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# Development and Standardization of a Short-Term Assay for Evaluating Polluted Estuarine and Coastal Environments: The Medaka Embryo-Larval Assay

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# DEVELOPMENT AND STANDARDIZATION OF A SHORT-TERM ASSAY FOR EVALUATING POLLUTED ESTUARINE AND COASTAL ENVIRONMENTS: THE MEDAKA EMBRYO-LARVAL ASSAY

by

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David J. Burdige (Director)

## ABSTRACT

**Development and Standardization of a Short-Term Assay for Evaluating Polluted Estuarine and Coastal Environments: The Medaka Embryo-Larval Assay**

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The eggs of the Japanese medaka (*Oryzias latipes*) were employed in a number of studies to develop a routine, standardized assay which can assess the acute and sublethal impacts of individual toxicants and complex mixtures. The eggs of this Cyprinodont minnow were topically treated with each toxicant or mixture dissolved in membrane permeable dimethylsulfoxide (DMSO) solvent. Nine chemicals were initially evaluated with two, pentachlorophenol (PCP) and tributyltin chloride (TBTCl), subsequently selected for extensive evaluations of salinity tolerance and the accuracy of the topical exposure method for representing a typical immersion exposure. The amount of material actually penetrating the eggs was determined for a number of these chemicals, as were the optimum rearing conditions for the eggs. To determine the usefulness of this assay in field studies, surveys of lower Chesapeake Bay tributary water and sediment samples were performed using the MELA approach.

The results indicate that the proposed assay is very useful for the evaluation of the developmental effects of individual toxicants as well as complex mixtures. The salinity tolerance studies indicated that salinity (up to 20ppt for PCP and 35ppt for TBTCl) elicited no significant effect on the toxicity of the two test substances on developing embryos. In addition, it appears that if the bioconcentration factor (BCF) is well established for a chemical, it may be possible to extrapolate to an equivalent medium concentration for a given topical dose. The permeability data indicate that

the toxicants penetrate into the eggs at levels well correlated to their octanol-water partition coefficients, suggesting that passive transport is occurring, even in the presence of a membrane permeable carrier.

Finally, the field studies demonstrate that the MELA approach is very useful for evaluating the relative toxic/teratogenic potential of numerous sites, simultaneously. The data from the MELA treatments often correlated well with other biological assays and with chemical data on a site specific basis.

## DEDICATION

I would like to dedicate this research to two individuals without whom this work would never have been possible. First, I would like to express my deepest appreciation to Dr. Raymond W. Alden III who unselfishly and graciously funded the majority of this project and who's knowledge, incite and endless motivation were the foundation of this research. Ray, without your guidance, patience and input this research would be incomplete, I thank you for all that you have provided. Second, I would like to thank my best friend, Cecilia Dacey Helmstetter, for her patience, perseverance and unending support over the duration of this project and during the many true tests of character and faith that we have encountered over the years. Cecilia, to you I dedicate this research and all of my future work as a scientist.

## ACKNOWLEDGEMENTS

A project of this magnitude requires the assistance and support of many individuals too numerous to mention here. To all those who participated in this venture, I express my sincerest gratitude. In particular, I would like to thank the University community in general for both material and educational support throughout my graduate education. To the members of my committee, Dr. Raymond W. Alden III, Dr. Alexander E. Maccubbin, Dr. David J. Burdige and Dr. John R. McConaugha, I wish to thank you for your helpful suggestions and guidance throughout the progress of this dissertation. I would like to express my appreciation to the faculty, staff and students of the Applied Marine Research Laboratory; expressly, Thomas L. Price, Jr. and Michael J. Ehret for the late night comradery and the attitude adjustments, Ann M. Dombrowski for assisting in the land use activity research and for keeping things afloat in my absence, and Peter C. Adolphson for technical advice and assistance in culturing of the medaka. I would like to thank my family and my wife's family for their support and tolerance of my absence over the last several years. And finally, I would like to thank my trusty companion, Tucker, who patiently stood by during the late nights and early mornings just to say 'hello'.

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## TABLE OF CONTENTS

## TABLE OF CONTENTS (Continued)

## LIST OF TABLES

## LIST OF TABLES (Continued)

## LIST OF FIGURES

## LIST OF FIGURES (Continued)

## LIST OF FIGURES (Continued)

## LIST OF FIGURES (Continued)

# CHAPTER ONE

## Overview and Purpose of Study

## Overview and Purpose of Study

Covering over seventy percent of the Earth's surface, the oceans and their numerous coastal zones and tributaries are often the final repository for many polluted wastes. Since the beginning of documented history, man has regarded the oceans' capacity to dilute and degrade polluted materials as enormous, and therefore has viewed the ocean as an acceptable reservoir for dumping of such waste. In addition, man has typically viewed the oceans as limitless with respect to their resources, being capable of accepting polluted materials without inducing a significant impact on the resource diversity or abundance. More recently, however, researchers are finding that the "self-purifying" abilities of the oceans are not unlimited and that continued global introduction of hazardous materials may impose permanent damages to the oceanic system (GESAMP, 1990).

Oceanic pollution is not only a global problem in magnitude but a complex problem with integrated economic, technological, political and legal aspects (Schachter and Serwer, 1970). For example, the oceans are a major source of food and water to many countries, and are therefore an international resource. They are also responsible for stabilizing the Earth's climate as well as the oxygen/carbon dioxide balance in the overlying atmosphere (Lerman, 1986; Thurman, 1991). The use of the oceans as a waste repository has the potential to conflict with its use as an economic and environmental resource. The oceans do demonstrate the ability to assimilate many of these wastes, often allowing the ecosystem to exist with little effect from this waste introduction (Goldberg, 1982; Champ and Duedall, 1989; Risebrough, 1989). However, long-lived substances, such as halogenated organics and radioactive materials can accumulate in the oceans and exceed the oceans ability

to accept and tolerate these materials (GESAMP, 1990). In other words, the ability of the marine environment to adapt to these changes may not be unlimited, and the adaptations may not necessarily be beneficial to marine ecosystems or to man. The capacity of the environment to assimilate wastes depends upon the rate of input of the waste materials and the environmental "life-time" of the wastes prior to processing and transformation into their structural components by natural biogeochemical cycles (Kester *et al.*, 1981). Of primary concern in the evaluation of this global marine pollution is the potential destruction of coastal ocean environments and associated animal species as well as health hazards to humans through direct contact or through ingestion of contaminated fish/shellfish or through consumption of, or exposure to, contaminated waters.

The coastal oceans comprise approximately ten percent of the total oceanic area and include the area from the shoreline to the edge of the continental shelf, estuaries, lagoons, inshore waters and numerous marginal seas. These coastal areas receive the bulk of polluted material through direct inputs of continental materials via rivers, terrestrial runoff, atmospheric input and a variety of industrial sources (e.g., effluents, ship discharge, dredge materials, etc.; Clark, 1989). The open ocean areas, with a few exceptions such as some productive equatorial regions, are the "marine deserts". However, materials in the open ocean generally have long residence times, and open ocean waters below about 100 meters often remaining out of contact with coastal waters for extended periods of time. For example, particle reactive metals (e.g., aluminum and iron) have open ocean residence times of hundreds of years, while water soluble metals (e.g., sodium) have residence times of hundreds of millions of years (Lerman, 1986; Albaiges, 1989; Risebrough, 1989). A situation

such as this may lead to a significant build-up of foreign materials through slow accumulation of man's wastes (Goldberg, 1976; GESAMP, 1990).

Although they comprise only a fraction of the ocean's area, the coastal waters are exceedingly important in terms of primary productivity and recreational use. The photosynthetic activity of marine phytoplankton which constitutes the base of the food chain that ends in marine fish, birds and mammals, takes place predominantly in these waters. Because of the high rate of nutrient turnover and biomass associated with this primary production, estuarine and coastal areas also serve as important breeding and nursery grounds for many commercially important and other species of fish and shellfish and as important habitats for many migrating species. The diversity of marine life in these coastal regions is interconnected in a web of inter-related food chains, all of which depend in the end on the chemical condition of the marine environment. This species diversity is an essential characteristic of food webs in these productive marine waters, for diversity is frequently associated with stability in ecological systems (Schachter and Serwer, 1970).

Coastal and estuarine areas are typically shallow and confined, with properties strongly influenced by associations with the continents and the sea floor. Reduced dilution and limited physical processes lessen the ability of these coastal areas to assimilate or eliminate foreign materials (Bishop, 1983). Moreover, most coastal environments are variable with regard to natural environmental factors, such as salinity and temperature, which can further enhance the deleterious impact of pollutants (see, for example, Weis *et al.*, 1981). Unfortunately, whether intentional or accidental, coastal waters are used as a major vehicle for the disposal of industrial, agricultural and urban runoff, and relocated wastes. The passive impacts created

through this relationship include not only damage to environmentally stable ecosystems and their associated biota, but also potential risks to human health through consumption and/or exposure (Black, 1984). The Intergovernmental Oceanographic Commission (IOC) has defined marine pollution as "the introduction by man, directly or indirectly, of substances or energy into the marine environment (including estuaries), resulting in such deleterious effects as: harm to living resources; hazards to human health; hinderance of marine activities including fishing; impairing the quality for use of seawater and reduction of amenities" (Gerlach, 1976). U.S. manufacturers were reported to have released 5.7 billion pounds of toxic chemicals into the water, air, and land in 1989 (Environmental Reporter, 1991), much of which either temporarily or permanently resides in the coastal oceans. The unremitting presence of these "substances" in coastal areas provides consistent exposure to indigenous species as well as a continuous trickle of wastes into the open ocean areas.

The ecological balance of the oceans can be upset in many ways. Some pollutants simply poison the animals and plants with which they come into contact (e.g., dibenzodioxins; Czuczwa and Hites, 1984; Waldron, 1990; Wisk and Cooper, 1990). Other pollutants (e.g., urban sewage and the accompanying excess nutrients) make such a demand on the seawater dissolved oxygen that the resident species are asphyxiated (Clark, 1989; Freedman, 1989). Finally, other pollutants accumulate in marine food webs because they are not readily metabolized (e.g., some halogenated organics; Phillips, 1980; Moriarty, 1988; Clark, 1989; Freedman, 1989). These biomagnified pollutants are therefore concentrated through successive levels of food chains, and can reach levels which upset biochemical and physiological function (Schachter and Serwer, 1970). Of primary concern is the possibility that levels of

toxic materials can magnify to the point where exposures of organisms to these materials in coastal waters, as well as in the open ocean, result in widespread mortalities of resident species (Goldberg, 1976). In addition, direct impact of these toxicants on humans may also occur through ingestion of contaminated foods and physical contact with impure waters.

Maintaining the quality of the marine environment will require an improved understanding of the fate and effects of wastes in the oceans. Poor waste disposal and effluent discharge practices can lead to a deterioration in environmental quality which our society now considers unacceptable. Kester *et al.* (1981) define four classes of techniques for assessment of impacts of these materials on aquatic life based on an International Council for the Exploration of the Seas (ICES) workshop on monitoring biological effects of pollution in the oceans:

1. Bioassay measurements
2. Physiological techniques
3. Biochemical measurements
4. Ecological assessments

This document will focus on the first of these four techniques, bioassay measurements, which entail determination of the toxicological effect of chemicals, mixtures of chemicals or *in situ* samples on test species. The study of these toxicological effects in the marine environment, through the use of biological assays, is defined as 'marine toxicology' - the branch of the marine sciences dealing with the chemical and biological assessment of known or suspect pollutants entering estuarine and coastal ocean waters (Wells, 1981). The assessment includes the physico-chemical fate of compounds, biological degradation, accumulation and magnification, lethal and sublethal toxicological effects, and derivation of "safe" toxicant

concentrations. Its prime goal is to determine levels of materials causing significant impacts on marine organisms and to assist regulatory agencies in establishing marine water quality criteria by defining acceptable threshold concentrations, if they exist, for recognized toxic substances or complex environmental mixtures (Wells, 1981). In other words, the goal of marine toxicity testing is to identify hazardous agents and environments, to define the conditions (dose, time, route of exposure, susceptible species, salinity, etc.) under which they will exert their toxic effect and to estimate the potential effects on resident marine populations and on human health (Brown and Fabro, 1982).

Much of the present research in toxicology is directed toward finding sensitive biological response parameters, which allow evaluation of the health of organisms exposed in the field or under experimental conditions in the laboratory (Lu, 1985; McCarthy and Shugart, 1990). In the former approach, the intention is to assess the impact of pollutant contamination under "natural" conditions; the latter evaluation is an attempt to define the lower "safe" threshold concentration of a representative species, such that the level of damage to a species or population can be predicted from the identities and concentrations of pollutant constituents (Anderson, 1979). Anderson (1979) conveys that the latter approach may be the only feasible method of assessing damage due to the profound variability of organisms in the natural environment. Whether conducted in the laboratory or in the field, biological evaluation studies serve as an early warning system to resident species and ultimately to man. These "biomonitoring" programs provide governmental agencies with a scientific basis to regulate the release of materials that may have a deleterious effect upon marine resources and ultimately to man, with the results of these studies being

used to assess the state of health of the system and the effectiveness of existing regulatory activities (Goldberg, 1976).

The relevance of these ecotoxicity studies to humans is that the quality of life in the marine ecosystem is, in many cases, more sensitive than the human system and thus serves to set thresholds of exposure which will coincidentally protect humans (Solomon, 1981). Further, though phylogenetically distant from mammals, many fish species commonly employed in these studies contain remarkably similar organ systems to humans. Moreover, there is a expanding foundation of data which indicates that fish species metabolize many known toxicants in a manner similar to higher vertebrates (Lech and Vodicnik, 1982; Cooper *et al.*, 1984). This metabolism is a requirement for a number of chemicals to elicit their toxicological and/or carcinogenic effects (e.g., benzo[a]pyrene and diethylnitrosamine). In order for a test organism to be a reliable model for both environmental and human health testing, it must be capable of activating (or deactivating) these types of compounds in a manner similar to those species (whether indigenous or consumers) which the test is designed to "protect".

