour makes chemical concentrations in the water a poor surrogate for the chemical concentrations at the site of toxic action and thus do not accurately reflect the potential risk to the organism.

The basic framework of the "toxic unit" model is considered acceptable but, in order to address the short-coming described previously, the exposure was redefined by a measure of the whole-body chemical residue. By expressing exposure in terms of body-residues and then relating the response of the organism to body-residues, problems such as bioconcentration and bioaccumulation cease to be quantitative issues and are simplified to qualitative ones. Using body-residues allows one to avoid many of the uncertainties associated with defining a potential level of risk based on environmental concentrations because, although still a surrogate, the whole-body-residue provides a direct measure of what is in the exposed organisms body.

A body-residue based model has been described by McCarty (1989) and has been used to examine the acute and sublethal toxicity of fluctuating concentrations of aquatic contaminants on fish. McCarty (1990) describes a deterministic approach utilizing this residue based model to deal with environmental mixture toxicity problems. Not only is such a model more readily adapted from the laboratory to the field, but it can yield a great deal of information about chemical interactions and the mechanisms of mixture toxicity.

4.1.1 Chemical Interactions

Chemical interactions refers to the way two or more chemicals behave, with respect to causing a response in an organism, relative to when they are present alone. The terms "strict addition","synergistic" and "antagonistic" are used to define types of interactions but mean many things to many people. Toxicology is a multidisciplinary science and between pharmacology, microbiology, biostatistics and aquatic ecology there are significantly different interpretations of what these terms mean. It is thus important to clearly define these chemical interactions. For the purposes of this document chemical interactions will be divided into four basic classes. These include the following:

> additivity - the effect of a combination is exactly what is expected.
> synergy - a positive interaction such that the response is greater than expected.
> antagonism - negative interaction such that the response is less than expected.
> no interaction - the response is limited to that of the most toxic component of the mixture

The different types of chemical interactions can be graphically illustrated with an isobole diagram (Loewe, 1953) as presented in Figure 4.1. Isoboles are lines of equal response and can be determined for different mixtures of two toxicants where the concentration of one toxicant represented by its toxic unit is plotted against the corresponding concentration of

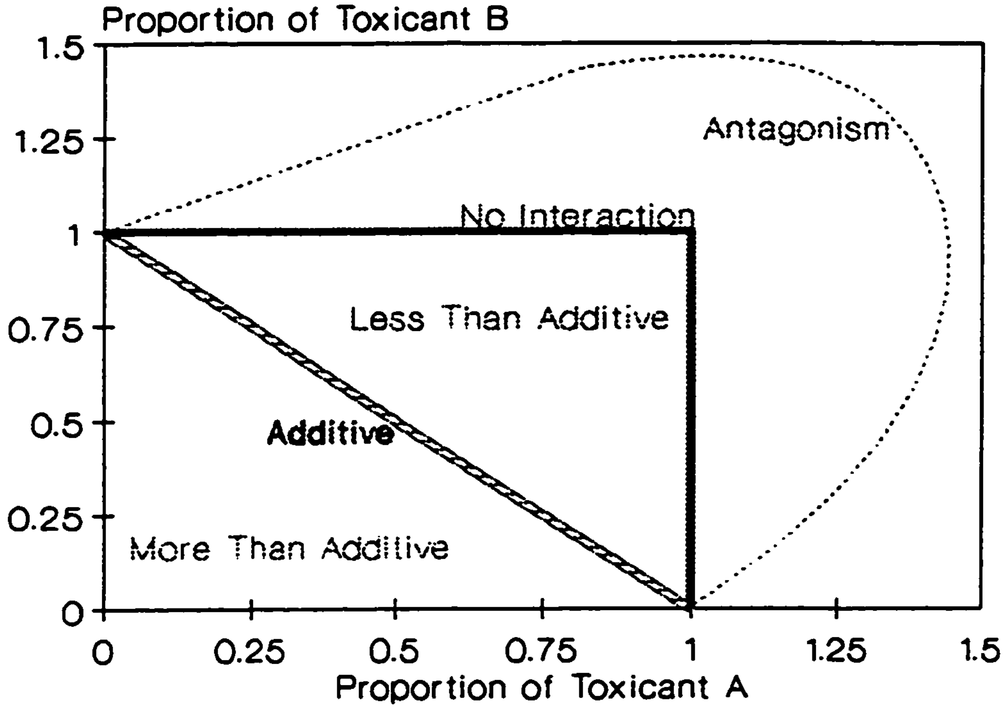


Figure 4.1 Interpretation of the data pattern on an isobole graph. The possible interactions between the toxicity of a two-component mixture (Gaddum, 1948, redrawn from Sprague, 1970.)

the other toxicant also represented by a toxic unit. A series of concentration response curves for the mixtures at different ratios of the component toxicants is derived.

The area outside the box frame is the area of antagonistic interaction, where the presence of one compound inhibits the toxicity of the other. The lines forming the box indicate the area of no interaction, that is, that the presence of one toxicant has no influence on the toxicity of the other. The area inside the box is where joint action occurs: both components contribute to the toxicity. The diagonal line indicates the situation where the toxicants are strictly additive; on either side negative and positive deviations from additivity are found (Sprague, 1970).

4.1.2 The Toxic Unit Model

Current approaches for dealing with the combined effects of toxicants on aquatic systems has been the topic of several reviews (USEPA, 1986; de March, 1987a; 1987b; Hermens, 1989). These approaches are based largely on results from the mixture toxicity methodology discussed by Sprague (1970) as the "toxic unit" model. A Toxic Unit (TU) is defined as the ratio of the water concentration of a chemical in the situation being examined to its LC50 value. Results for bioassays where ratios are less than 1.0 indicate that the concentration is less toxic than the LC50; ratios greater than 1.0 indicate greater toxicity. As toxic units are dimensionless values representing the ratio of the exposure concentration and the lethal effect concentration, mixtures of toxicants can be examined as the total of the toxic units contributed by each component i.e.,

$$Toxicity(TU) = \frac{Cw_1}{LC50_1} + \frac{Cw_2}{LC50_2} + \ldots \frac{Cw_i}{LC50_i}$$

Where Cw_i is the exposure concentration in question and $LC50_i$ is the respective threshold LC50 for chemicals 1 to i.

One drawback of the toxic unit approach is that it can be used to predict only those situations where strict toxic additivity in the mixture occurs, although the nature of the deviations from additivity can be used to classify experimental results in the categories discussed above. As a result, efforts have been made to increase the sophistication of the methods of dealing with mixtures (Placket and Hewlett, 1967, Konemann, 1981, de March, 1987). Most of this work on mixture toxicity has been directed towards statistical aspects still employing the toxic unit approach to deal with multiple toxicity problems.

A more serious limitation of the toxic unit approach is that it ignores partitioning behaviour of the chemicals in question. All of the current schemes employ a bioassay endpoint, such as the LC50, as the starting point for analysis. The LC50 is an index derived from external exposure concentration and therefore may not accurately represent the true chemical concentration at the site of toxic action. Since the kinetics of accumulation of the component chemicals in a mixture may vary significantly, the ratio of actual body-residues at a given time may not be proportional to their relative waterborne concentrations. Thus, unless all the chemicals in a mixture have reached a steady-state, it is not valid to assume a proportional internal dose.

In general, exposure concentration based toxicity data is difficult to extrapolate to the environment where concentrations of toxicants in the water may fluctuate greatly and numerous modifying factors must be considered. This makes mixture toxicity difficult to incorporate into water quality guidelines.

In order to address some of the caveats presented for the traditional toxic unit approach, the "toxic unit" model was modified to include body-residues by redefining the toxic unit as the quotient of the concentration of the chemical in the organism and the concentration in the organism at which a response is observed. When mortality is the response, the concentration in the organism is referred to as the critical body-residue (cBr). As the body-residue of a chemical in an organism approaches the cBr, i.e the TU ~ 1, mortality is expected in the average organism. As with any biological response, the cBr is actually represented by a distribution with animals displaying more or less tolerance then others. Like the traditional toxic unit, the body-residue based one is also unitless allowing toxic units to be summed to account for each chemical in a mixture.

4.1.3 The 1CFOK Model with Mixtures

A 1CFOK model describes the movement of a chemical between the water and the organism. Within a controlled setting like a laboratory, it can be used to accurately predict the body-residues in aquatic organisms exposed to known concentrations of chlorinated benzenes and phenols. As long as the appropriate rate constants are known, the model can also be applied to each chemical in a mixture, thus simultaneously providing a prediction of the expected body-residues for each over time. By expressing each predicted body-residue as a toxic unit and summing them it is possible to predict both when and if mortality will occur for any given mixture scenario.

In order to apply the 1CFOK model, a number of assumptions have to be made. First, it was assumed that the rate constants, k_1 and k_2, derived for each chemical are still valid when the compound is part of a mixture. Chlorinated benzenes and phenols are taken up from the water passively through the gill. Although the gill surface area is of finite size, the relatively low concentrations of the contaminants in the water reduce the possibility of saturation. Competitive inhibition is therefore considered insignificant. On the other hand, the physiological effect of one compound may alter the rates of uptake and depuration of another. The compounds studied here all act via narcosis except PCP which is a metabolic inhibitor (Hodson and Blunt, 1981). Whether it be direct or indirect, changes in fish activity may translate into a change in chemical kinetics between the water and the fish tissue. It was previously shown that, in the absence of mortality, the values of k_1 and k_2 for these compounds remain constant.