The impact of water-borne pollutants on marine life has traditionally been assessed using standard laboratory bioassay techniques often employing freshwater and marine fish species. In these assays, the major criterion used to determine an organism's response to a toxicant stress is the measurement of an $LC_{50}$ value or the concentration of a pollutant or pollutant mixture which results in a lethal response in 50% of the test population. These quantal results are used by regulatory agencies to establish maximum allowable concentrations of contaminants in discharge effluents or waste disposal materials. Hence, data on lethal exposure are useful for detecting the

sensitivity of an organism to a particular waste input, comparing the relative toxicities of various pollutants, and establishing guidelines for the input of materials into the environment. However, important physiological, behavioral, and ecological changes that may occur due to chronic, sublethal exposure are overlooked in these standard all-or-nothing acute assays.

Birge and Black (1985) suggest that the techniques for rapid assessment of chronic effects on aquatic biota fall primarily into three general categories: 1) predictions based on physico-chemical structure-activity relationships, 2) extrapolations from acute data (e.g., employing application factors), and 3) development and use of appropriate short-term chronic ("mini-chronic") tests. Because of changing research needs of both scientists and regulatory agencies, and time and cost constraints associated with conventional life cycle chronic studies, high priority has been placed on the development and validation of sensitive, short-term tests and other means of estimating or measuring the chronic effects of chemicals in the environment. Though often neglected, chronic toxicity is typically a primary element in determining the effects of toxicant stress on biota. However, chronic data are in short supply, and this often precludes a complete assessment of the impact of environmental toxicants on marine ecosystems. Further, short-term chronic tests should prove equally important in many areas of fundamental toxicological research: including the evaluation of the similarities (and dissimilarities) in response of different species to a given toxicant or toxicant mixture; synergistic or antagonistic interactions and net effects of complex mixtures; laboratory to field extrapolations of toxicity data; and structure-activity relationships (Birge and Black, 1985).

The life cycle toxicity test is considered by most toxicologists to be the

ultimate chronic test in establishing long-term "safe" environmental concentrations of toxic chemicals (McKim, 1985). Mount and Stephan (1967) conducted the first aquatic vertebrate life cycle study with the fathead minnow (*Pimephales promelas*). This type of life cycle test demands a minimum lab exposure from embryo to embryo, which for most aquatic species, requires a minimum of 6-12 months of concentrated effort (McKim, 1985). In order to expedite chronic toxicity testing, the 30 to 90 day fish embryo-larval test was introduced in the mid-1970s (McKim, 1977). While this has become an acceptable and more cost-effective approach for deriving chronic data for environmental toxicants, the duration of exposure and dependence on flow-through test conditions render this technique impractical for extensive testing and research efforts or for use in field biomonitoring programs (Birge and Black, 1985). Several investigators proposed that chronic toxicity to fish might be predicted by use of even shorter tests with early developmental stages. In studies with selected toxicants, these early stages were shown to be among the most sensitive in the life cycle (McKim, 1985 and references within). This demonstrated sensitivity of the early life stages compared to the later developmental stages provides an accurate and efficient tool for predicting chronic effects of environmental pollutants in less than one month of testing. In addition, the rapid proliferation of cells and resulting rapidity of growth and morphological changes during early fish development is a prime factor in the usefulness of these early life stage tests to the marine toxicologist seeking a short, sensitive, predictive toxicity test.

At present, standardized early life stage tests focus primarily on overall growth and survival. Alternatively, these tests can provide data which could incorporate all of the processes of growth and differentiation, so that any teratogenic agent would

produce a response, regardless of its mechanism of action. Therefore, *in vitro* test protocols are needed which incorporate the processes of cell multiplication, determination/induction, aggregation, organization, morphogenetic movement, cell death, etc. Brown and Fabro (1982) indicate that only two types of systems satisfy these criteria: those using developing embryos (or portions of embryos) and those employing regenerating tissue (e.g., limb bud studies).

Embryonic and larval stages of fish present a broad spectrum of target sites for trace contaminants, including mechanisms associated with fertilization; cellular differentiation, proliferation, and growth; basic metabolism and systematic functions; the hatching process; and the acclimation to post-hatch survival. In addition, because of their mobility, adult fishes can avoid or emigrate from polluted areas. Such is not the case for the embryonic and larval stages which are either planktonic or demersal. Planktonic eggs are often directly exposed to the sea surface micro-layer which has been demonstrated to concentrate many organic and inorganic pollutants (Hardy *et al.*, 1985; Cleary and Stebbing, 1987; and others) while demersal eggs may be particularly susceptible to contaminated bottom sediments which may gradually leach pollutants into the overlying water column. Accordingly, protective environmental standards and pollution abatement policies, which have primarily been based on substance effects on adult stages, may not provide adequate protection for embryonic development of many species (Birge *et al.*, 1979).

Early life stage toxicity tests are conducted from the period post-fertilization up to hatching and are designed to establish the minimum concentration of a chemical or chemical mixture, which produces a statistically significant observable effect on a number of endpoints including: hatching and survival rates, developmental processes

and growth (U.S. EPA, 1988a). The developing embryo is thus a highly complex and balanced physio-chemical system, which displays alterations in normal development as toxicity, teratic effects and delayed growth following exposure to test substances (Laale and Lerner, 1981). The question then becomes, how well do impacts on these early life stages predict or represent effects on the complete or partial life cycle of a given fish species? McKim (1977) compiled the data on 56 life cycle toxicity tests with four species of fish with 34 different organic and inorganic chemicals and showed that embryos, larvae and early juvenile life stages were the most sensitive, or among the most sensitive stages in life cycle toxicity tests. Eighty-two percent of the time the maximum acceptable toxicant concentration (MATC) estimated by the embryo-larval or early juvenile exposures was identical to the MATC established for longer, more involved and costly partial or complete life cycle toxicity tests. The remaining 18% showed increased or decreased sensitivity as compared to the life cycle tests by a factor of two. However, a factor of two variation is relatively insignificant, since the MATCs for specific toxicants, species, and water combinations can easily vary by a factor of two (McKim, 1985). Further, toxicity tends to manifest itself as a function of the log of the treatment concentration, therefore, differences of a factor of two are relatively small (on a log scale). If the above is true for individual toxicants, it should also be true of toxicant mixtures or complex effluents present in estuarine and other coastal ocean areas.

These research efforts focused on sensitive early life stages in search of an effective, efficient and less costly approach to predicting the acute and chronic toxicity of chemicals to fish in estuarine, coastal and open ocean environments. In addition to the demonstrated representation of species life cycles, the evaluation of

these early life stages are essential, as the biological effects of an environmental chemical include not only its toxicity, mutagenicity, and carcinogenicity, but also the toxic effects which the substance may have on the development of embryos. Therefore, the first goal of this study was to develop an assay to be employed for determining embryotoxic effects which satisfies both ecological and economic criteria. From the ecological point of view, a test system should be as representative as possible of a specific ecosystem and of a specific trophic level. The relevant economic criteria are fulfilled if sufficient quantities of the organism can be made readily available for standardized tests at a limited expense (Dumpert and Zietz, 1984), and the test duration can be reduced while maintaining critical toxicity end points.

The second goal of these studies was to develop a test protocol which could be employed throughout the range of salinities from the tidal fresh waters of the upper reaches of an estuary (salinity $\approx$ 0ppt) to open ocean waters (salinity $\approx$ 35ppt). Though there are an abundance of short-term toxicological tools available to the researcher, to date, no single, routine assay has been developed which can evaluate the "health" of a system which may cover the full range of salinity. An assay of this versatility is essential to determine the fundamental differences in the effects of sea salt concentration on the toxicokinetics of a given chemical or mixture of chemicals. For example, some chemicals and elements may exhibit reduced toxicity with an increase in sea salts due to osmotic changes (see, for example, Phillips, 1980). On the other hand, it may be that organisms reared in high salinity environments display increased sensitivity to toxicant stress due to their adaptation to the near isotonic conditions in high salinity systems (Gerlach, 1976). Because of marked physiological

differences between freshwater and marine organisms, there is a need for a bioassay protocol utilizing sensitive life stages of a representative species which can be used to evaluate complex and variable marine and estuarine systems.

The Japanese medaka (*Oryzias Aplocheilus latipes* Temminck & Schlegel) has demonstrated the potential to be an appropriate surrogate species for such studies through established tolerance to a wide range of salinities (Kirchen and West, 1976; Tachikawa *et al.*, 1991) and demonstrated sensitivity to a numerous toxicants, mutagens, teratogens and carcinogens (Solomon, 1979; Hatanaka *et al.*, 1982; Klaunig *et al.*, 1984; Takimoto *et al.*, 1984a&b; Maccubbin *et al.*, 1987; Hinton *et al.*, 1988; Shigeoka *et al.*, 1988a&b; Hawkins *et al.*, 1990; Wisk and Cooper, 1990; Cooper *et al.*, 1991; and others). Adult medaka have been implemented in a single study of salinity sensitivity to pentachlorophenol (Tachikawa *et al.*, 1991) which indicated a significant effect of changing salinity on the accumulation, elimination and toxicological effects of this toxicant. The few other recent studies which have been conducted on the salinity-related sensitivities of fish species to toxicants have employed seawater-acclimated guppy sensitivity to tributyltin (Tsuda *et al.*, 1990) and tilapia (*Tilapia nilotica*) and mullet (*Mugil cephalus*) sensitivities to chlordane and pentachlorophenol (Tachikawa *et al.*, 1987a and Tachikawa *et al.*, 1987b, respectively). Few studies have been conducted where the eggs of these or other fish species have been extensively evaluated in a similar manner; most have dealt with a narrow range of salinity and with inorganic elements (Eisler, 1971; von Westernhagen *et al.*, 1974; von Westernhagen and Dethlefsen, 1975; Weis *et al.*, 1981). No salinity-related sensitivity toxicity studies have been reported utilizing the eggs of the

medaka.

Eggs of the medaka were employed in this study to meet the following objectives:

1. To develop a short-term, mini-chronic, eukaryotic toxicological assay which demonstrates sensitivity to known toxicants, in a cost-efficient fashion, and which can be used across a wide range of salinities, from freshwater to full-strength seawater.

2. To determine the specific acute and short-term sublethal effects of nine known or suspect toxicants, mutagens, teratogens and carcinogens, commonly detected in coastal ocean areas, on developing medaka eggs through range-finding studies, and to evaluate two of these toxicants in extensive salinity-related sensitivity evaluations.

3. To determine the amount of toxicant actually penetrating the egg chorion to elicit an effect on the developing embryos.

4. To determine the optimum rearing conditions for this assay including test of the variables - culture container type, solution renewal and aeration.

5. To evaluate water and sediment extracts from the Elizabeth River, Norfolk, Virginia and the Chesapeake Bay in pilot biomonitoring studies of the usefulness of this assay in determining spatial and temporal toxicity trends.

The complex nature of coastal ocean ecosystems and pollutant impacts on these regions necessitates the development of a sensitive assay which can evaluate the lethal and sublethal effects of toxicants and toxicant mixtures in environments crossing many salinity regimes. This document reports the results of studies which employ the eggs of the Japanese medaka as a surrogate early life stage system to determine the effects of nine known or suspect toxicants on embryological development of a fish species and the impact of varying salinities on a pair of these chemicals.

# CHAPTER TWO

## The Japanese Medaka and Egg Sensitivity to Nine Chemicals

# INTRODUCTION

## The Japanese Medaka

The Japanese medaka (*Oryzias (Aplocheilus) latipes*) is a small teleost fish native to Asian countries, measuring 2 to 4 centimeters (cm) in length and weighing between 0.3 and 0.6 grams (g). An oviparous killifish from the family Cyprinodontidae (Kirchen and West, 1976), the medaka has a short life cycle, however, individuals reared under captive conditions have been shown to live in excess of four years (Egami *et al.*, 1988). Sexual dimorphism is both internally and externally evident. Internal evidence is based on the fact that the female contains a single, large ovary (Rugh, 1962); external indications are based on the fact that the male has a larger anal fin than the female; the central margin of this fin is convex in the male and the fin rays are rarely branched; and the dorsal fin of the male is smaller than the female and rounded with no notch in the posterior rays (Figure 2.1; Kirchen and West, 1976).

Oocytes mature in the female medaka ovary primarily in the dark during periods of low activity. Ovulation occurs at dawn coincident with the presence of light and an associated increase in overall metabolism (Weber and Spieler, 1987), however, feeding schedules, temperature and pH may also play a significant role in the stimulation of the shedding of eggs (oviposition). First light also inspires the male medaka into a brief courtship followed by spreading of the milt which, in turn, appears to encourage oviposition (Kirchen and West, 1976). Copulation takes less than two minutes during which time the male holds the female with its dorsal and anal fins while spreading milt over the eggs with its pectoral fin (Rugh, 1962). A single

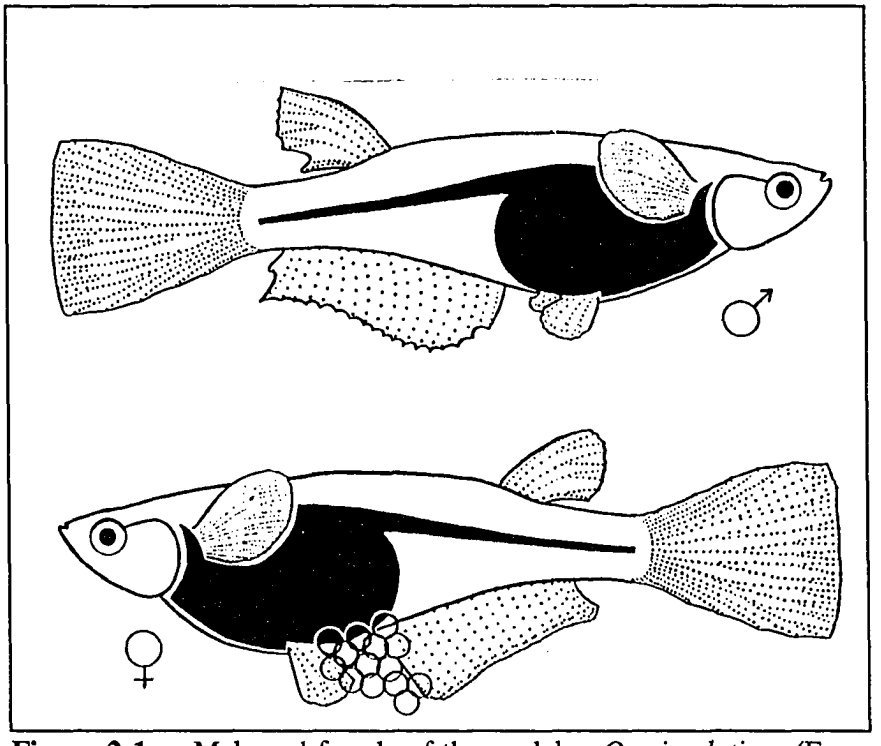


**Figure 2.1** Male and female of the medaka, *Oryzias latipes* (From Kirchen and West, 1976).

female may produce from 1-120 eggs per day, the average being between 20-40. Breeding females employed through the duration of this study indicated that an average healthy female under simulated natural breeding conditions can produce in excess of 4200 eggs in a four to six month period.