A second assumption was that the interaction between the chemicals in the mixture was strictly additive. Strict addition is used in modelling not because it is necessarily valid but because it represents the simplest scenario and lends itself well to mathematical equations. Although, it is fair to say that much of the concern over synergistic interactions is unfounded. True examples of synergy are rare and strict addition provides a good degree of conservatism when trying to predict the potential impact of chemicals with similar chemical structures and modes of toxic action.

In order to test both assumptions, the time of death was recorded during the mixture toxicity studies and the body-residues in the dead organisms was quantified. Based on the time of death. one can compare the predicted residue concentration with the measured thus validating the kinetics. Plotting the derived toxic units for each chemical in the dead fish on an isobole allows one to determine the exact nature of the chemical interaction with binary mixtures. As shown in Figure 4.1, the surface of the plot is divided into zones defined by the properties of the interaction. If the data points fall on the diagonal representing strict addition then the assumed interaction will be validated. If the data points deviate significantly from this line, then the classification changes accordingly.

4.1.4 Purpose

The purpose of the studies is to test the applicability of body-residue based kinetic modelling for predicting the potential toxicity of binary mixtures of chlorinated benzenes and phenols. In addition, by expressing the dose in a more appropriate manner it is possible to more accurately assess the chemical interaction and comment on any deviations from "strict addition".

4.2 Materials and Methods

The chemicals used in the rainbow trout binary mixture toxicity tests, including 1,2,4-trichlorobenzene, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol, were all purchased from the Sigma Chemical Co. and assayed to be 99% pure by GC-MS. Stock solutions of the chlorinated phenols were made by dissolving a known amount of the chemical into 0.1 NaOH while those for the chlorinated benzene used a minimum amount of DMSO as a solvent. An appropriate amount of these stock solutions was added to the water in the primary stock tanks and fed into the diluter in order to provide the necessary dose in each exposure tank. The final pH of the exposure water was checked in order to ensure that NaOH addition did not significantly alter the hydrogen ion concentration. The amount of DMSO was restricted in order to ensure that the concentration never exceeded 0.2 % in the exposure tanks.

Rainbow trout (*Oncorhynchus mykiss*) were purchased as "eyed-up" eggs from the Rainbow Springs Trout Farm in Thamesford, Ontario. The eggs were incubated in the lab, and once hatched, they were reared until the fry had completely absorbed their yolk-sack (2-3 weeks post hatch). At this stage they were transferred to an artificial stream system where water was continually fed into the troughs at one end and syphoned off at the other. This system was found to maintain the trout in excellent health while making them readily accessible (thus reducing stress during capture and transfer) for subsequent experimentation. Fish were fed twice a day ***ad libidum*** with a mix of 0.5- and 1-point starter chow purchased from Richies Seed and Feed located in Elmira, Ontario.

4.2.1 Binary Mixture Toxicity Tests

A continuous-flow nine-dose diluter was modified in order to deliver a binary mixture of the following chemicals, 1,2,4-trichlorobenzene, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol, to the exposure tanks. For one of the chemicals a geometric range of concentrations of 9 concentrations was produced while the second was held at a constant concentration in each of the exposure tanks. For the chemical that was applied over a range, the system was set-up to provide a 20-fold difference between the highest and lowest concentration plus a zero level. Two control tanks were also set up which received neither compound. The concentration of the chemical held at a constant concentration was chosen to supply a body-residue of roughly half of the cBr for that compound based on the toxicity studies conducted with the individual chemical. The concentration range of the chemical that varied was chosen such that significant mortality (> 50%) was expected in at least half of the exposure tanks within the 96 hour exposure period assuming that its contribution to the toxicity was additive with the fixed-concentration chemical. Thus the median concentration of the varying chemical was set to provide a final body-residue (within 96 hours) which was half of its respective cBr.

Of the four chemicals chosen for this study, either 2,4,5-trichlorophenol or pentachlorophenol was administered as a range of concentrations with the other three held constant. Every possible binary combination was performed giving a total of six scenarios. These two compounds were of primary focus because they had different modes of toxic action and they were easy to use. They were relatively water soluble, toxic at low concentrations and well quantified with the Cryo-Extraction procedure. The point of the study was not to exhaustively explore the interactions between the four compounds but to test the applicability of the model.

Twenty fish were placed in each exposure tank and there were two tanks for each exposure regime. The tanks were checked for mortalities every 4 hours for 7 days. Dead fish were removed immediately and stored at -80°C until their critical body-residues could be quantified. Mortality was assessed based on gill movement. Both the mortality and the "time to death" were recorded for the fish in each exposure tank. The temperature of the exposure water was maintained at 15°C and only ambient lighting was used.

4.2.2 Tissue and Water Analysis

All tissue samples were weighed and the chemical residues quantified using the Cryo-extraction protocol outlined in Section 2.0. In order to improve peak resolution, samples containing pentachlorophenol were methylated as described by Levitt (1973) prior to GC-ECD analysis.

Water samples from the exposure tanks were routinely taken during the course of the toxicity experiments. Chemical quantification was done on these samples immediately in order to verify the predicted exposure concentration. All analysis was accomplished using the Cryo-extraction protocol outlined in Section 2.1 followed by GC-ECD analysis.

4.2.3 Modelling and Statistics

For each exposure concentration within the mixture toxicity studies, the individual body-residues for the chemicals in the mixture were predicted over the entire time of the of the exposure. The prediction of chemical uptake by the fish was accomplished by using the 1CFOK model and using the kinetic parameters derived for the individual compounds. The body-residues were subsequently divided by the respective critical body-residue in order to obtain toxic units. A plot of the toxic units versus time for each chemical provided a visual representation of the contribution of each component of the mixture to the potential lethal impact of the mixture to the rainbow trout larvae over the course of the exposure. By summing the two "uptake" curves, the total impact of the mixture was predicted. The time of death for the average fish was defined by the point when the uptake curve representing the total of the two toxic units intersected with a horizontal line set at a toxic unit of 1. The predicted time for lethality was compared to the observed time of lethality by plotting the two against each other and testing whether the slope of the scatter graph deviated significantly from zero. A paired-sample t-test was also used to test the similarity of the observed with the predicted.

Body-residues in the fish at the time of death were predicted based on the chemical exposure concentration and the time of death and by assuming that chemical uptake followed 1CFOKs. The predicted body-residues at the time of death were plotted against the observed body-residues at the time of death and the slope tested for significant deviations from zero. A paired-sample t-test was also used to test the similarity of the observed values with the predicted.

Isoboles were constructed for each mixture scenario using the body-residue based toxic units in order to determine the chemical interaction between the two components of the chemical mixture. Since the sum of the toxic units at death were assumed to add to 1, the assumption of strict addition was tested by defining the distribution of the "total toxic unit" and testing for significant deviations from the value of 1.

4.3 Results

During the toxicity tests using binary mixtures of 1,2,4-trichlorobenzene, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol, and pentachlorophenol, rainbow trout mortality occurred at chemical concentrations that were significantly lower than those required to cause the same response when either chemical was present alone. The response of the fish to the chemical stressors, however, was the same i.e. hyperactivity, followed by a loss of equilibrium, curving of the body, immobilisation and finally death. Both the chemical residues in the dead fish and the time at which the death occurred were determined. This data was compared to predictions made using a body-residue based 1CFOK model in order to further evaluate the observed chemical interactions.

Modelling of the chemical exposure concentrations, used in the binary mixture toxicity tests, included the prediction of body-residues for each chemical over the entire time of the test. Chemical residues were expressed as toxic units. By assuming that the chemicals interacted by strict addition, the time of death was predicted when the sum of the toxic units for the chemicals in the mixture equalled 1. Significant deviations between the observed and the predicted values indicated the more appropriate description of the interaction i.e. more than additive or less than additive.

The graphical representation of the 1CFOK model and its application to binary mixtures is presented in Figure 4.2. The graphs provide examples of the three possible scenarios with respect to a lethal response to chemical exposure. Actual data from the toxicity tests were overlaid onto the graphs in order to provide a comparison of the modelled and observed response of the rainbow trout larvae. In the first graph (A), the exposure concentrations of pentachlorophenol and 2,4,5-trichlorophenol and resulting body-residues were predicted to be insufficient to cause mortality. The sum of the toxic units was well below the value of 1 and only the most sensitive fish were expected to display an adverse response. During the toxicity test, only 3 fish died (15%) after 36 hours of exposure. The second graph (B), depicts the situation where the chemical exposure was sufficient to reach the threshold of lethality. The sum of the toxic units for pentachlorophenol and 1,2,4-trichlorobenzene approached the value of 1. Significant mortality was expected to occur after 50 hours of exposure but the more tolerant fish should survive indefinitely. Results from the toxicity test showed 3 fish dying at around hour 30, 9 at hour 46 and 3 more at hour 60, giving a total mortality of 75%. The third graph © provides an example of exposure concentrations which are clearly lethal. The sum of the toxic units for 2,4,5-trichlorophenol and 2.4.6-trichlorophenol intersect with the threshold level at hour 6. By the end of the exposure time, the sum is nearly 2-fold greater than the level predicted to cause lethality. The overlaid data suggest that the lethal potential of this mixture was somewhat overestimated. Only 4 fish had died by hour 6 and the majority did not succumb until after hour 12. The total mortality was 100% though.

A. Sublethal

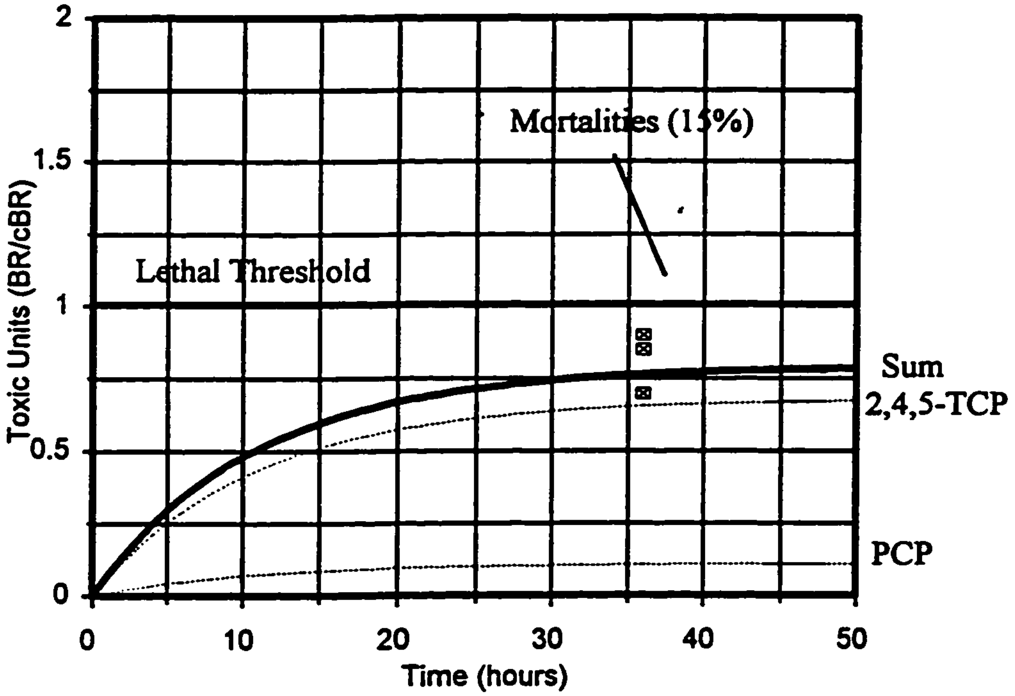
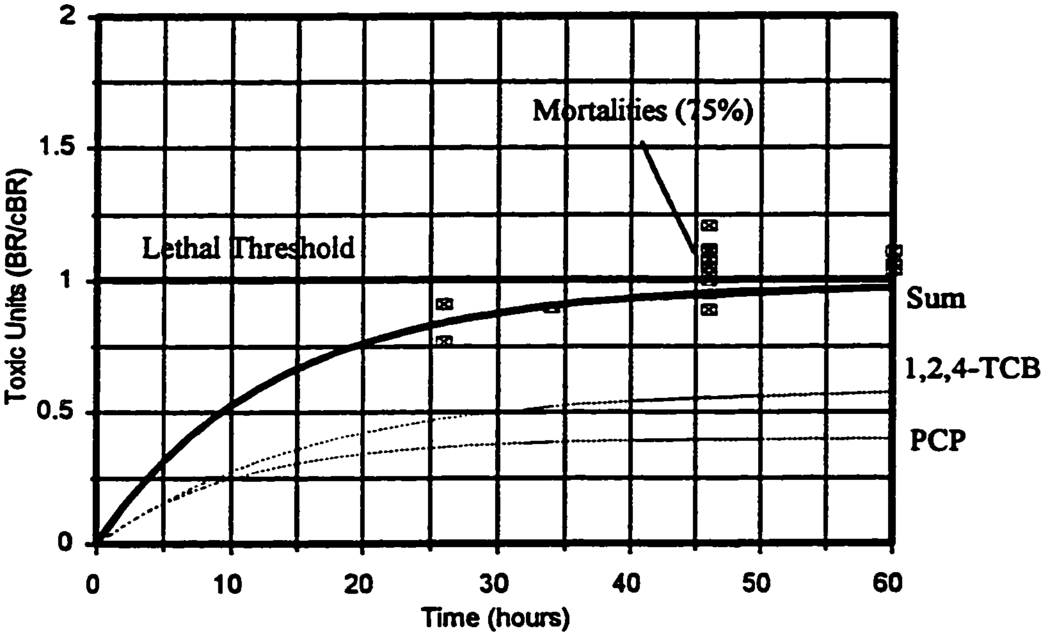
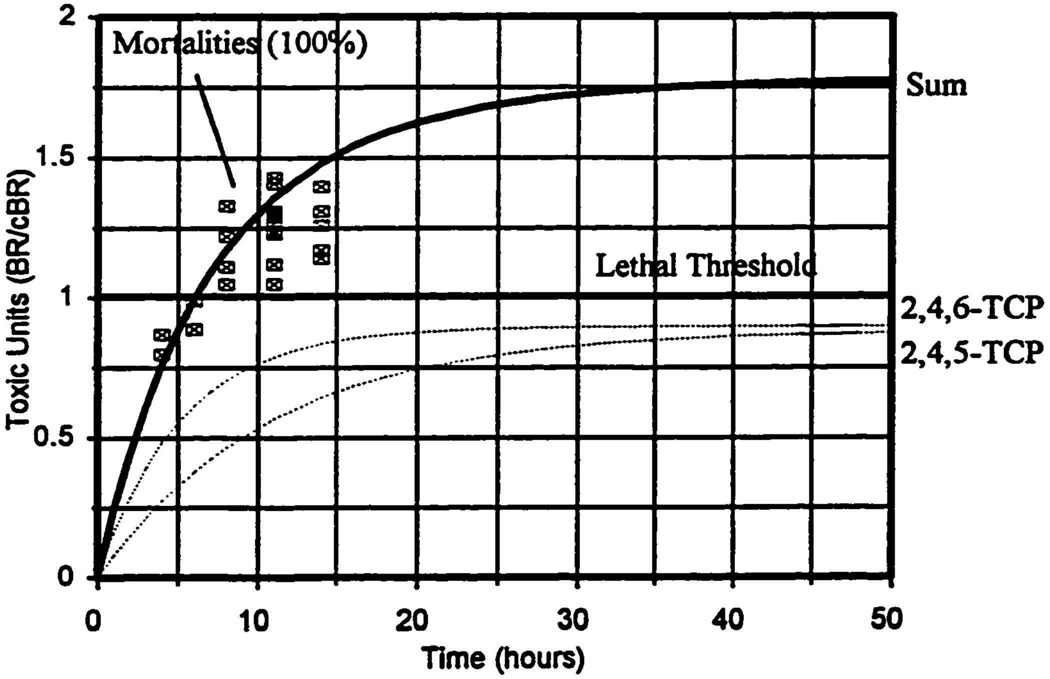


Figure 4.2 Modelling of chemical mixtures for three typical response scenarios: sublethal (A), lethality threshold (B) and lethal (C) (continued on next page). The predicted uptake of chemicals (expressed relative to their respective critical body-residues) by rainbow trout larvae exposed to binary mixtures of 1,2,4-trichlorobenzene, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol. Predictions derived from 1CFOK modelling and assume strict addition. Observed mortalities and body-residues (indicated by ⊠) were over-laid onto the modelled values.

B. Lethality Threshold



C. Lethal



A comparison of the modelled data with the observed data and an assessment of the chemical interaction was conducted for each exposure in the binary mixture toxicity tests. The results are summarized for each chemical mixture in the following sections.

4.3.1 Pentachlorophenol and 1,2,4-Trichlorobenzene

For the mixture toxicity test with pentachlorophenol and 1,2,4-trichlorobenzene, the pentachlorophenol ranged from 0 to 927 ug/L while the trichlorobenzene was held constant at 692 ug/L. Significant mortality (>50%) was observed when the concentration of pentachlorophenol exceeded 93 ug/L. The time at which 50% mortality occurred (cT) correlated well with the predicted times using the 1CFOK modelling approach (see Figure 4.3a). The relationship between the two was relatively linear with the slope of the line being 1.24 (SE of 0.08) and an r^2 of 0.90. Significant deviations from the line were noted at the lower concentrations when the mortality occurred during the latter stages of the exposure time. In these cases, the model underestimated the time-to-death. Body-residues quantified in the dead fish demonstrated the usual high variability but correlations between the measured critical body-residues and the predicted, for both chemicals, were good (see Figure 4.3b). An isobol generated from the contributing toxic units from each of the two chemicals did not suggest a significant deviation from the strict addition interaction mechanism (see Figure 4.3c). There was considerable scatter about the diagonal line but the mean of the sum of the toxic units was 1.01 (SD of 0.14).