The transparent, demersal medaka egg measuring 1 to 1.5 mm in diameter (Kirchen and West, 1976) has a large, central membrane-bound yolk compartment surrounded by a thin layer of cytoplasm, which is in turn bounded by the plasma membrane and a tough protective non-cellular membrane, the chorion (Figure 2.2a; Gilkey, 1983). The chorion has a tiny opening at the animal pole, the micropyle, which is the site of sperm entry. Within minutes after oviposition, this micropylar opening closes eliminating the potential for subsequent fertilization (Kirchen and

West, 1976).

At oviposition numerous oil globules may be seen throughout the yolk material of the egg. During early development these decrease in number as they gradually coalesce (Figure 2.2b) to form a single, large oil globule at the vegetal pole. The oil globule remains present through the duration of embryonic development and is gradually absorbed during the early fry stage as a nutritive substance simultaneously with the absorption of the yolk material (Rugh, 1962). The yolk "sphere" is uniform throughout the egg, except at the animal pole where the germ disc (or blastodisc) will form during early development (Figure 2.2c-d). Yolk itself is non-living and inert and plays no active part in cleavage, but does serve as a mechanical impediment to the cleavage process in the egg containing a large amount of yolk (megalecithal), allowing only a limited area at the animal pole for initial cell division and development (i.e., meroblastic cleavage; Swartz, 1983). Following extensive development, a distinct polarity occurs whereby the cytoplasm and dividing cells remain at the animal pole and the yolk stays at the vegetal pole (Figure 2.2e). Additional stages of development are shown in Figure 2.2f-g; more extensive assessments of embryonic development in the medaka may be seen in reviews by Rugh (1962), Yamamoto (1975) and Kirchen and West (1976).

Hair-like filaments are distributed throughout the surface of each egg. These filaments are small ($\approx$ 0.025 mm in length) over the entire surface of the egg except at the vegetal pole where they are nearly 1 mm in length. These longer filaments are coated with adhesive material and serve to bind neighboring eggs, thereby forming large egg clusters (Figure 2.2h; Rugh, 1962).

The fully developed egg undergoes a hatching event at anywhere between 11

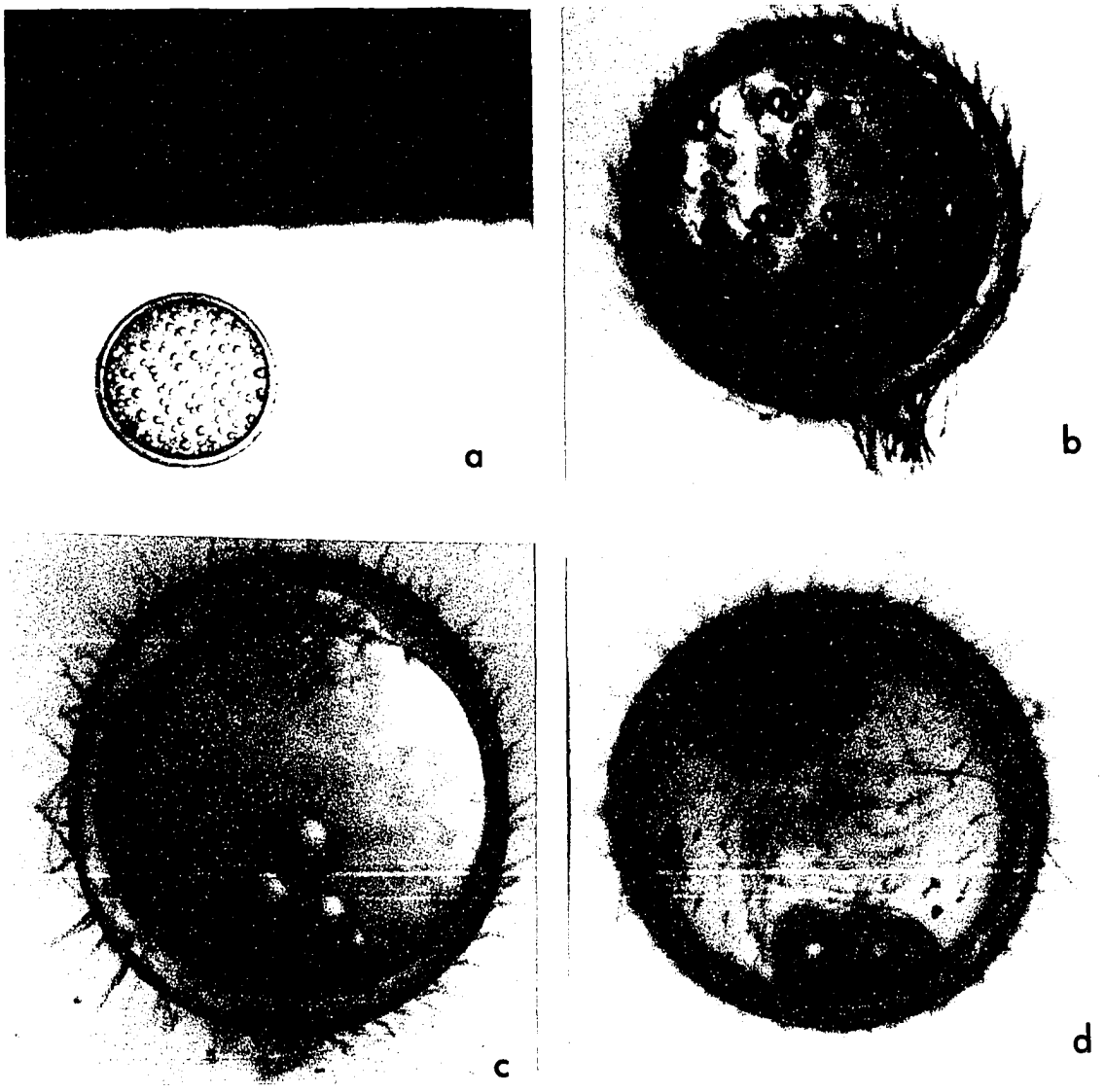



**Figure 2.2.** Stages of embryological development of the medaka, *Oryzias latipes*. See legend for phase descriptions.

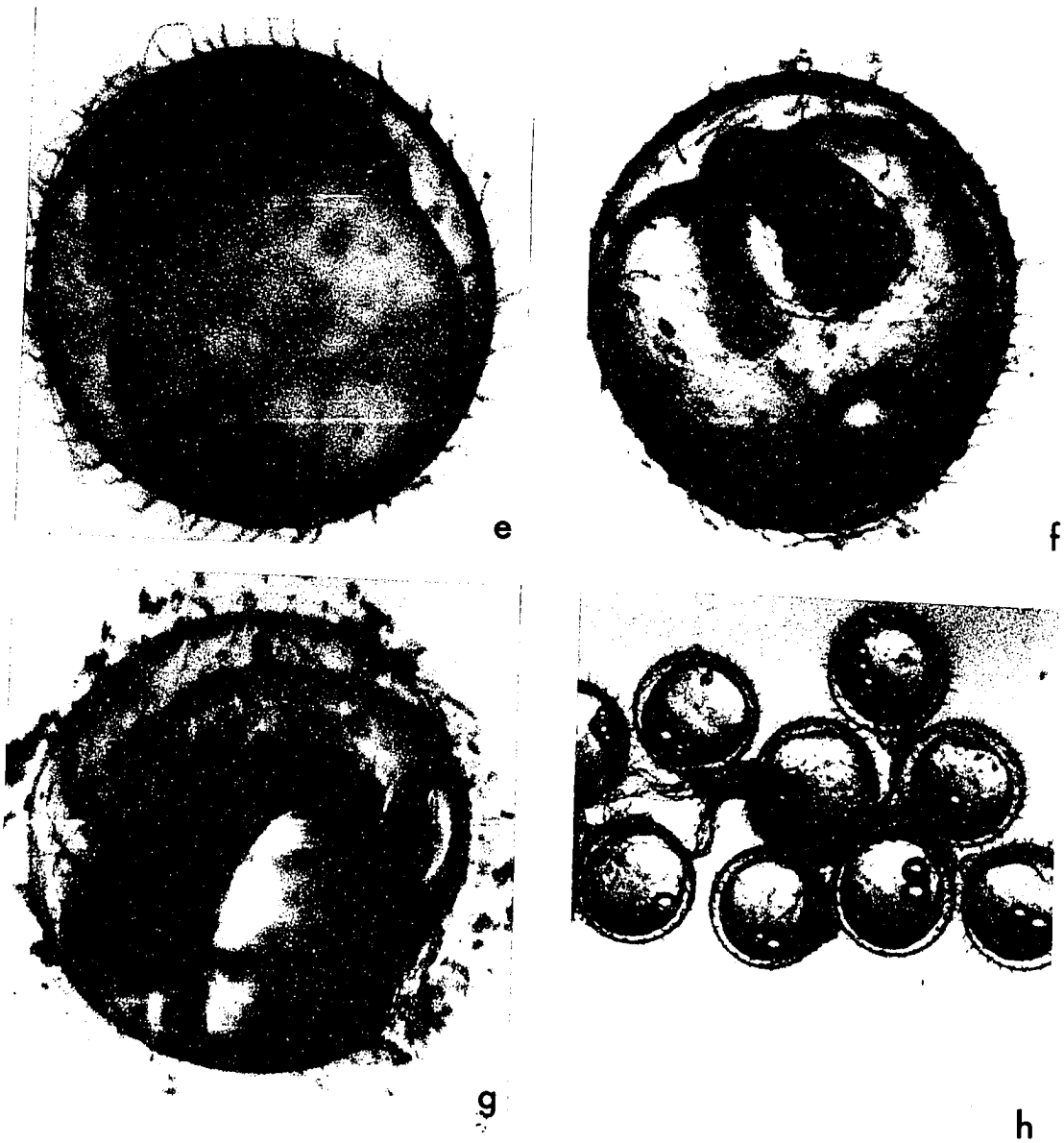
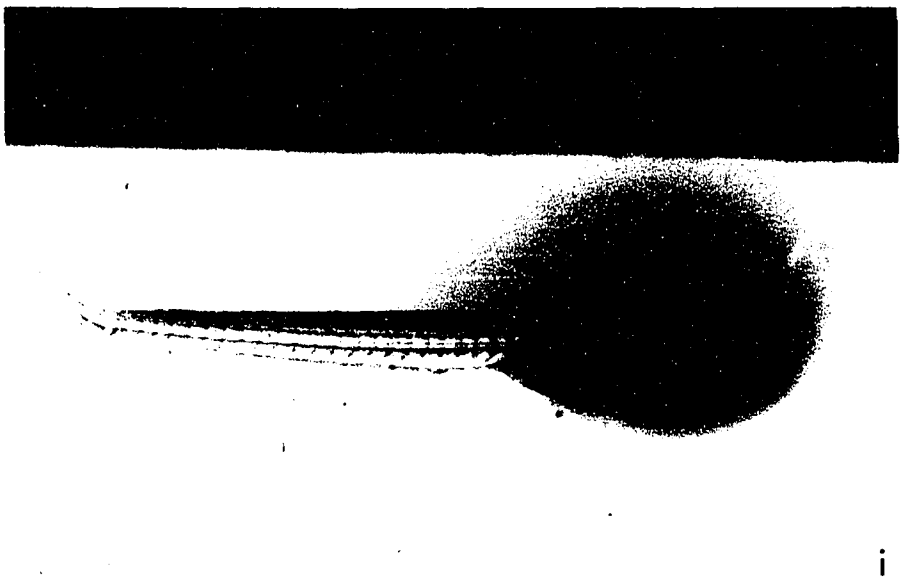



**Figure 2.2.** (Cont'd).



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**a** - Freshly fertilized egg. Perivitelline space apparent and oil globules evenly distributed throughout egg. (x15)

**b** - (1hr egg) Oil globules migrating toward vegetal pole (longer filaments). (x40)

**c** - (4-6hr egg) 32-64 cell stage. Oil globules are larger and fewer and have migrated to vegetal pole. Blastodisc well formed at animal pole. (x50)

**d** - (12hr egg) Mid-blastula stage. Multi-layered blastoderm. (x50)

**e** - (24hr egg) Mid-gastrula. Blastoderm expanding to cover yolk sphere. Germ ring no longer visible. (x55)

**f** - (48hr egg) Keel apparent at animal pole. Brain sections forming; pericardial cavity defined. Weak contractions of the heart. Optic lens and cup present. Oil globules coalesced into a single droplet. No circulation present. (x50)

**g** - (216hr) Circulation present. Optic and mandibular movement apparent. Liver rudiment, bile duct and swim bladder formed. Significant movement observed in embryo, including tail, mouth and eyes. Circulation more rapid and extensive. Golden chromatophores observed over dorsal surface. (x50)

**h** - Cluster of eggs, displaying adhering function of longer, vegetal pole filaments. (x15)

**i** - Newly hatched fry. 4-5mm in length. (x10)

**Figure 2.2.** (Cont'd).

and 20 days post-fertilization, releasing a 4 to 5mm fry from the confines of a weakened chorion (Figure 2.2i). Adult medaka mature in two to six months under normal laboratory conditions and have a life span of four or more years (Kirchen and West, 1976). However, breeding individuals, stressed by consistent exposure to simulated mating conditions, tend to exhibit a significantly reduced life expectancy.