4.3.2 Pentachlorophenol and 2,4,6-Trichlorophenol

For the mixture toxicity test with pentachlorophenol and 2,4,6-trichlorophenol, the pentachlorophenol ranged from 0 to 927 ug/L, while the trichlorophenol was held constant at 1380 ug/L. Significant mortality (>50%) was observed when the concentration of pentachlorophenol exceeded 114 ug/L. The time at which 50% mortality occurred (cT) correlated well with the predicted times using the 1CFOK modelling approach (see Figure

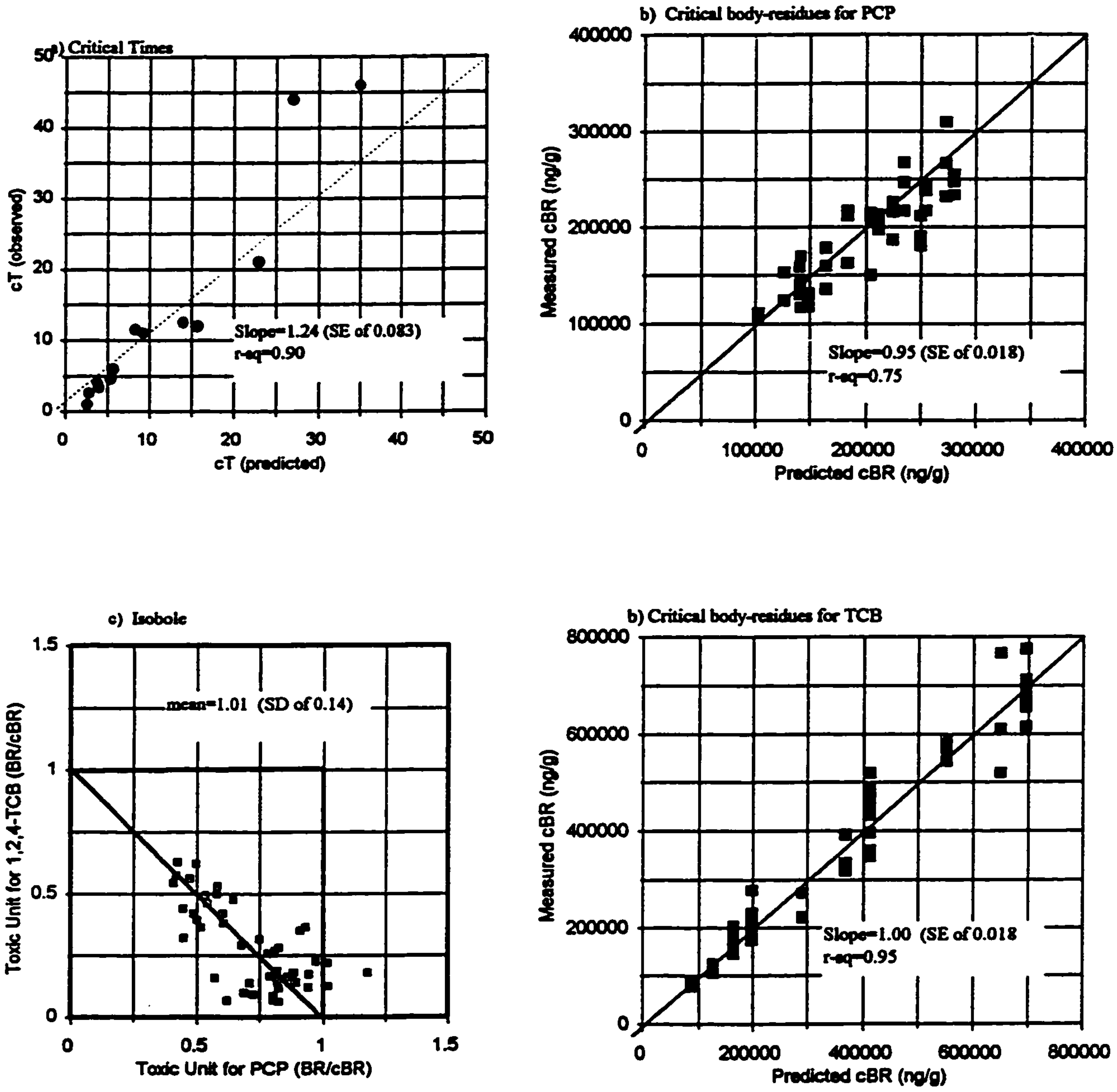


Figure 4.3 The comparison of the modelled and observed values for the critical times (a) and the critical body-residues (b) from the binary mixture toxicity tests with pentachlorophenol and 1,2,4-trichlorobenzene. All slopes were shown to be significantly different from zero (p-values < 0.05). The chemical interaction was assessed with an isobole (c).

4.4a). The relationship between the two was relatively linear with a slope of 0.85 (SE of 0.05) and an r^2 of 0.93. No significant deviations from the line were noted. Body-residues quantified in the dead fish demonstrated the usual high variability but correlations between the measured critical body-residues and the predicted, for both chemicals, were good (see Figure 4.4b). Linear regression indicated that the slopes for both pentachlorophenol and 2,4,6-trichlorophenol were close to 1 and the r^2 values were around 0.8. An isobol for pentachlorophenol and 2,4,6-trichlorophenol did not suggest a significant deviation from the strict addition interaction mechanism (see Figure 4.4c). There were several outliers generated with the higher pentachlorophenol exposures but the mean of the sum of the toxic units was 1.02 (SD of 0.15).

4.3.3 Pentachlorophenol and 2,4,5-Trichlorophenol

Two mixture toxicity tests were conducted with pentachlorophenol and 2,4,5-trichlorophenol. In one, the pentachlorophenol ranged from 0 to 464 ug/L while the trichlorophenol was held constant at 736 ug/L. In the second the trichlorophenol ranged from 0 to 2890 ug/L and the pentachlorophenol was held constant at 123 ug/L. The data from the two studies were combined into one data set for subsequent analysis and evaluation. The time at which 50% mortality occurred (cT) correlated well with the predicted times using the 1CFOK modelling approach (see Figure 4.5a). The relationship between the two was relatively linear with the slope of the line being 0.75 (SE of 0.06) and an r^2 of 0.77. One outlier was noted at the lower concentrations when the mortality occurred during the latter stages of the exposure time. In this case, the model underestimated the time-to-death. Body-residues quantified in the dead fish demonstrated the usual high variability but correlations between the measured critical body-residues and the predicted, for both chemicals, were good (see Figure 4.5b). The isobol generated from the contributing toxic units from each of the two chemicals revealed considerable scatter, particularly at the extreme chemical concentrations i.e. when either pentachlorophenol or 2,4,5-trichlorophenol were at high

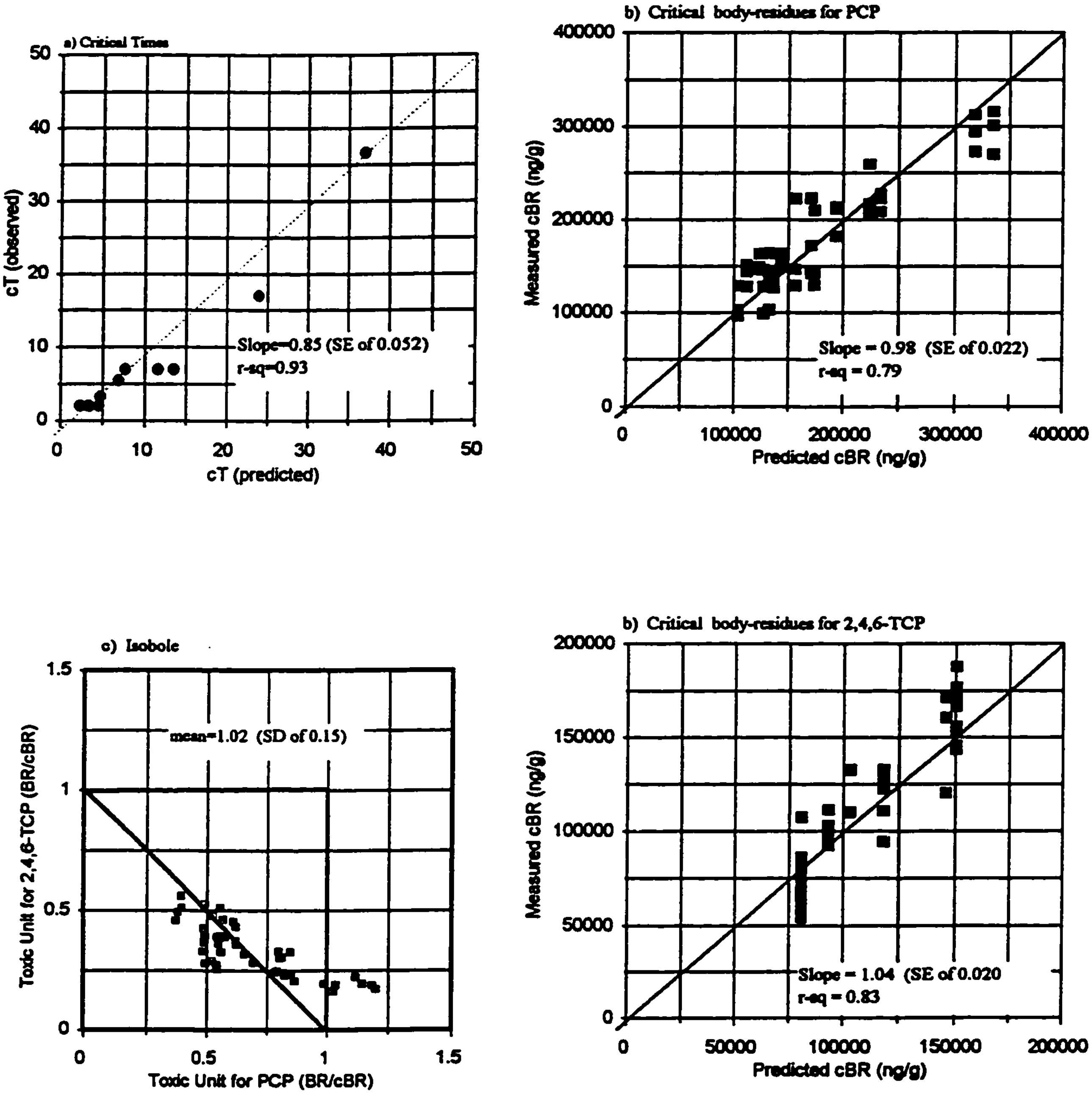


Figure 4.4 The comparison of the modelled and observed values for the critical times (a) and the critical body-residues (b) from the binary mixture toxicity tests with pentachlorophenol and 2,4,6-trichlorophenol. All slopes were shown to be significantly different from zero (p-values < 0.05). The chemical interaction was assessed with an isobole (c).