There are numerous advantages to the use of medaka in studies of normal or stressed embryology including the following:

1. The medaka is a small vertebrate species, therefore, large numbers of breeding individuals can be maintained in a limited space and at an acceptable cost. Further, sexual dimorphism is easily distinguishable through external features.

2. It is a hardy fish and easy to sustain under normal conditions in the laboratory. It is omnivorous and can be maintained on freshly hatched, dried, or synthetic foods.

3. The medaka is easy to breed under favorable laboratory conditions, therefore, large numbers of test eggs can be obtained with relative ease on a daily basis, any time of the year. Further, eggs typically remain attached to the female for several hours after fertilization, during which time the cluster can be removed; or the breeding chamber can be modified to provide "collectable structures" on which the females can scrape/deposit their eggs.

4. Time of oviposition can be predicted so that the earliest developmental stages may be employed in studies of normal or altered embryology.

5. The adult mouth is such that only the larger fish in the aquarium can eat the smaller eggs; eggs are rarely eaten until they are brushed free of the female. However, the eggs are large enough to be useful in most experimental procedures.

6. The egg is nearly transparent, except for oil globules in the yolk which soon coalesce at the vegetal pole away from the developing embryo. Hence, all of the internal organs of the medaka can be observed *in vivo*.

7. The various medaka life stages can tolerate and thrive in a wide range of culture temperatures (5-30 °C) and, therefore, can be employed in studies of temperature effects. The tolerance to low temperatures allows for the slowing of normal development allowing close, detailed observation of particular features or anomalies.

8. The medaka has been shown to be highly susceptible to a wide range of toxic, mutagenic, teratogenic and carcinogenic agents, making it an ideal species for the evaluation of field samples or new chemicals.

9. The medaka has a low incidence of spontaneous, background anomalies and spontaneous neoplasia and a short latency period for induction of such anomalies and/or tumors through treatment with test substances/mixtures.

10. All tissues can be assessed histologically through observation of transverse, sagittal or serial sections of eggs, fry or adults with comparative ease.

11. Conceivably the greatest advantage of using the medaka egg and other fish egg species in studies of toxicity and teratology is the fact that the egg is separated from the maternal surroundings allowing the investigator the ability to directly observe the developing embryo (Weis and Weis, 1987).

12. The adults of the medaka have previously been shown to be tolerant to a wide range of salinities in assays of toxicity and bioconcentration (Tachikawa *et al.*, 1991).

**Embryologic Assays**

The eggs of the medaka and many other fish species have been used for a number of studies, from examination of basic processes and biochemical mechanisms of embryological development to research involving the determination of the tumorigenic potential of known or suspect carcinogens. The majority of the assays with fish embryos involve their exposure to known or unknown substances through immersion tests which entail the submersion of the test eggs in a medium containing the chemical or mixture of chemicals of concern in an aqueous solution. Eggs of the medaka have been exposed to a number of organic and inorganic substances (employing numerous variations of this immersion technique) including: tolbutamide (Smithberg, 1962), mercuric chloride (Heisinger and Green, 1975; Shin *et al.*, 1986), 2,4,5-trichlorophenoxyacetic acid (Schreiweis and Murray, 1976), paraquat (Murray and Schreiweis, 1977), aflatoxin B1 (Llewellyn *et al.*, 1977), carbaryl, malathion and

parathion (Solomon, 1979), diethylnitrosamine (Klaunig *et al.*, 1984), fenitrothion (Takimoto *et al.*, 1984a&b), hexachlorobenzene (Huang *et al.*, 1986), chlorophenols (Shigeoka *et al.*, 1988a&b), 2,3,7,8-tetrachlorodibenzodioxin (Wisk and Cooper, 1990), and cod liver oil (Cooper *et al.*, 1991). Extensive employment of medaka eggs in these and other studies have made this species one of the most commonly used species in tests of fish embryology.

Immersion exposures with medaka eggs and eggs of other frequently tested species (e.g., *Salmo spp.* and *Xenopus laevis*) prove quite useful in studies of field-collected samples, *in situ* exposures and evaluations of moderately hydrophilic components (e.g., diethylnitrosamine, and aflatoxin B1), providing a realistic expression of exposure which most closely simulates passive exposure observed in nature (Llewellyn *et al.*, 1977; Wales *et al.*, 1978; Hendricks *et al.*, 1984; Cooper *et al.*, 1991). These exposures may be under static, semi-static, static renewal or flow-through medium conditions. However, the evaluation of the toxicological properties of many substances employing the immersion technique is often complicated by the physical and chemical properties of these compounds. One of the most common complicating factors is high or low water solubility which may inhibit adequate, consistent exposure concentrations to a test population (Hendricks *et al.*, 1984; Hawkins *et al.*, 1990). High water solubility requires extensive exposure to high concentrations of the test substance in order to achieve an adequate fraction partitioning into the egg (see, for example, Hendricks *et al.*, 1980). For large molecular structure, low solubility compounds, transport across the egg envelope is a problem. Investigators have approached this predicament through filtration of residual

particulate test substance or by simply exposing the test species to the particulate fraction in addition to the soluble fraction (Hawkins *et al.*, 1990). Carrier solvents may be also be employed to "solubilize" the substance in the aqueous medium, however, many substances rapidly come out of solution upon introduction into the medium and large volumes of many carrier solvents may introduce variables which are not desirable in a sensitive assay for toxicity and teratogenicity (Birge *et al.*, 1979). Moreover, due to these solubility problems and the uncertainty of the amount chemical partitioning into the egg, exposure using immersion tests is inferred, being based on the nominal or absolute medium concentration. This inference inhibits the development of true dose response data on a chemical weight to egg weight basis (Black *et al.*, 1985).

To circumvent the problems associated with immersion exposure, investigators developed a technique whereby the chemical was dissolved in a carrier solvent, most commonly dimethylsulfoxide (DMSO) or saline solution, and injected directly into the perivitelline space or yolk sac of the early embryonic stages of salmonids (Metcalfe and Sonstegard, 1984; Black *et al.*, 1985). This approach limited the volume and type of solvent to which the embryo was exposed and eliminated inconsistent exposure problems associated with refractory, water insoluble and very water soluble substances and their ability to permeate the egg envelope during short-term tests. This type of approach has numerous additional advantages including: reduced quantity of chemical required (advantageous for availability/cost as well as for safety and disposal), the use of a single, uniform exposure, and cost advantages (Black, 1988).

However, this approach is limited to the use of large eggs which in many

cases (e.g., *Salmo gairdneri*) do not provide many of the features available employing the medaka in short-term studies. The eggs of these salmonid species require exacting rearing conditions, they are difficult to obtain outside of the natural breeding period (Black *et al.*, 1988), and their opaque character makes observation of embryological development difficult.

The amount of chemical actually retained in the egg shortly after injection appears to be quite high at approximately 85-90% (Black, 1988; Metcalfe *et al.*, 1988). The loss is suspected to be due to leakage from the egg after the fine-gauge needle is withdrawn. A recent study has evaluated the potential of sealing the injection point with an adhesive to reduce the loss of test material to approximately seven percent (Walker *et al.*, 1991).

An additional and seemingly more significant predicament associated with these microinjection protocols is the high rate of injected control mortality apparently associated with the trauma of the injection procedure. Previous investigations indicated that early stage embryo lethality in the negative (carrier injected) controls ranged from 34.0 to 69.9% (Metcalfe and Sonstegard, 1984 and 1985; Black *et al.*, 1985; Grizzle and Putnam, 1987; Black *et al.*, 1988). In an attempt to reduce these mortalities, Black *et al.* (1985) injected at a later embryonic stage and, though still high, control mortalities at this egg stage were significantly reduced ($\approx$ 10-20%). In addition, Metcalfe *et al.* (1988) modified the embryo injection assay through injection of trout at the sac-fry stage, immediately following hatch. This approach reduced the injected mortalities to 16-22% (adjusted for untreated controls), however, this technique becomes ineffective if the early embryonic stages are of concern as an

indicator of teratogenesis.

In order to eliminate the problems of loss of treatment material and mortalities associated with the microinjection procedure, Maccubbin and Black (1986) developed a protocol for the direct application of the DMSO/toxicant mixture to the surface of the egg. The authors describe that the impetus for the technique was induced by accident after an egg inadvertently received material leaking from a microinjection apparatus and subsequently exhibited motor responses in the developing embryo. DMSO itself has been shown to be membrane permeable and apparently was capable of crossing the chorion of the test egg. A complete discussion of the properties of DMSO will follow in a subsequent section of this Chapter.

Following the discovery of this trans-chorionic property of DMSO, Maccubbin and Black (1986) developed a non-invasive procedure whereby a 1$\mu$l droplet of the test substance dissolved in DMSO was topically applied to each test *Salmo gairdneri* egg (i.e., perchorionic exposure). When radiolabelled $^{3}$H-benzo[a]pyrene ($^{3}$H-BaP) was used, these authors determined that approximately 30% of the $^{3}$H-BaP was present in the egg one minute after treatment. Though this represents a smaller fraction of chemical actually crossing the chorion than indicated using the microinjection technique, the use of absolute chemical exposure determinations through radiolabelling or other analytical procedures or the development of a model for predicting the level of permeability of a given chemical (see Chapter 3 Discussion) allows the determination of a true dose response curve, while maintaining a low negative control mortality (0% mortality at the egg stage in Maccubbin and Black, 1986).

In a single published study by Maccubbin *et al.* (1987), the eggs of the

medaka were employed using this topical, perchorionic exposure technique. This study evaluated the carcinogenicity of sediment extracts from the Black River, Ohio, the Buffalo River, New York and Times Beach, New York and neat standards of two known carcinogens, benzo[a]pyrene (BaP) and n-methyl-n'-nitroso-n-nitrosoguanidine (MNNG) in the eggs of the medaka as well as in the eggs of the rainbow trout (*S. gairdneri*). The trout exposure system was analogous to that employed in the Black *et al.* (1985) microinjection system; for the medaka exposure, the dosing system was adjusted to topically deliver approximately 0.1$\mu$l of the test mixture to each egg. The dose was given 30 seconds to cross the chorion, followed by washing of excess material from the eggs and the incubation of the treated specimens through hatching and to the later stages of growth to allow adequate time for potential tumor formation. As in the Maccubbin and Black (1986) perchorionic study, $^{3}$H-BaP was used to "calibrate" the retention of the dose. The radiolabelled material indicated that 0.11 $\pm$ 0.01$\mu$l of the dose was delivered to each egg and resulting mortality of the DMSO control was $< 1\%$ at the egg stage. The level of absorption of the material was reported as similar to that seen in the Maccubbin and Black (1986) topical treatment study with *S. gairdneri* (i.e., $\approx 30\%$). The results of this preliminary study indicated that the medaka topical treatment protocol proved to be a useful assay for single carcinogens. However, the complex mixtures obtained from the sediment extracts did not indicate the usefulness of this approach in complex mixture evaluations. Both individual chemicals (this Chapter and Chapters 5 and 6) as well as complex chemical mixtures (Chapter 7) were evaluated in this study for teratogenic and embryotoxic effects and indicate that a protocol similar to that developed by Maccubbin *et al.*

(1987) is useful for the evaluation of individual or mixtures of substances for several endpoints, however, further studies are needed to support this conclusion with respect to carcinogenesis.

Prior to describing the specific methodologies employed in the pilot phase of this research, a brief discussion of the carrier solvent and the test chemicals employed in the pilot study, is warranted.

**Carrier Solvent**

The first chemical to be discussed, dimethylsulfoxide (DMSO), is used as a carrier solvent to deliver the test chemicals across the egg chorion to the developing embryo. DMSO is a dipolar, aprotic solvent, and a weak base (see Willhite and Katz [1984] for an extensive review of DMSO; Figure 2.3). DMSO is also very miscible with water which may account for its ability to permeate protein-based and other biological structures (Rammler and Zaffaroni, 1967) and to penetrate eggs of the medaka (Arii *et al.*, 1987).