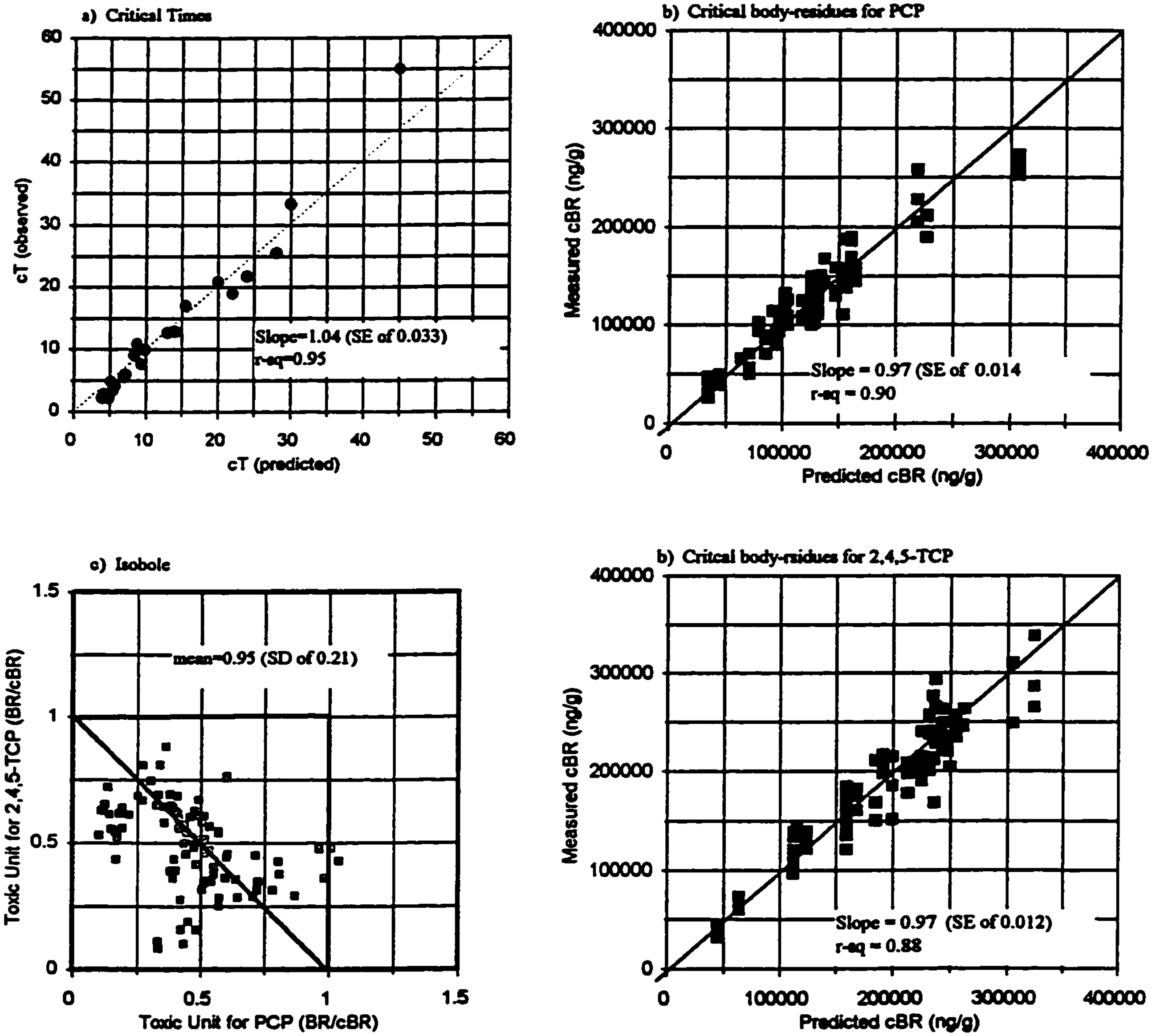


Figure 4.5 The comparison of the modelled and observed values for the critical times (a) and the critical body-residues (b) from the binary mixture toxicity tests with pentachlorophenol and 2,4,5-trichlorophenol. All slopes were shown to be significantly different from zero (p-values < 0.05). The chemical interaction was assessed with an isobole (c).

concentrations. However, the mean (0.95 with a SD of 0.21) did not suggest a significant deviation from the strict addition interaction mechanism (see Figure 4.5c).

4.3.4 2,4,5-Trichlorophenol and 1,2,4-Trichlorobenzene

For the mixture toxicity test with 2,4,5-trichlorophenol and 1,2,4-trichlorobenzene, the trichlorophenol ranged from 0 to 2500 ug/L while the trichlorobenzene was held constant at 720 ug/L. Significant mortality (>50%) was observed when the concentration of trichlorophenol exceeded 455 ug/L. The time at which 50% mortality occurred (cT) correlated well with the predicted times using the 1CFOK modelling approach (see Figure 4.6a). The relationship between two was linear with the slope of the line being 1.06 (SE of 0.02) and an r^2 of 0.99. No significant deviations from the line were noted. Body-residues quantified in the dead fish demonstrated the usual high variability but correlations between the measured critical body-residues and the predicted, for both chemicals, were good (see Figure 4.6b). The isobol did not suggest a significant deviation from the strict addition interaction mechanism (see Figure 4.6c). As with the other quantified body-residues, there was considerable scatter about the diagonal line but the mean of the sum of the toxic units was 0.99 (s.d. of 0.11).

4.3.5 2,4,5-Trichlorophenol and 2,4,6-Trichlorophenol

For the mixture toxicity test with 2,4,5-trichlorophenol and 2,4,6-trichlorophenol, the 2,4,5-trichlorophenol ranged from 0 to 2800 ug/L while the 2,4,6-trichlorophenol was held constant at 2760 ug/L. Significant mortality (>50%) was observed when the concentration of 2,4,5-trichlorophenol exceeded 134 ug/L. The time at which 50% mortality occurred (cT) correlated well with the predicted times using the 1CFOK modelling approach (see Figure 4.7a). The relationship between two was relatively linear with an r^2 of 0.76 but the slope of the line (1.43 with a standard error of 0.10) was significantly different from the value of 1. Thus the model consistently underestimated the time-to death. Body-residues quantified I