The goal of a toxicant carrier is to provide the required physical and/or chemical properties to deliver a test substance to a test organism, but without induction of any extraneous variables, such as structural alteration of the test substance or acute or sublethal effects. As discussed previously in this Chapter, several studies have been conducted where the primary carrier solvent was DMSO and the effects (due to the solvent itself) on developing salmonid and medaka embryos were minimal (Black *et al.*, 1985; Maccubbin and Black, 1986; Maccubbin *et al.*, 1987; Grizzle and Putnam, 1987; Black *et al.*, 1988; Metcalfe *et al.*, 1988). Other investigators have found that in low concentrations, DMSO was relatively non-toxic to

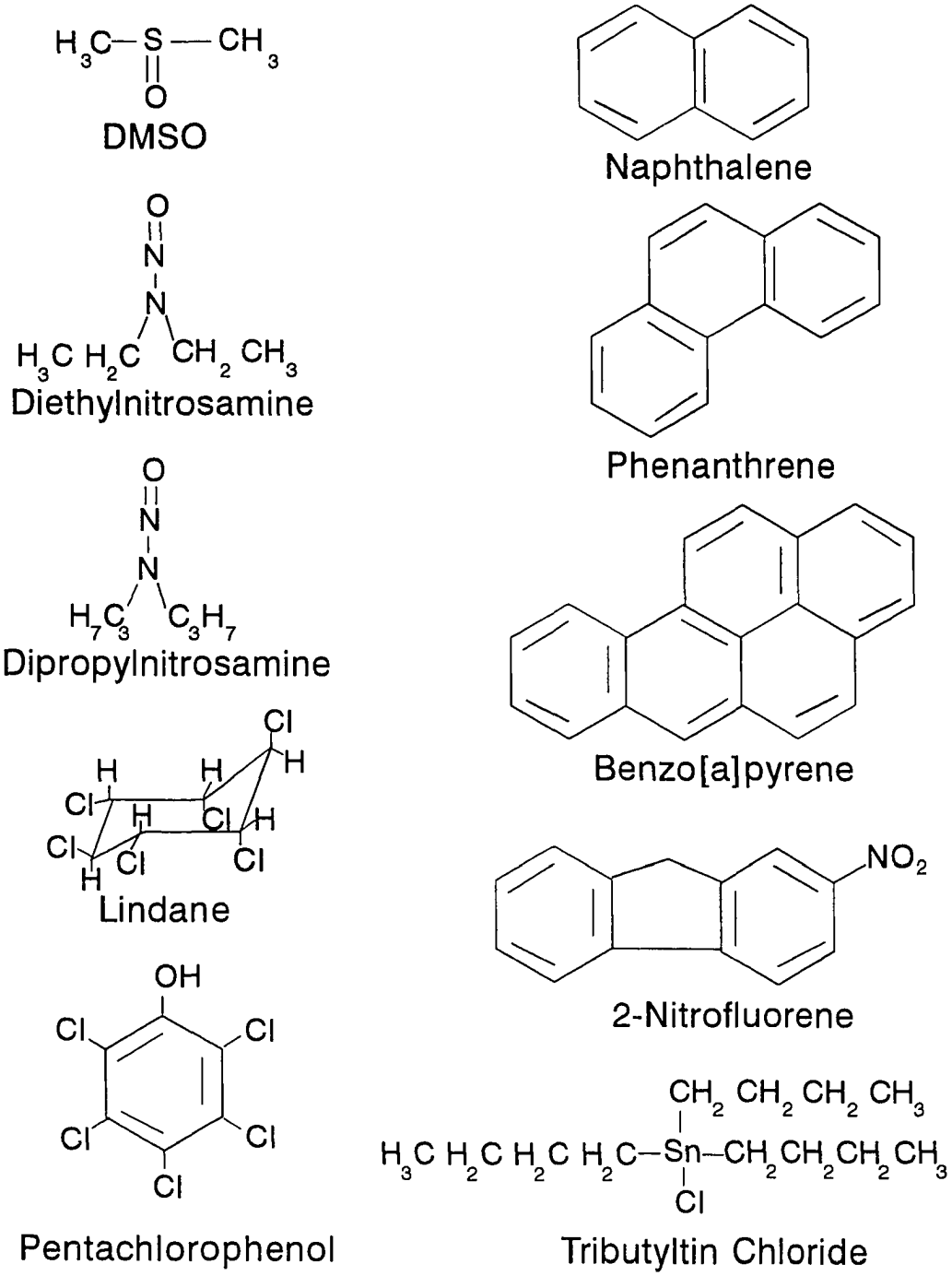



**Figure 2.3.** Chemical configurations of the carrier solvent (DMSO) and the nine test substances.

developing red drum eggs (toxicity results were nearly equivalent to those observed for artificial seawater; Robertson *et al.*, 1988) and *Xenopus laevis* eggs (Anderson and Prahlad, 1976). Further, Landauer and Salam (1972) found that following injection of DMSO or water into the yolk sac of White Leghorn foul, embryo mortality was much lower for DMSO than water and that DMSO played no teratogenic role in these embryological tests. Benville *et al.* (1968) found that DMSO was relatively harmless to four species of salmonids through intraperitoneal injection, immersion and dietary exposures (safe levels were shown to be very high at approximately 2.8g per kg of body weight). Finally, Arii *et al.* (1987) found that greater than 90% of medaka embryos exposed to a 2M DMSO solution in Ringer at the gastrulation stage showed no effect of the treatment regardless of the length of exposure. Hence, it is apparent that DMSO can serve as an adequate carrier for test substances, and unlike other commonly used carriers such as methanol or acetone, DMSO has the ability to permeate biological membranes without damage to the structural integrity of these "barriers".

## Test Chemicals

The structures of the chemicals employed in this phase of the research are presented in Figure 2.3. There were nine test chemicals representing six chemical classes selected for evaluation in the pilot, range-finding portion of this study. Three polynuclear aromatic hydrocarbons (PNAHs) of differing ring number were included in the pilot study. A two-ringed representative, naphthalene, was included as a representative of the acutely toxic diaromatic PNAHs (Anderson, 1979). Phenanthrene, a three-ringed PNAH is also considered to be acutely toxic, but to a

lesser degree than the naphthalene class, therefore, reduced toxicity should be observed in this treatment. The final PNAH, benzo[a]pyrene (BaP), has a five-ringed structure and is the most studied of the PNAHs in biological assays of adverse effects. This compound requires activation (typically to more polar epoxides or hydroxides; Dipple *et al.*, 1984) by an enzymatic system, the mixed function oxidase system, to elicit its pernicious effects. However, many findings have indicated that BaP is strongly carcinogenic, but rarely acutely toxic to fish early life stages (see, for example, Maccubbin and Black, 1986 and Maccubbin *et al.*, 1987), therefore, minimal effects are anticipated during the short duration of this study.

Two nitrosamines, diethylnitrosamine (n-nitrosodiethylamine) and dipropylnitrosamine (n-nitroso-di-n-propylamine) were evaluated employing the medaka embryo-larval assay (MELA) technique. The former has received a significant level of attention with respect to effects on many life stages of several fish species including the medaka (Egami *et al.*, 1981; Kimura *et al.*, 1981; Hatanaka *et al.*, 1982; Klaunig *et al.*, 1984; Hinton *et al.*, 1988; and others) and is considered one of the most potent known carcinogens (Preussmann *et al.*, 1979). Like many PNAHs, nitrosamines are chemically stable compounds requiring metabolic activation (characteristically, hydroxylation) to elicit their adverse response, which is typically carcinogenic (Preussmann and Stewart, 1984).

A single chlorinated hydrocarbon, lindane (gamma-1,2,3,4,5,6-hexachlorobenzene) was studied employing the MELA protocol. This compound has previously been shown to be acutely toxic to a number of life stages of both vertebrates and invertebrates at concentrations similar to those used in this study

(accounting for the bioconcentrating ability of lindane; Macek *et al.*, 1976). Therefore, one would expect an acute response in the range of study concentrations. A nitro-polynuclear aromatic hydrocarbon, 2-nitrofluorene, was included as a compound which has previously demonstrated a strong mutagenic response in the Ames bacterial assay (Butler, 1985), however, this component has received limited attention with respect to effects on aquatic species, and therefore, the anticipated results on the eukaryotic medaka eggs are unknown.

The eighth test chemical, pentachlorophenol (PCP), has received considerable attention in tests with on various life stages of fish (Glickman *et al.*, 1977; Holcolmbe *et al.*, 1982; Trujillo *et al.*, 1982; Johansen *et al.*, 1987; Shigeoka *et al.*, 1988b; Tachikawa *et al.*, 1991; and others), however, the eggs of the medaka have not previously been employed as a test species for PCP. The previous studies of this chemical indicate that the effects on the medaka should be primarily acute, but may be present as more subtle, sublethal responses to the treatment. The final chemical of study was tributyltin chloride (TBTCl), an organotin commonly used in anti-foulant paints (Hall and Pinkney, 1985). This chemical (TBTX, where X, the alkyl or aryl group, may vary between studies) has recently received significant attention with respect to effects on target and non-target invertebrate species, however, few studies have focused on the effects of this substance on marine or freshwater vertebrates, particularly early life stages. Therefore, the effects of this toxicant were evaluated using the MELA protocol, to determine the acute and sublethal (mini-chronic; Birge and Black, 1985) effects on the medaka.

The chemical selection process attempted to cover several chemical classes, to evaluate a wide range of water solubilities and to evaluate chemicals with a diverse

scope of biological effects from acutely toxic to carcinogenic. Though carcinogenicity was not an endpoint of the study during these experiments, nitrosamines and benzo[a]pyrene were selected for evaluation as a comparison of short-term effects with previously conducted embryological assays, employing similar exposure techniques.

The primary purpose of this phase of the study was to evaluated a number of chemicals over a wide range of concentrations (Table 2.1) in order to select two for more extensive evaluations in the salinity-related response evaluations (Chapters 5 and 6). The major selection criterion was a dramatic and yet distinct effect(s) on the medaka eggs. A secondary purpose was to determine the effects of the nine toxicants on the eggs of the medaka. The test eggs were scored daily and the data were evaluated employing probit analysis to determine $LD_{50}$ (lethal dose to 50% of the test population) and $ED_{50}$ (effective dose to 50% of the test population) values for each chemical.

## MATERIALS AND METHODS

### Maintenance of Breeding Cultures and Eggs

Adult medaka were obtained from Carolina Biological Supply Company (Burlington, N.C.) and were subsequently sexed and maintained in ten gallon aquaria. Due to their polygamous nature, the medaka adults were maintained at six females and four males per aquarium (this 3:2 ratio has been previously demonstrated to be ideal for optimum egg production - Kirchen and West, 1976; Klaunig *et al.*, 1984; Weber and Spieler, 1987). In addition, this polygamous property allows for rapid gene frequency changes reducing the variations of susceptibility between eggs from

**Table 2.1.** Test chemicals, purities and standard concentrations (in DMSO).

| Test Chemical | Purity (%) | Test Concentrations ($\mu$g/egg) |
|---|---|---|
| Naphthalene | >99 | 0.0025, 0.025, 0.250, 2.50, 25.0 |
| Phenanthrene | >98 | 0.0005, 0.005, 0.050, 0.500, 5.00, 33.3 |
| Benzo[a]pyrene | 98 | 0.0025, 0.025, 0.250, 2.50, 10.0 |
| Diethylnitrosamine | >98 | 0.100, 1.00, 10.0, 50.0 |
| Dipropylnitrosamine | >99 | 0.005, 0.050, 0.500, 5.00, 50.0 |
| 2-Nitrofluorene | 98 | 0.005, 0.050, 0.500, 2.50, 5.00 |
| Lindane | 97 | 0.005, 0.050, 0.500, 5.00, 50.0 |
| Pentachlorophenol | >99 | 0.012, 0.125, 1.25, 12.5 |
| Tributyltin Chloride | 96 | 0.0000187, 0.0000375, 0.000075, 0.00015, 0.0003, 0.0006, 0.0012, 0.012, 0.120, 1.20, 12.0 |

differing adult pairs (Weis *et al.*, 1982). Breeding adults were maintained in conditioned (aerated for at least 24 hours prior to use) synthetic freshwater of pH 7.5 to 7.9 and medium hardness (120$\pm$3 mg/l as $CaCO_3$). This water was prepared by deionizing city tap water to remove contaminants and then reconstituted with the salts (Table 2.2). The breeding sets were maintained at 24 to 28°C and artificial light was provided by fluorescent bulbs with a photoperiod of 16 hours light/8 hours dark to mimic their natural breeding environment. These conditions have been demonstrated to increase egg laying behavior, without increasing antagonistic behavior (Weber and Spieler, 1987). Each aquarium contained an undergravel bottom filter unit with two aerated stand pipes to generate circulation through the gravel containing microbial filtering activity.

Two groups of five breeding sets (one set = a ten adult assembly) were

**Table 2.2.** Recipe for reconstituted, synthetic freshwater. Salt weights are for ten gallons of solution.

| Salt | Weight (g) |
|---|---|
| $NaHCO_3$ | 1.82 |
| $CaSO_4 \cdot 2H_2O$ | 1.14 |
| $MgSO_4$ | 1.14 |
| KCl | 0.08 |

simultaneously maintained. One group was reared under the simulated breeding conditions while the other was exposed to a 12 hour light/12 hour dark cycle and reduced temperatures ($\approx$ 20°C) to allow these breeders to "rest" from the stress of daily reproduction. These sexually inactive sets were maintained in a black poly-chamber and exposed to a separate lighting system from the actively breeding sets. All adults were fed twice daily; the first feeding was coincident with the onset of light and the second feeding took place in the early evening. The adult diet included freshly hatched brine shrimp (*Artemia salina*; $\approx$3ml concentrated shrimp per aquarium per feeding event) supplemented with Tetra-Min artificial flake food ($\approx$0.3g per aquarium per feeding event), depending on availability of the shrimp.

Eggs were stripped from each female by removing the female from the tank with a small dip net followed by the gentle removal of the egg cluster while she remained in the net. Slight finger pressure to the cluster resulted in a spontaneous response in the female to "instinctively" remove herself from the egg mass. The female was then returned to the tank. The procedure was repeated for all productive females in the five breeding sets. This approach to stripping seemed to be less stressful on the female and more efficient than previously described techniques where

the female was removed from the net and placed in an egging bowl or where the eggs were physically stripped with forceps or a pipet (Kirchen and West, 1976).

The eggs were placed in plastic petri dishes (90mm x 15mm) containing 20ml of embryo rearing solution (Table 2.3) as described in Kirchen and West (1976) at no more than 40 eggs per dish. Methylene blue was included in this solution to reduce the growth of bacteria and molds and to stain dead eggs blue for easy identification. Each egg was separated from the neighboring cluster (to ensure uniform development) by gently teasing at the long adjoining filaments with stainless steel watchmakers forceps until the filaments detached. Each egg was evaluated under a dissecting scope to ensure successful fertilization. Unfertilized eggs, those lacking a raised vitelline membrane (i.e., appearance of the perivitelline space; see Figure 2.2a), were discarded and the remaining fertilized eggs were placed in fresh rearing solution at a density of $\leq$ 20 eggs per dish. The dishes were placed in an air-tight incubator maintained at 25 $\pm$ 1°C. Eggs were evaluated daily for mortalities and latent development prior to use in the chemical assays. Any eggs found to be dead (as indicated by their blue color), or underdeveloped (based on microscopic examination), were removed to avoid possible fungal or bacterial contamination of the remaining eggs in a dish. Egg envelopes of embryos carried to term for use in future breeding sets were also removed daily, as these egg cases provide an optimal surface for rapid bacterial growth. Fry hatching from eggs to be used in future breeding sets were placed in conditioned ten gallon tanks with reduced flow from the aerated standpipes. From the time of transfer, the diet of endogenously feeding fry was supplemented with freshly hatched *Artemia*. Exogenous feeding animals were fed a diet of *Artemia* and finely ground flake food. Following a three to four month period, the juveniles

**Table 2.3.** Recipe for embryo rearing solution (modified from Kirchen and West, 1976). Solution is brought to 500ml volumetrically with reagent grade bottled water.

| Constituent | Percentage Solution (Wt./Vol.) | Volume (ml) per 500ml |
|---|---|---|
| NaCl | 12.500 | 5 |
| $MgSO_4 \cdot 7H_2O$ | 2.040 | 5 |
| KCl | 0.375 | 5 |
| $CaCl_2$ | 0.500 | 5 |
| Methylene Blue | 0.05 | 1 |

were sexed and separated into six female:four male breeding sets. As new tanks were developed for additional breeders, a portion of well conditioned water from other breeding sets was included to induce rapid biological and algal activity in order to maintain a well conditioned environment for the breeding adults.