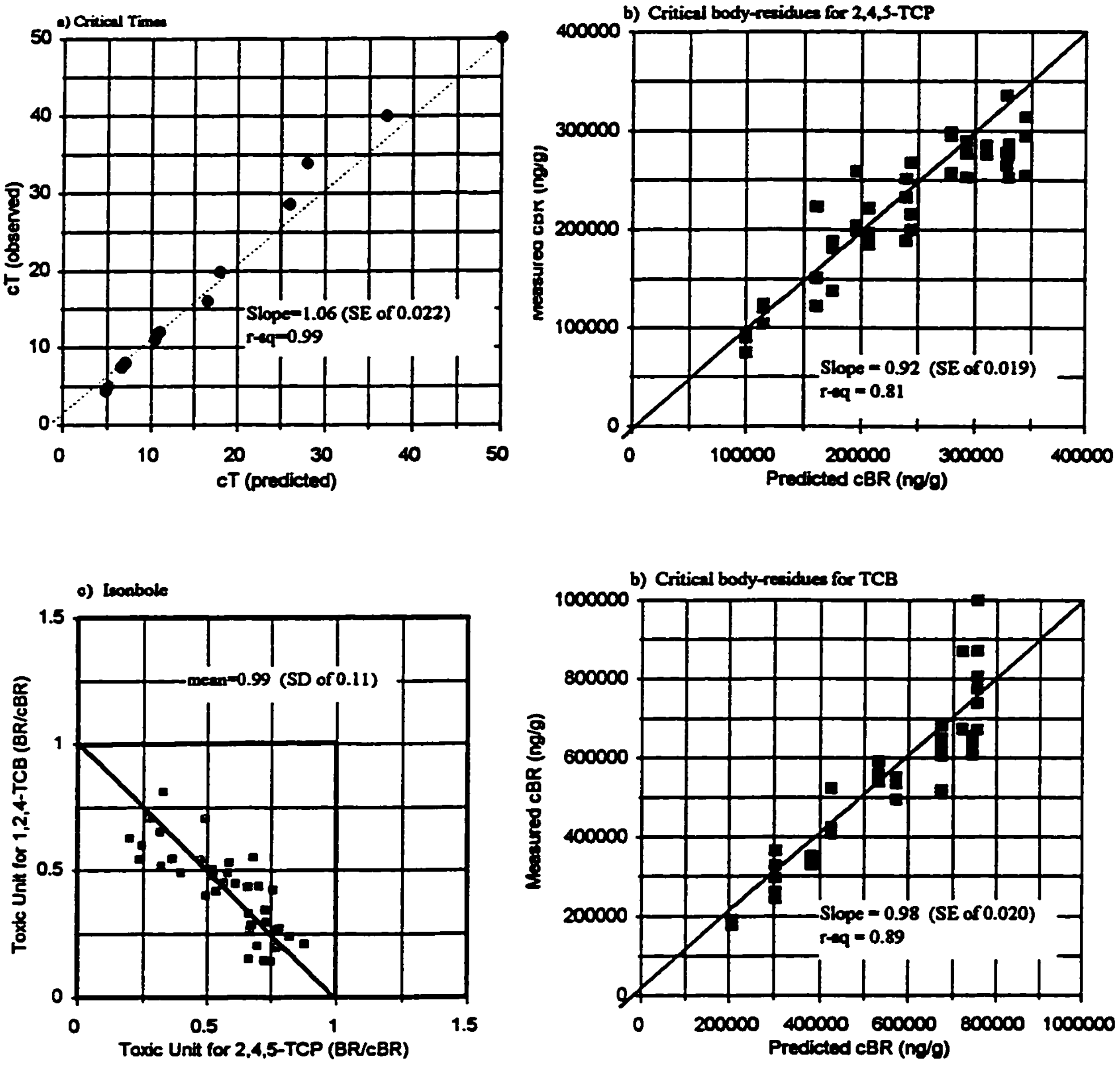


Figure 4.6 The comparison of the modelled and observed values for the critical times (a) and the critical body-residues (b) from the binary mixture toxicity tests with 2,4,5-trichlorophenol and 1,2,4-trichlorobenzene. All slopes were shown to be significantly different from zero (p-values < 0.05). The chemical interaction was assessed with an isobole (c).

n the dead fish demonstrated the usual high variability but correlations between the measured critical body-residues and the predicted, for both chemicals, were good (see Figure 4.7b). The isobol for 2,4,5-trichlorophenol and 2,4,6-trichlorophenol demonstrated a significant deviation from the strict addition interaction mechanism (see Figure 4.7c). There was considerable scatter but the majority of the points were situated above the diagonal line. The mean of the sum of the toxic units was 1.18 (SD of 0.15). The interaction between 2,4,5-trichlorophenol and 2,4,6-trichlorophenol would be more accurately described as less-than strictly additive.

4.4 Discussion

The purpose of conducting the mixture toxicity studies was to evaluate the ability of the body-residue based 1CFOK model to accurately predict both the kinetics and toxicity of chemical mixtures. Comparisons of the observed or quantified results, from each study, with predicted values indicated that the model performed quite well. For those instances where the model seemed to fail, the approach taken, in terms of the data that was collected, also allowed for a more comprehensive evaluation of the mechanism behind these deviations.

Whole-body-residues of 1,2,4-trichlorobenzene, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol predicted in the rainbow trout larvae at the observed time-of-death demonstrated good agreement with the body-residues quantified in the dead fish. The observed and predicted values were compared by way of a scatter plot with the expected on the x-axis and the observed on the y-axis. If the observed values matched the predicted values perfectly then the slope of the line through the points would have a value of 1. The slopes of the critical body-residue plots, for the binary mixtures studied, ranged from 0.92 to 1.04 with standard deviations no greater than 0.02. The body-residues showed the usual scatter but the closeness of the observed with the predicted values was not surprising given that the predicted residues were derived using the observed time-of-death, the exposure concentration in the water and by assuming that each of the chemicals in the mixture would

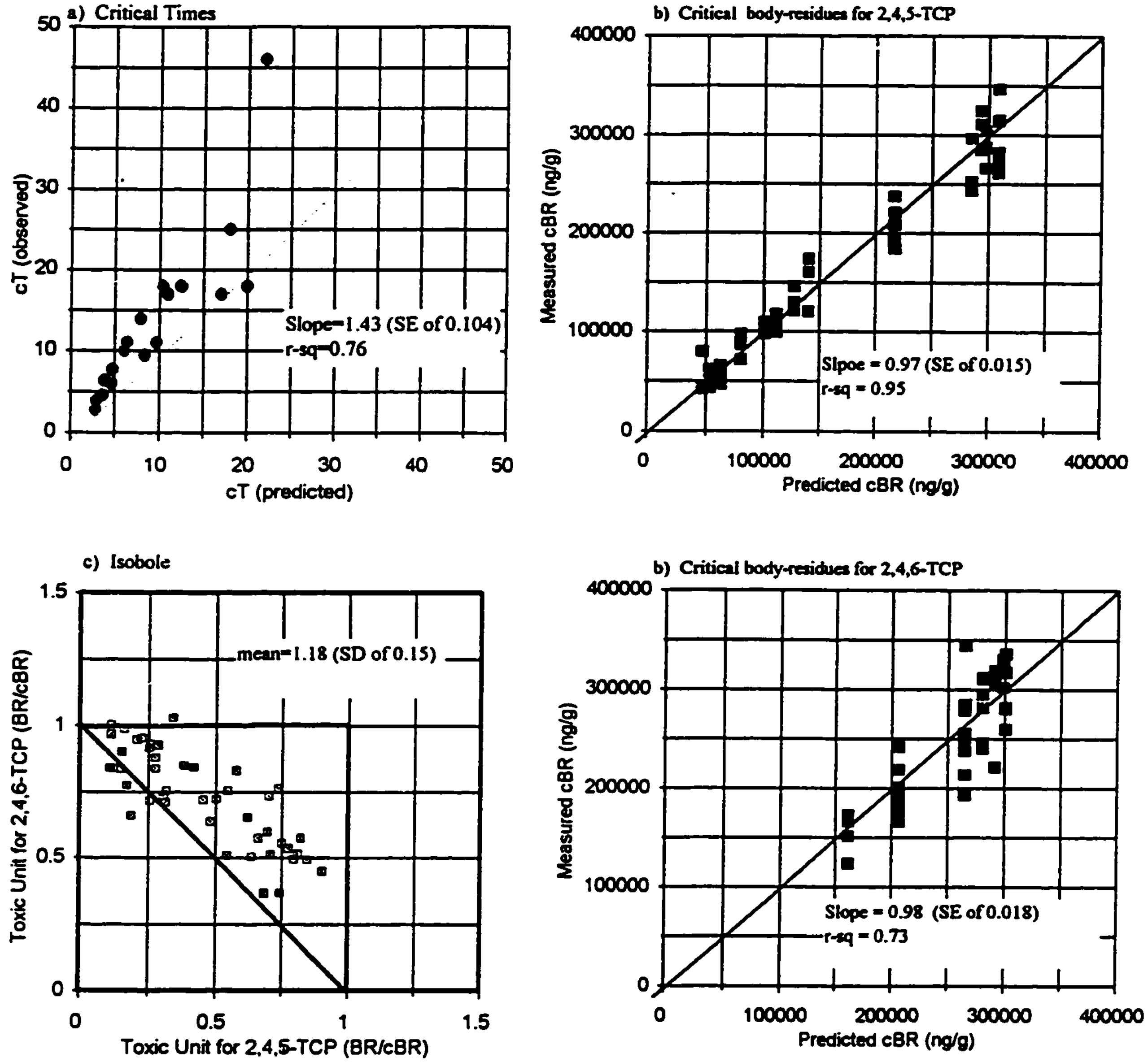


Figure 4.7 The comparison of the modelled and observed values for the critical times (a) and the critical body-residues (b) from the binary mixture toxicity tests with 2,4,5-trichlorophenol and 2,4,6-trichlorophenol. All slopes were shown to be significantly different from zero (p-values < 0.05). The chemical interaction was assessed with an isobole (c).

be taken up by the fish in accordance with one-compartment first-order kinetics. Previous studies clearly showed that this assumption was valid for each of the chlorinated organics used in the mixture toxicity tests (see Section 3.0).

The model also assumed that the two chemicals in the mixture would not influence each other's kinetics. Thus the rates of uptake and depuration derived for each compound singly also applied when they were present in a mixture. Studies with mixtures of mercury and selenium raised concerns with respect to this assumption when it was demonstrated that an antagonistic interaction existed between these two metals (Pelletier, 1985). The root of this antagonism seemed to be competitive uptake where the presence of selenium inhibited the uptake of mercury resulting in lower than expected body-residues of the latter metal. No evidence of this type of mechanism has been presented in the literature for organic compounds but it remains an interesting hypothesis for explaining a biological response to chemical mixtures which is less than that which would have been predicted if the exposure concentrations in the water were strictly additive. This is not surprising given that the movement of organic chemicals from the water to the fish tissue is not regulated, as it is for many metals, and is primarily achieved via passive diffusion (Hunn and Allen, 1974). Active transport across the gill membrane is unnecessary and thus competitive inhibition between organic compounds at this site is unlikely. The potential for the narcotic effects of the chemical with the faster rate of uptake to influence the rate of uptake of the second compound was also ruled out. Studies comparing the kinetics of live and dead fish indicated that rates of chemical uptake and depuration were affected only after extreme disruption of fish activity and that for the most part the values of k_1 and k_2 were independent of fish activity and health. There are some interesting examples of compounds whose activity is altered by a second chemical which acts by modifying the biological processes which are involved with the metabolism, activation or excretion of the first compound (Greco *et al.*, 1995; Calabrese, 1991). This essentially increases or decreases the exposure, or the potency of the compound at the site of toxic action. However, many of these interactions are used with pharmaceuticals, cancer treatment etc, requiring a high level of human control and are thus not very relevant under environmental conditions. In addition, the modes of toxic action of the two compounds would have to be different yet compatible or linked in some way.