## Preparation of Toxicant Standards

Table 2.1 contains the nine toxicants employed in the pilot phase of this study and their respective standard concentrations. The standards were developed to cover a wide range of concentrations which should hypothetically cover an equally wide range of potential effects (i.e., range-finding studies) and which should encompass potential natural environmental concentrations. One test substance, tributyltin chloride, had several more concentrations than the other chemicals, as a no observable effects concentration (NOEC) was not obtained with the initial range. Several serially-diluted concentrations were added in an attempt to achieve a NOEC.

The test chemicals were obtained in neat form from several commercial suppliers (Aldrich - tributyltin chloride, 2-nitrofluorene, benzo[a]pyrene, lindane;

Supelco - dipropylnitrosamine, pentachlorophenol; ChemServe - diethylnitrosamine; U.S.EPA Standards Division - naphthalene, phenanthrene); purities are listed in Table 2.1. As "pure" pentachlorophenol (PCP) has previously been shown to contain several toxic contaminants (Cleveland *et al.*, 1982; Hamilton *et al.*, 1986), even in high purity standards, the 99% neat material obtained from a commercial supplier was evaluated for the presence of several of these substances. Approximately 1g of pentachlorophenol was evaluated by gas chromatography/mass spectrometry (GC/MS) for the presence of chlorinated dibenzodioxin, dibenzofuran and phenoxyphenol impurities. These impurities, when present, have been suggested to be responsible for a significant amount of the PCP toxicity (Hamilton *et al.*, 1986). No impurities were detected in this evaluation at an approximate detection limit of 500ng/g PCP, therefore, this neat material was considered pure and acceptable for use in the tests of PCP toxicity and teratogenicity.

DMSO was obtained as Burdick and Jackson high-purity, distilled in glass. All standards were stored in amber containers at 4 $\pm$ 2°C in the dark to reduce any natural degradation. DMSO was stored in the dark at room temperature ($\approx$20°C). The neat chemicals were weighed on a Sartorius 2434 balance to 0.00001g and diluted volumetrically with the DMSO. Each standard was placed in a Branson 12 ultra-sonic water bath for one minute to ensure homogeneity of the mixture. Successively lower standard concentrations were developed through serial dilution from the stock standards with DMSO diluent. All standard preparation took place under reduced light and under a negative pressure hood. All standards were stored at 4 $\pm$ 2°C in the dark until needed for the topical treatment experiments.

## Medaka Embryo-Larval Assay (MELA) Topical Treatment Method

Forty-eight hour old medaka eggs (see Figure 2.2f) were used in the topical treatment procedure of the medaka embryo-larval assay (MELA). In the majority of the test eggs, this was the stage at which the early heart was being formed and began contracting, the tail bud was formed, the optic lobes were forming and the oil globules had coalesced into a single drop. The exposure of eggs after the cleavage stages were completed was employed to eliminate any naturally occurring, spontaneous abnormal eggs or non-viable eggs from the experimental and control groups.

Ten eggs were used as a single replicate for each toxicant in this range-finding study. For a given standard concentration, ten eggs were randomly selected from all rearing dishes for a given date of collection and placed on the back of a glass crystallizing dish cover (approximately 10cm in diameter). Each egg was subsequently blotted dry of rearing solution with a piece of glass fiber filter paper. Each egg then topically received a 0.1$\mu$l droplet of the DMSO/chemical mixture dispensed using a Hamilton push-button repeating dispenser (Model No. PB-600-1) equipped with a Hamilton 5$\mu$l syringe (900 Series, Model #95) and a 10cm x 0.17mm O.D. fused silica gas chromatography on-column injection needle and a 0.17mm bore teflon ferrule (Supelco). This ultra-fine gauge needle produced a defined droplet at its end with volumes less than 0.1$\mu$l of solution. The repeating dispenser was calibrated using naphthalene as a standard and gas chromatography/flame ionization detection (GC/FID). A known number of repeated dispenses were made into a 1ml aliquot of methylene chloride and the solution was subsequently analyzed by GC/FID. Five such replicates were evaluated and the amount dispensed ($\pm$ standard deviation) was

determined to be 0.10 $\pm$ 0.01$\mu$l. Metcalfe *et al.* (1988) used a comparable system equipped with a 25$\mu$l syringe and a 31 gauge needle in their salmonid microinjection study and obtained similar results.

After receiving the dose, each egg was allowed to stand for 1min to permit the dose to penetrate the chorion. At 1min each egg was washed with rearing solution into a 100ml beaker containing clean rearing solution. Once all eggs of a given replicate were transferred to this beaker, they were washed of any external residual test material through vigorous swirling of the solution for 0.5 min. The eggs were then transferred to a new Petri dish containing 20ml of fresh rearing solution and placed back into the air-tight and light-free culture incubator. The rearing solution was not changed for the duration of the assay.

Each egg was evaluated daily under a low power dissecting scope for developmental abnormalities, mortalities and hatching. Any such occurrences were recorded in a logbook. Eggs were considered viable if development progressed normally and fry were considered normal when free of their chorions, morphologically normal as compared to controls, and able to maintain postural equilibrium and swim successfully. Otherwise they were recorded as deformed if the heart was still beating and, if not, they were recorded as dead. Dead eggs, dead fry and egg envelopes of hatched fry were removed daily to avoid bacterial and fungal contamination of the remaining eggs in a given treatment.

The experiment was terminated at 21 days post-treatment with the dose or when all eggs and/or fry of a given treatment were dead. Fry which hatched during the course of the experiment were transferred to a 90mm x 50mm crystallizing dish containing 150ml of the conditioned synthetic freshwater used in the breeding aquaria.

The fry dishes were lightly aerated with clean breathing-quality air. No feeding was necessary during the "swim-up" period as endogenous feeding of the yolk material typically takes place for five to seven days following hatching (Takimoto *et al.*, 1984a). Any surviving deformed hatch fry were preserved in 10% buffered formalin for subsequent photographic records.

DMSO controls served as negative controls for all experiments and were evaluated in conjunction with the test chemicals. Each egg was treated in the same manner as the test eggs except that DMSO alone was used. Untreated controls were also evaluated in conjunction with all tests. These eggs went through an identical procedure (i.e., drying, static for 1min, and washing), however, they were not treated with any DMSO or DMSO/chemical mixture. If more than ten percent of the eggs for any given control died, the test chemical experiments evaluated in conjunction with these controls were terminated and repeated with new controls. The control treatments were randomly distributed throughout the rearing incubator.

## Statistical Methods

The data produced by these range-finding studies were interpreted for egg and fry viability as well as incidence levels of developmental anomalies and hatching success. In addition, the data were evaluated using probit analysis (SAS Institute, Inc., Cary, N.C.) of acute effects, sublethal effects and all adverse effects. This analysis was used to determine the $LD_{50}$ (lethal dose to 50% of the test population) and $ED_{50}$ (effective dose to 50% of the test population) for each of the nine compounds. When plotted on an arithmetic scale, cumulative percentage data tends to produce a sigmoid curve. Plotting the data on a probit scale linearizes the cumulative

percentage affected data for statistical analyses (e.g., $ED_{50}$ and $LD_{50}$ determinations). These values were utilized to estimate an approximate threshold concentration for both lethal and all adverse effects and as a basis for developing a geometric concentration series (around these lethal and effective concentrations) for the two chemicals selected for more extensive study (see Chapters 5 and 6). One of the limitations of probit analysis is the requirement for at least two observations which are not 0 or 100% affected. Therefore, it was not possible to calculate $ED_{50}$ and $LD_{50}$ values for all chemicals. Finally, the pre-established control criteria of $\leq 10\%$ of the group adversely affected eliminated the need for tests of statistical significance relative to the controls.

## RESULTS

For all of the substances discussed in this phase of the research, four general effective endpoints were evaluated. Dead eggs were those that died during the embryonic development phase. Deformities were those embryos which exhibited at least a partial hatch, but resulted in an abnormally developed medaka fry, or an unsuccessful hatching event due to some abnormality (e.g., swollen abdomen). Unhatched eggs were those eggs that did not successfully hatch within the prescribed 21-day period and are considered a sublethal effect of the treatment. Finally, dead fry were those fry which successfully hatched, but death occurred before the termination of the test. The results discussed below for each individual compound are based on a cumulative index of these endpoints at day 21 post-exposure. Growth was not used as an index for effect of the test substances as previous investigations, with similar test chemicals, revealed that growth was a comparably insensitive

indicator of teratogenesis (Ward and Parrish, 1980; Shigeoka *et al.*, 1988a).

DMSO and untreated controls were evaluated in conjunction with these chemicals. The mean response for two replicates of these control types is presented with the data for each toxicant. In general, both control types averaged 0.5 unhatched eggs per ten egg group, but exhibited no mortalities or developmental anomalies. Chi-squared analysis of a 2x2 contingency table indicated that the two control types were not significantly different from each other.

The only chemical which provided data capable of probit determination of an $LD_{50}$ was TBTCl, primarily due to the low level of acute mortality observed for the remainder of the test substances. PCP elicited extensive acute toxicity, however, the "all-or-nothing" results prohibited the effective determination of a $LD_{50}$. $ED_{50}$ values were calculated for most chemicals, however, the sporadic, and often infrequent, effective data precluded the ability to establish confidence levels for many of these substances. Little confidence can be placed on these $ED_{50}$ values without 95% fiducial limits, therefore, only $ED_{50}$ values for those data producing confidence limits are reported.

**Naphthalene**

The results for naphthalene are probably the most surprising of all chemicals evaluated. Though this chemical has previously been shown to be quite toxic to marine organisms (Anderson, 1979), the effects on medaka were less pronounced. The results indicate that naphthalene does not appear to induce a strong toxic or teratogenic, concentration-dependent response in medaka eggs to a maximum dose of 25$\mu$g/egg (Figure 2.4). The most significant effect appeared to be delayed hatching,

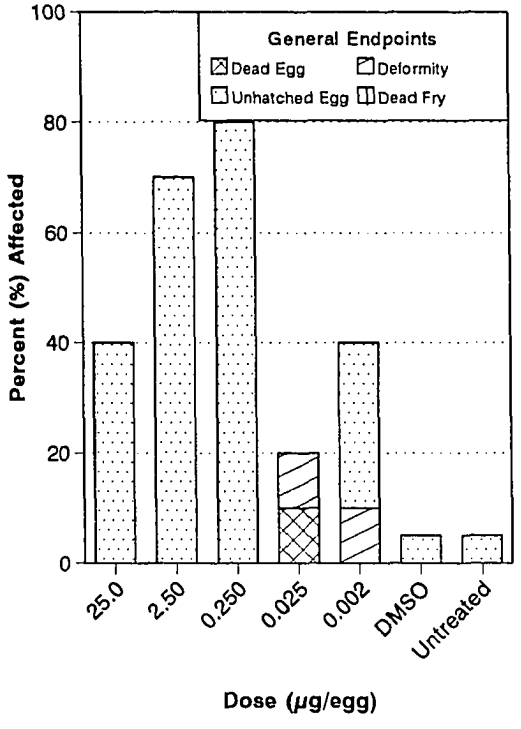



**Figure 2.4.** Effects of naphthalene on the eggs of the medaka.

however, this response did not appear to be concentration dependent. Further, the only deformities and mortalities encountered were present in the lower naphthalene concentrations (0.002-0.025$\mu$g/egg). This may be the result of a reduction in the delayed hatch effect seen in the higher concentrations. The deformities were fry which hatched with enlarged abdomens/yolk sacs and, therefore, were unable to completely free themselves of the restrictive chorion. The inability to complete the hatching process may have been due to 1) a chemical-induced effect on the ability of the developing embryo to osmoregulate, resulting in an enlarged abdominal cavity, 2) an alteration of the mechanism responsible for induction of the movement to, or hatching enzyme required for, rupture of the chorion, 3) an alteration of the chorion structure, reducing the ability of the embryo to successfully complete the hatching process, or a combination of these effects. Moreover, investigators have previously

demonstrated that aromatic hydrocarbons (particularly the 1 and 2 ringed members) can induce changes in the surface organization of membranes which may interfere with the processes of neurotransmission, muscle contraction and osmoregulation (Neff, 1979).

**Phenanthrene**

Similar to naphthalene, this three-ringed PNAH did not produce a defined concentration dependent response (Figure 2.5). The initial concentration series, 0.0005-5.00$\mu$g/egg produced such sporadic results that a sixth concentration, 33.3$\mu$g/egg, was added in an attempt to induce a more significant response. Though no mortalities occurred in this high concentration, none of the test eggs hatched within the experimental period. However, a similar response was produced in the lowest concentration (0.0005$\mu$g/egg), where 80% of the eggs did not hatch. The intermediate concentrations had variable responses of deformities, mortalities and delayed hatching ranging from no adverse response (0.050$\mu$g/egg) to a combination of dead fry, deformed fry and delayed hatches in the 0.005$\mu$g/egg treatment. The deformed fry in the 0.005 and 0.500$\mu$g/egg doses were identical to those observed for naphthalene, with distended abdominal regions, preventing successful, complete hatching from the egg envelope.