An examination of the observed and predicted chemical residues in fish that died after exposure to 2,4,5-trichlorophenol and 2,4,6-trichlorophenol further rules out the hypothesis that changes in chemical kinetics are responsible for deviations from strict addition observed with mixtures of organic chemicals. The isobole for the toxicity test with these two chlorinated phenols (see Figure 4.7c) indicated that the interaction was significantly less than strictly additive. The mean of the sum of the toxic units contributed by each chemical was 1.18 (s.d. of 0.15). The same conclusion with respect to the chemical interaction would have been reached with the use of the more traditional toxic units based on water exposures but no explanation of the mechanism would have been possible. It could have been hypothesized that either the chemical residues in the fish or the potency of the compounds were depressed. However, with the current approach, it is clear that the observed body-residues were not significantly different from what was expected. In fact, the predictions were quite good, with plots showing the relationship between the predicted critical body-residues and the measured, having slopes of 0.97 (SE of 0.01) and 0.98 (SE of 0.02) for 2,4,5-trichlorophenol and 2,4,6-trichlorophenol, respectively. Consequently, it can be stated that the combined toxicity of these two chlorinated phenols to fish was less than additive, not because of reduced exposure but rather because the potency of the compounds, once in the body, was reduced. It is tempting to say that the potency at the site of toxic action was reduced but this would be over-stepping the limitations of the body-residue approach. The measure of whole body-residue is still a surrogate for the concentration of the chemical at the site of toxic action and it is quite possible that the observed antagonism was actually the product of a reduced exposure concentration at the site of toxic action.

Further study would be required in order to determine the exact nature of the interaction mechanism observed for 2,4,5-trichlorophenol and 2,4,6-trichlorophenol. Although chemical interactions are defined based on the response of an organism, they actually occur at an atomic or molecular level. For example, chemicals compete for transport sites across membranes or for binding sites at the site of toxic action. Different metabolic pathways are interrupted or enhanced as chemicals bind to key enzymes or act as chemical messengers themselves. These metabolic pathways may be directly or indirectly dependent on one another. Other chemicals interfere with membrane structure and function by their very presence and it is the sheer numbers of contaminant molecules that determines the response. The numbers and types of mechanisms are as numerous and varied as the interacting chemicals themselves and the systems on which they effect. Like chemical toxicity, chemical interactions are both chemical and organism specific. Although knowledge of the mechanism is of great value, it is often difficult and/or impractical to elucidate.

The reduced potency, displayed by 2,4,5-trichlorophenol and 2,4,6-trichlorophenol relative to what was expected based on critical body-residues derived for the individual compounds, dictates the need for a higher tissue concentration to induce mortality that subsequently required more time to achieve. A comparison of the average times-to-death observed during the mixture toxicity test for these two chlorinated phenols with the predicted time further illustrates this (see Figure 3.7b). The observed critical times are consistently higher than the predicted values with the slope of the line being 1.43 (SE of 0.10).

The critical time represents the time required for a given exposure concentration necessary to cause a response in the average fish. In this case the response is mortality and "average" is defined as 50% of the population. Thus the observed critical time defines the point during the toxicity test when half of the fish have died. The expected critical time is a prediction of when the average fish will die and is defined as the point when the sum of the predicted toxic units is equal to one. Thus the expected critical time is based on the critical body-residues and the predicted residues in the fish defined by the 1CFOK model and the exposure concentration. Where predicted residue concentrations in the fish, at the time of death, was defined for the individual and based on the the individuals time-of-death, the critical time was defined for the sample population and was not biased by the results of the mixture toxicity test in any way.

The observed critical times for the mixture toxicity studies, like the critical body-residues, were also in good agreement with the expected critical times. A comparison of the two was also done graphically, using linear regression to fit a line to the points and determine by how much the slope deviated from the value of 1. Excluding the toxicity test with 2,4,5-trichlorophenol and 2,4,6-trichlorophenol, which was discussed previously, values of the slope ranged from 0.85 to 1.24 with the standard error not exceeding 0.08. Outliers, when they existed, tended to be associated with the longer critical-times. At the high levels of exposure (short critical times), the fish died quickly and they died on schedule but as the exposure concentration dropped towards the threshold level (longer critical times), the response of the fish was less predictable and the observed time-of-death was typically underestimated. A reason for this non-linearity is not immediately apparent. It is likely that modifying factors which alter the response of the fish to the chemical exposure have a greater impact during threshold exposures. At high chemical concentrations, mortality occurs so quickly that changes in pH, temperature or even minor fluctuations in the exposure concentration have little chance to impact on the outcome. For lower chemical concentrations, the fish ends up close to the threshold of mortality. If the body-residues in the fish are above the threshold the fish dies on time but if the modifying factors pull it below, then the fish will live far past when they were predicted to.

Isoboles were used to assess the nature of the interaction between the chemicals in the mixture toxicity studies. For the most part, the interactions between binary mixtures of 1,2,4-trichlorobenzene, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol were shown to be strictly additive. Based on the similarity in the physical and chemical properties of these compounds and because they share a common mode of toxic action, the interaction was expected to be strictly additive for the trichlorophenols and the chlorinated benzene. The chemical interactions with pentachlorophenol had potential to be more or less than additive due to its different mode of toxic action.

There is general agreement within the scientific community that chemicals which share a common mode of toxic action will be additive when present together in a mixture. Quantitative structure activity relationships are often used to predict which chemicals are expected to behave the same so that an appropriate model can be used. Initially, pentachlorophenol was chosen because it was similar in structure but was known to be a metabolic inhibitor rather than a narcotic. It was anticipated that this difference in its mode of toxicity would result in an interaction that strayed from strict addition. However, it has been shown that pentachlorophenol, when present at high concentrations, actually acts as a narcotic (McCarty, 1993). It was unexpected that 2,4,5-trichlorophenol and 2,4,6-trichlorophenol were not additive. These two compounds differ by the position of one chlorine atom and yet somehow inhibited each others potency. It was originally hoped that these two compounds would serve as a model for strict-addition but, as it turned out, they were the only two that exhibited an interaction that significantly deviated from additivity.

Much of the concern regarding chemical mixtures in the aquatic environment stems from the possibility that chemicals will interact to produce potential toxicities that significantly exceed what is predicted. The consequence of this is that aquatic life would be at risk under conditions which were considered safe. However, despite all of the hype about the potential for synergistic interactions, very few examples of true synergy between chemicals in the environment can be found in the literature. A large number of aquatic mixture toxicity tests have been conducted over the years by a number of researchers (summarized in Alabaster and Lloyd, 1992.). Chemical interactions were quantified for mixtures of a variety of compounds including metals, chlorinated organics and pesticides. Contrary to what was expected, the results of these experiments were quite consistent and can be summarized as follows:

i) Mixtures were never less toxic than the most toxic component.

ii) The vast majority of the mixtures were less toxic than could be predicted by assuming concentration addition.

iii) Mixtures exhibiting an interaction which was greater than additivity were infrequent and involved primarily metals and organics with modes of toxic action which were significantly different.

The phenomenon of concentration addition was much more common than expected and was an excellent approximation for chemicals that had comparable modes of toxic action.

4.5 Conclusion

The affects on larval rainbow trout of binary mixtures of 1,2,4-trichlorobenzene, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol were accurately modelled, both in terms of exposure and toxicity, using the 1CFOK model and a body-residue based toxicity endpoint. For the most part, the interaction of the chemicals in the mixture could be described as strictly additive. Deviations from this were noted for the mixture of 2,4,5-trichlorophenol and 2,4,6-trichlorophenol but were marginal. The exact nature of the interaction was not determined but changes in the rates of chemical uptake and depuration by the fish were not responsible. The potential toxicity to fish of mixtures of chlorinated phenols and benzenes should be adequately predicted by assuming strict addition.

5.0 SUMMARY AND CONCLUSIONS

The use of a body-residue-based one-compartment first-order kinetic (1CFOK) model to predict the lethal toxicity of binary mixtures of chlorinated phenols and benzenes on juvenile rainbow trout (*Oncorhynchus mykiss*) was examined. Although the 1CFOK model is relatively well established, only recently have technological advances permitted an extensive analytical evaluation. The results of the research presented in this thesis demonstrated that the body-residue approach, with the assumption that the chemicals will interact additively, accurately predicted the response of the fish to binary mixtures of 1,2,4-trichlorobenzene, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol.