**Benzo[a]pyrene**

Like phenanthrene, benzo[a]pyrene (BaP) produced a significant response in the highest (10.0$\mu$g/egg) and lowest (0.002$\mu$g/egg) treatment concentrations (Figure 2.6). However, this response was restricted to unhatched eggs. No mortalities or

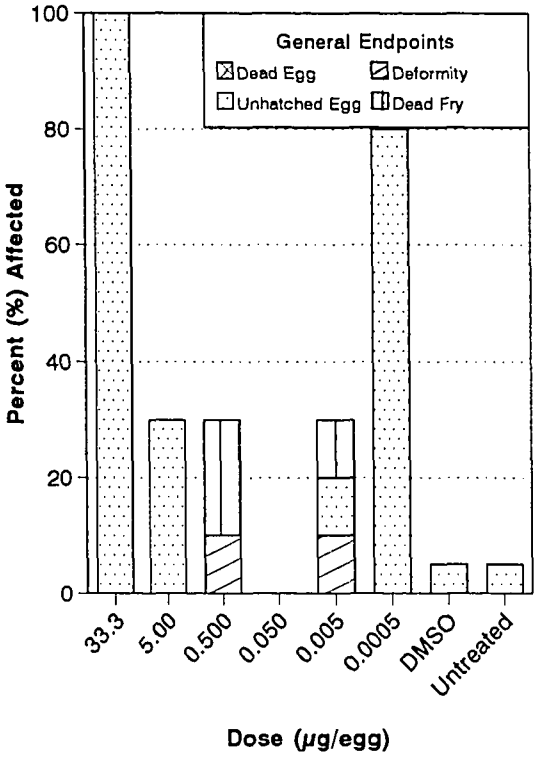



**Figure 2.5.** Effects of phenanthrene on the eggs of the medaka.

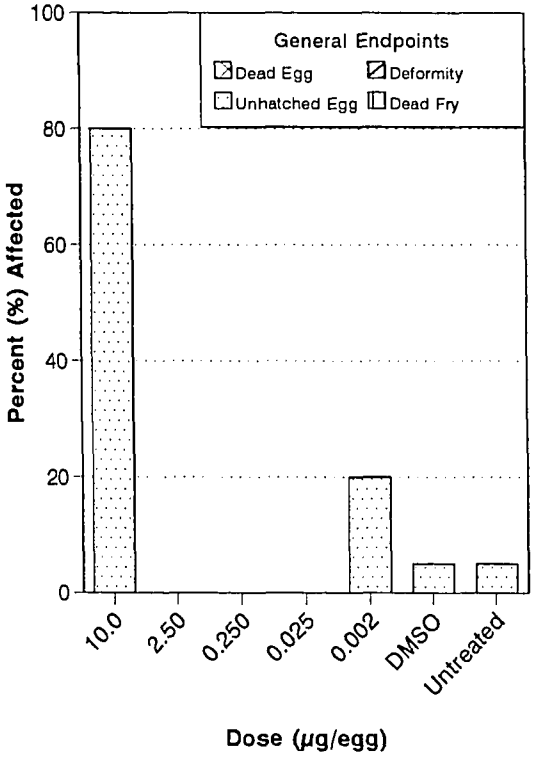



**Figure 2.6.** Effects of benzo[a]pyrene on two day old eggs of the medaka.

deformed fry were observed. BaP has been shown to be carcinogenic in fish treated at the embryo stage (see review by Black, 1988). However, it has been established that this carcinogen produces little acute or abnormal response in the early embryonic stages and that in order to produce a carcinogenic response, BaP must be activated by the mixed function oxidase system (i.e., BaP is a procarcinogen). The liver has been shown to be the primary organ responsible for this metabolic activity (see, for example, Ahokas *et al.*, 1977). To determine whether the presence of the liver during treatment of medaka eggs would enhance the short-term toxic response, ten day old eggs were treated with several concentrations of BaP. A response was detected in all concentrations including the presence of deformed fry, identical to those observed for naphthalene and phenanthrene, however, the response was still minimal (Figure 2.7). This confirms the results of the previous studies employing similar exposure techniques with BaP-treated fish eggs, where this compound did not produce a significant embryotoxic or teratogenic response (Maccubbin and Black, 1986; Maccubbin *et al.*, 1987).

**Diethylnitrosamine**

Diethylnitrosamine (DENA), a water soluble carcinogen requiring activation to induce a response, produced an effect in the medaka eggs similar to those seen for the three PNAHs (Figure 2.8). An increase in mortality in the fry stage was observed, indicating that this chemical may be sequestered in the yolk material in a form that may be moderately toxic to the medaka fry upon endogenous feeding. However, this response was not observed in the highest concentration (50.0$\mu$g/egg), but rather in the two intermediate concentrations (0.100 and 10.0$\mu$g/egg), indicating a non-dose-dependent response. A single fry with spinal curvature, dorsal lordosis, was observed

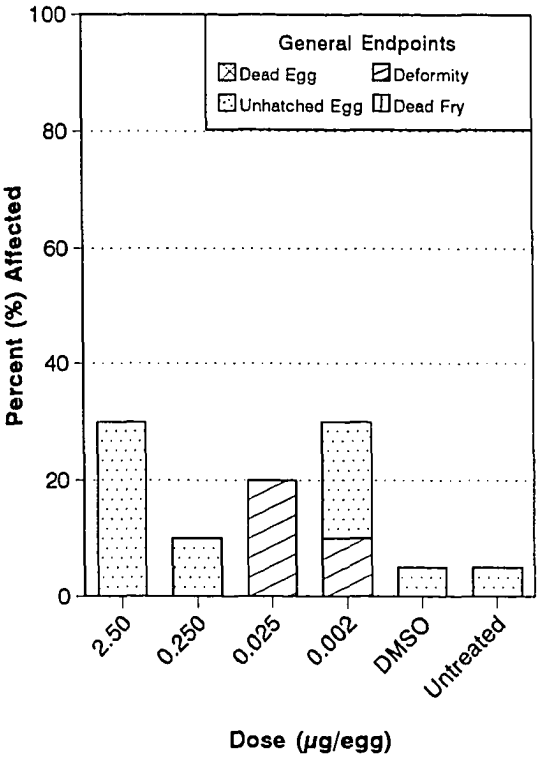



**Figure 2.7.** Effects of benzo[a]pyrene on ten day old eggs of the medaka.

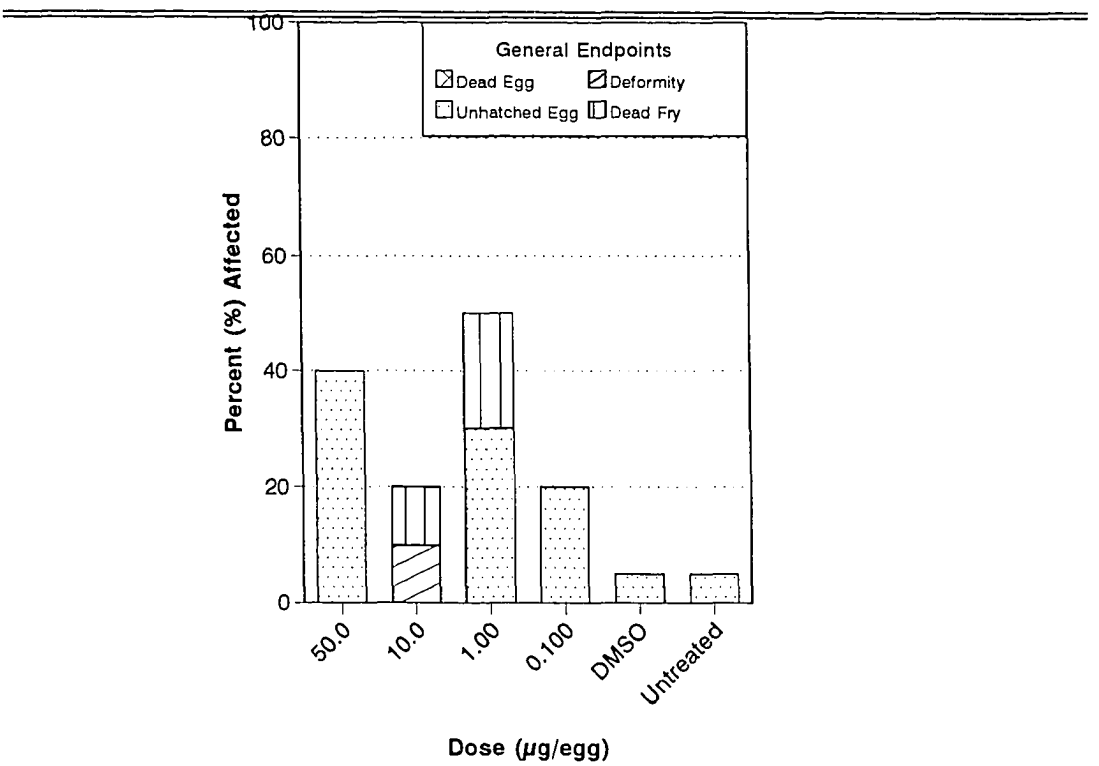



**Figure 2.8.** Effects of diethylnitrosamine on the eggs of the medaka.

in the 10.0$\mu$g/egg concentration group.

**Dipropylnitrosamine**

Dipropylnitrosamine (DPrNA) did not produce any acute response in medaka eggs (Figure 2.9). The sole response, unhatched eggs, did appear to be concentration dependent and significantly greater than the control treatments. As with BaP, DPrNA was evaluated with ten day old eggs. The response (Figure 2.10) was very similar to that observed in the treatment of the two day old eggs, with unhatched eggs being the only adverse response and the general trend of decreased effect with lower concentration. These results indicate that DPrNA is not acutely toxic to the eggs of the medaka, but that it significantly affects their ability to hatch, whether the treatment is on early or late embryonic stages.

**Lindane**

Lindane is known to be chronically toxic to invertebrates and fishes (Macek *et al.*, 1976). The present study indicated that lindane causes sublethal effects to the eggs of the medaka and that deformities and delays in hatching were generally concentration dependent (Figure 2.11). Deformities were primarily elongation of the heart and associated poor circulation, often resulting in blood islands scattered throughout the surface of the yolk sphere. The heart rate was significantly depressed as compared to that of controls of identical age, apparently resulting from "tube-heart" syndrome previously described by Weis and Weis (1979). The second most common deformity in the higher concentrations was the inability of seemingly normal fry to successfully swim, even when provoked. The appearance of a well formed swim bladder in these fry suggests that lindane may have an effect on the motor

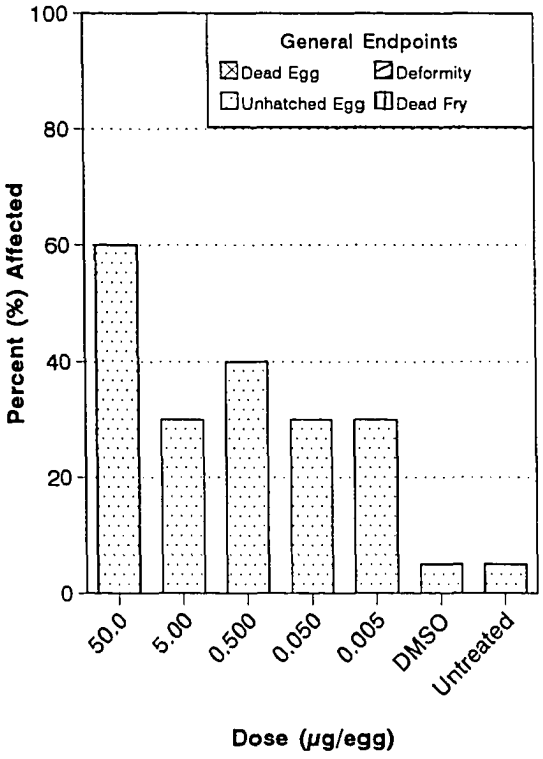



**Figure 2.9.** Effects of dipropylnitrosamine on two day old eggs of the medaka.

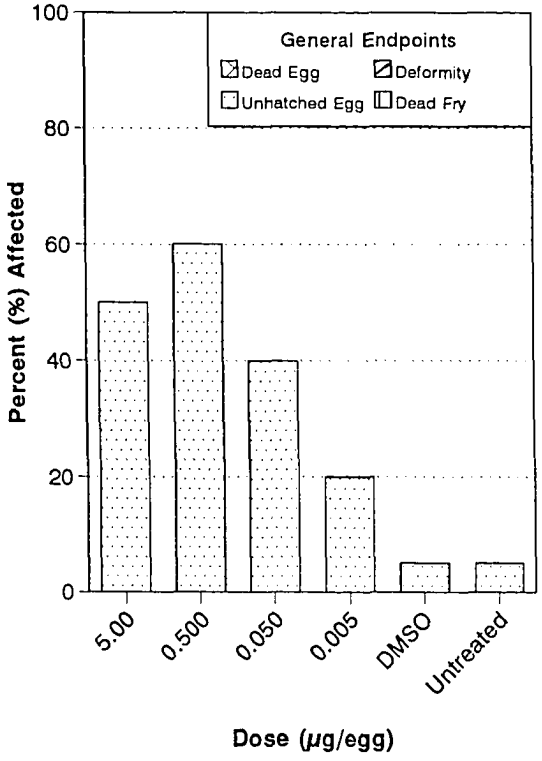



**Figure 2.10.** Effects of dipropylnitrosamine on ten day old eggs of the medaka.

response system responsible for the actual swimming activity. Swollen abdominal regions and the inability to successfully hatch from the egg chorion dominated the deformed hatches in the lower concentrations.

**2-Nitrofluorene**

At the highest test concentration (5.00$\mu$g/egg), 2-nitrofluorene produced a lethal response in 30% of the test eggs, however no other mortalities were observed for any of the lower concentrations (Figure 2.12). In spite of this lack of mortality in the lower concentrations, delays in hatching seemed to increase relative to the highest concentration, however, the pattern did not suggest a concentration dependent response.