For each mixture scenario, the body-residue-based 1CFOK model was used to predict the concentration of each chemical in the fish over time. By expressing this predicted body-residue as a ratio of the chemicals respective critical body-residue and summing these ratios for each chemical in the mixture, an attempt was made to predict both if and when death would occur. Subsequent validation of the models predictions was accomplished by performing the mixture experiments and noting the response of the rainbow trout larvae, i.e. the time to death, the number of mortalities and the chemical concentrations in each of the organisms at the time of death. A comparison of the results of the mixture toxicity tests with those predicted from the model demonstrate that the effects on larval rainbow trout of binary mixtures of 1,2,4-trichlorobenzene, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol were accurately modelled, both in terms of exposure and toxicity, using the 1CFOK model, a body-residue-based toxicity endpoint, and by summing the summing the body-residue-based toxic units for each chemical in the mixture. For the most part, the interaction of the chemicals in the mixture could be described as additive. Deviations from this were noted for the mixture of 2,4,5-trichlorophenol and 2,4,6-trichlorophenol but were marginal.

In order to use the 1CFOK model to predict chemical loading into fish tissue and the potential effects of those residues on the organism, a number of biological and chemical parameters were determined for each of the chemicals to be studied. These parameters include: rate constants for chemical uptake and depuration, and critical body-residues defining the chemical concentration in the fish at which death occurred. The rate constants for chemical uptake and depuration were derived using kinetic experiments in which the body-residues resulting from waterborne exposure were plotted over time. The critical body-residues and times-to-death were defined using a standard toxicity test format with the addition of quantifying chemical residues in samples of the dead fish. Based on the results, the 1CFOK model was shown to adequately describe the uptake and depuration of 1,2,4-trichlorobenzene, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol in rainbow trout larvae. Suitable measures of the rate constants, k_1 and k_2, were derived for each compound and were shown to be independent of the chemical exposure concentration which could subsequently be used to predict the chemical concentration in the organism at any time for acute exposure to multiple chemicals. A critical body-residue was used to define the chemical toxicity in terms of the chemical concentration in the fish. The critical body-residues for 1,2,4-trichlorobenzene, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol were shown to be independent of the exposure time and water concentration, and thus could be used, in conjunction with the 1CFOK modelling to accurately predict lethality in an exposed group of organisms.

In order to accurately and rapidly accomplish tissue residue analysis, the Cryo-extraction protocol was developed. This extraction technique took advantage of the higher freezing point of the aqueous sample (water or tissue) relative to the solvent in order to eliminate the need for a separatory funnel. After freezing the denser, aqueous layer, the liquid organic layer (hexane), floating on top, was simply decanted. Through the systematic assessment of the extraction efficiency, reproducibility and linearity, the cryo-extraction protocol was shown to meet or exceed the requirements for an acceptable analytical tool for quantifying the chemical body-residues of chlorinated benzenes and phenols in fish. The cryo-extraction protocol provided a faster, more efficient and more reliable approach.

Much of the environmental concern surrounding the impacts of mixtures is related to the potential for synergistic interactions. Where a chemical significantly enhances the toxic activity of another, the current regulatory system will fail to adequately protect environmental receptors. Recent work by McLachlan (Arnold *et al.*,1996) appeared to demonstrate this phenomenon with chemicals which mimic hormones leading to endocrine disruption and reproductive impairment. Claims were made that individually the potency of these compounds was low but, when present together, the activity was enhanced by orders of magnitude. In the wake of other studies which claimed that male sperm counts in humans were declining (McLachlan and Korach, 1995) and that alligators in Lake Apopka, Florida were incapable of reproduction due to micro-penises (Guillette *et al.*, 1996), the finding of significant synergy among endocrine disruptors shook the very foundation of the approach used to assess the potential impacts of anthropogenic chemicals in the environment. Although the claims by McLachlan were retracted after other researchers failed to reproduce this synergistic interaction, the damage was done and the misconceptions and fears related to chemical mixtures was further engrained in the minds of the scientific community and the public.

An effective way of correcting these misconceptions is through improved understanding of chemical interactions generated by continued research efforts. The study of chemical mixtures and their interactions is a large field and it is very easy to dwell on the vastness of what is not known rather than that which is known. However, an examination of the available published data reveals that synergistic interactions are rare under environmental conditions. The majority are found with therapeutic drug applications or are purposely engineered by people making synergy a positive quality. Most chemical interaction in the environment are, at most, additive and assuming this level of interaction generally provides a good estimate of the potential impact of two or more compounds. For compounds with differing modes of toxic action, the predicted toxicity is often considerably over-estimated with this model, providing a significant level of conservatism to the assessment. Thus, the use of an additive model to assess chemical mixtures in the environment is no longer done out of mathematical convenience but because the data supports this as being a realistic worse-case description of the potential interaction between chemicals. The results of the research presented in this thesis further support this approach to estimating the potential toxicity of mixtures of chemicals to aquatic receptors.

There is also considerable advantage to using a chemical body-residue, instead of the chemical concentration in the water, as a measure of chemical exposure to aquatic organisms. The data in this thesis supports the concept of a critical body-residue (a specific chemical concentration in the whole organism above which a biological response will be noted) and its utility in the toxic unit model for predicting the potential toxicity of a chemical either individually or when present with others. The expansion of this approach, beyond the laboratory, to include field applications is long over due. Quite often the potential environmental risks to aquatic ecosystems within a site contaminated with organics such as PCBs, chlorinated phenols, PAHs and methyl mercury are defined based on chemical concentrations in the water and sediment. Exposures defined in this way can be misleading. Considerable uncertainty is introduced by having to make assumptions with respect to the bioavailability of these chemicals to the biota. Often the factors which modify this bioavailability are too complex to define or model. Having site data which quantifies chemical residues in a variety of aquatic organisms (macrophytes, invertebrates and fish) and, laboratory data which defines the biological significance of these residues, would be invaluable for predicting the potential environmental risks associated with the contaminants. However, this data is not typically collected and even if it were, there are very few body-residue-based toxicity studies available from which to derive benchmarks of exposure.

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IMAGE EVALUATION
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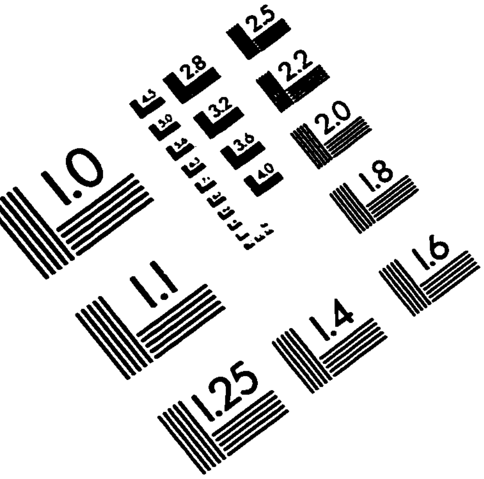

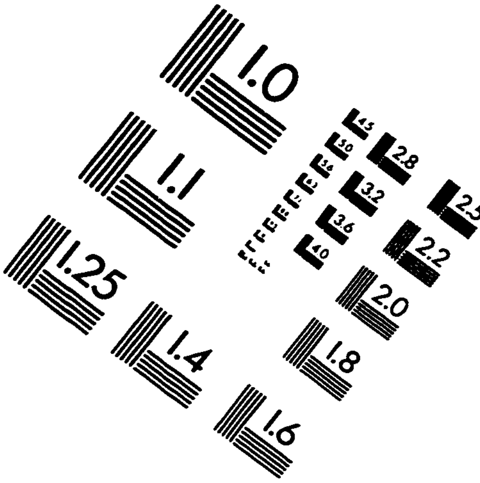

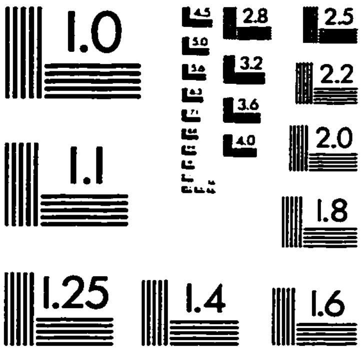

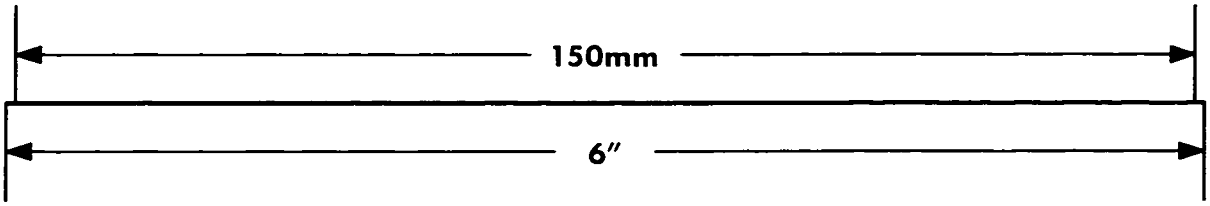

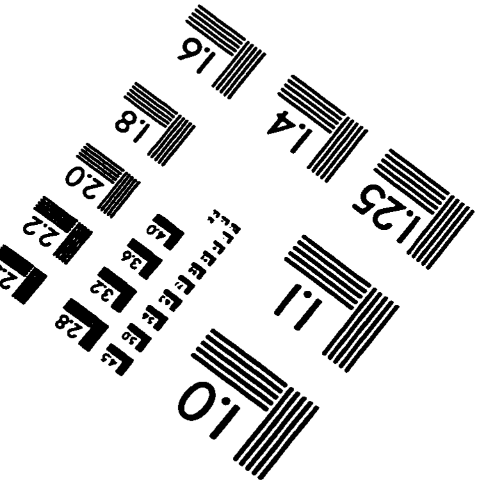

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1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989