Because of the lack of information relative to 2-nitrofluorene's effects on fish early life stages, ten day old eggs were treated with similar doses to determine if the presence of a primary metabolic system would increase (or decrease) the response. This treatment resulted in a decreased response to this substance, with the mortalities in the 5.00$\mu$g/egg concentration being partially replaced with a single deformity and a reduction in the level of unhatched eggs in the lower concentrations (though still high - up to 60% in the 0.005$\mu$g/egg treatment; Figure 2.13). Though inverse of typical dose-response data, these lower concentrations displayed a more defined pattern of response relative to concentration, with a steady increase in unhatched eggs with a decrease in concentration.

**Pentachlorophenol**

Pentachlorophenol (PCP) showed a strong concentration-dependent response in

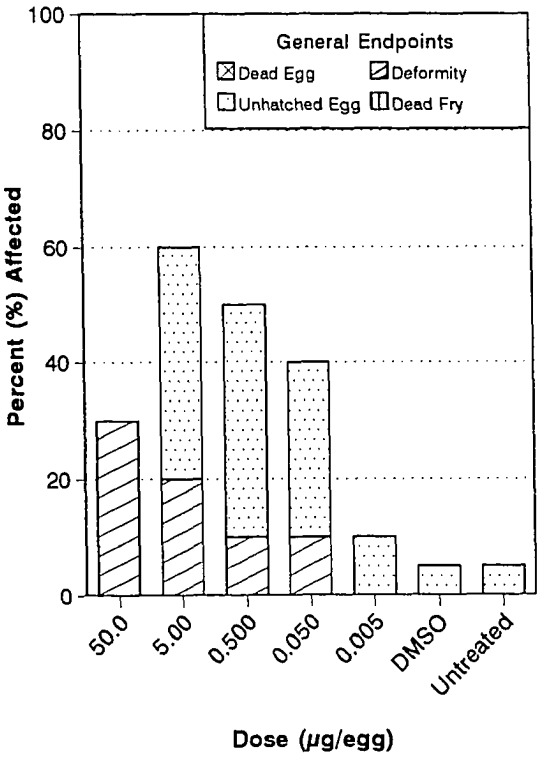



**Figure 2.11.** Effects of lindane on the eggs of the medaka.

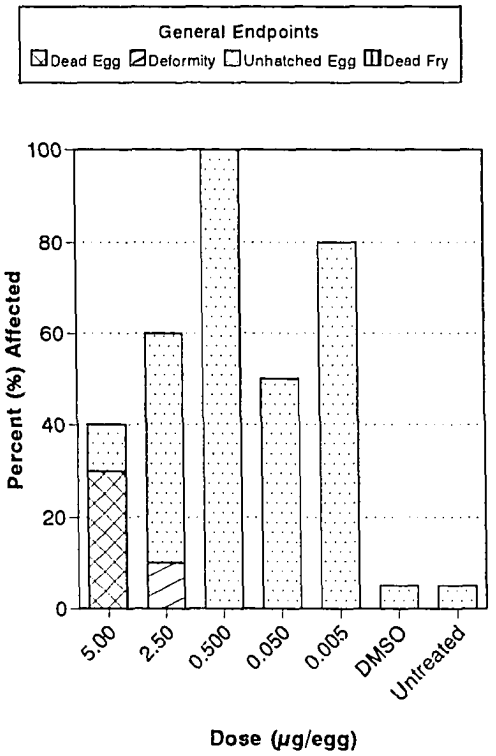



**Figure 2.12.** Effects of 2-nitrofluorene on two day old eggs of the medaka.

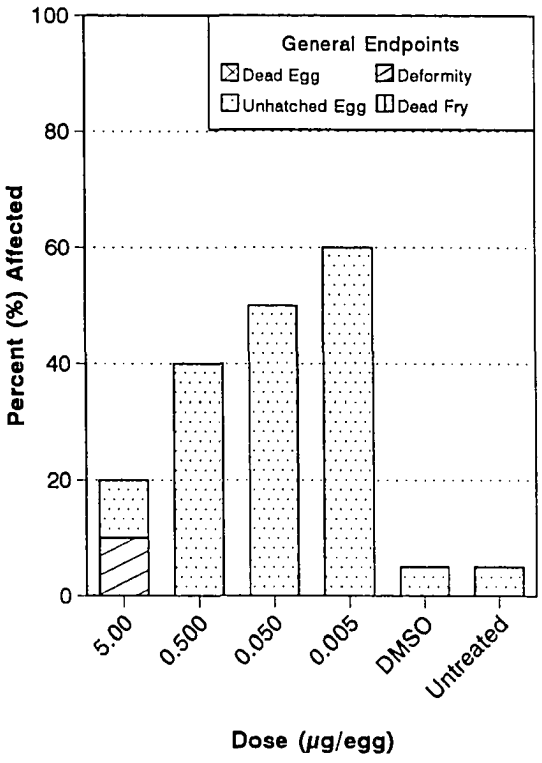



**Figure 2.13.** Effects of 2-nitrofluorene on ten day old eggs of the medaka.

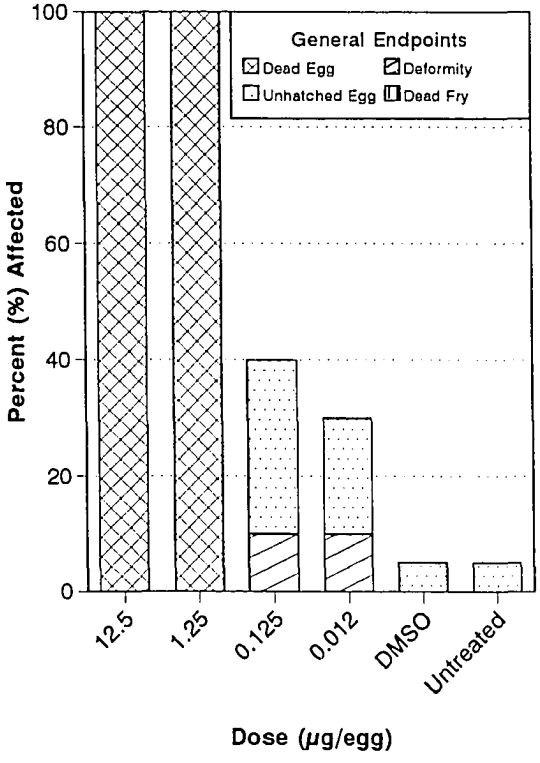



**Figure 2.14.** Effects of pentachlorophenol on the eggs of the medaka.

topically treated medaka eggs (Figure 2.14). Complete egg mortality was observed for the two higher concentrations (12.5 and 1.25$\mu$g/egg) while more subtle sublethal effects were observed in the lower two concentrations (0.125-0.012$\mu$g/egg). The deformities observed were primarily associated with swollen abdomens, preventing successful hatching or swimming activity. Further, several of the unhatched eggs in these lower concentrations were seen to have significantly elevated heart rates relative to the control eggs. Probit analysis of these data revealed a 21-day $ED_{50}$ value of 0.056$\mu$g/egg, with 95% confidence limits of 0.012 and 0.172$\mu$g/egg. A $LD_{50}$ could not be calculated for PCP, as the necessary criteria of two responses that are not 0 or 100% for lethality were not present. Due to the profound, defined effective response (primarily acute) to PCP in the medaka eggs, this toxicant was chosen for further evaluation in the higher salinity and immersion comparison studies (Chapter 5).

**Tributyltin Chloride**

Tributyltin chloride (TBTCl) displayed the most dramatic response in this study (Figure 2.15). Though little research employing TBT has focused on the embryonic stages of aquatic vertebrates, it is apparent that this chemical has a significant lethal and sublethal effect on the eggs of this species. The response is very concentration dependent, with acute toxicity in the eggs dominating the higher concentrations and sublethal effects governing the lower TBTCl doses. The initial concentration series (0.0012-12.0$\mu$g/egg) resulted in 100% response in all concentrations. Therefore, a decreasing geometric series was appended below this logarithmic series in an attempt to obtain a no observable effects concentration (NOEC). The data indicate that even at a concentration as low as 18.7 picograms per

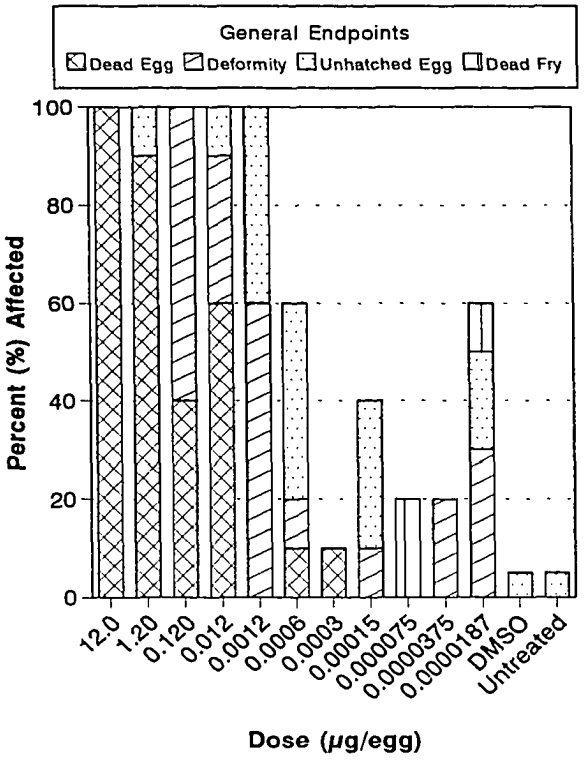



**Figure 2.15.** Effects of tributyltin chloride on the eggs of the medaka.

egg, there was still a significant effect and that the anomalies tend to increase at the lower concentrations. Deformities observed primarily included skeletal anomalies of varying magnitude in those embryos which did not hatch as well as those that did successfully hatch. Numerous blood islands were seen throughout many of the treated eggs and many embryos were observed to display severe and rapid tremors of the mouth and pectoral fins. Blood flow throughout the embryos was observed to be reduced in association with the static blood islands. A complete discussion and examples of these anomalies will be provided in the extensive evaluation of TBTCl discussed in Chapter 6.

Probit analysis indicated that the $LD_{50}$ for TBTCl was 0.047$\mu$g/egg, with 95% confidence limits of 0.017$\mu$g/egg and 0.163$\mu$g/egg. The $ED_{50}$ was determined to be 0.0002$\mu$g/egg, with lower and upper confidence limits of 0.000005 and 0.011$\mu$g/egg, respectively.

## DISCUSSION

Previous investigations have indicated that adults and eggs of *Oryzias latipes* can provide a model system for screening the carcinogenicity of chemicals (Llewellyn *et al.*, 1977; Egami *et al.*, 1981; Klaunig *et al.*, 1984; Maccubbin *et al.*, 1987; Hinton *et al.*, 1988; Hawkins *et al.*, 1990; and others), however, development of a similar model system, through establishment of a standardized assay for teratogenicity and embryo-larval toxicity, has received limited attention employing this species. A short-term, mini-chronic assay has been developed here to evaluate the embryotoxic and teratogenic effects of individual chemicals (chemical mixtures will be addressed in Chapter 7 using a similar procedure). The procedure allows for the administration of low levels of known or potentially toxic/teratogenic substances and provides a solitary investigator the ability to assay hundreds of eggs in a single hour, followed by limited rearing maintenance. The viability of negative controls (95%) relative to untreated controls (95%) indicates that the DMSO carrier solvent elicits no adverse effects on medaka eggs with respect to morphological endpoints responsible for embryo or fry lethality, abnormal development and retarded hatch rate.

This investigation suggests that perhaps the most sensitive test endpoint with respect to the majority of the chemicals evaluated was the effect on hatching. This may be due to overall retarded embryo development, reduced embryo activity resulting in the inability to successfully rupture the chorion, or a reduction of chorionase (or enzymatic cofactors for chorionase) activity necessary for dissolution of the chorion simultaneous with the increased activity during a hatching event (Chulakasem *et al.*, 1989).

Twenty-one day $ED_{50}$ values could only be obtained for TBTCl and PCP with

95% confidence limits and TBTCl was the only chemical producing data compatible with probit analysis requirements to generate a 21-day $LD_{50}$ with limits. Lindane also showed a strong dose-dependent response, where deformed fry dominated the highest concentration and dropped off with lower doses as did the number of unhatched eggs. Visual inspection of the data for many of the test substances indicated that those $ED_{50}$ and $LD_{50}$ values generated with no fiducial limits or with a single upper or lower limit were insignificant, as the data possessed no particular dose-dependent trend with respect to specific endpoints or total effects. However, it did appear that all nine test substances had significant effects on the medaka eggs. The least deleterious chemical appeared to be BaP, which has previously been shown to have little acute effect on eggs of the medaka and rainbow trout employing a similar exposure technique (Maccubbin and Black, 1986; Maccubbin *et al.*, 1987).

Exposure of later embryonic stages has previously been demonstrated to increase sensitivity to a number of chemicals, presumably due to the formation and increased volume of the liver (Hendricks, 1981; Wisk and Cooper, 1990). Therefore, two chemicals known to require liver-associated metabolic activation (BaP and DPrNA) were evaluated on eggs containing fully formed livers. Though the type of effects did not significantly change, the level of adversity seemed to increase in the later stage exposures. 2-Nitrofluorene was also evaluated at a later stage of development, however, unlike the two carcinogens, the adverse effect appeared to decrease in the later exposed stages, indicating that the liver may have the ability to detoxify this substance.

One other significant finding from the pilot, range-finding study was the overall delayed hatching rates for all treated and untreated eggs as compared to

historical assays with the medaka. The average rate in control eggs tended to be approximately 16-18 days post-treatment (or 18-20 day old eggs) as compared to the rates from previous investigations which tended to be between 10 and 15 days. It is speculated that this may be due to the type of culture vessel used in this study (i.e., Petri dish) and/or the absence of aeration and solution changes. This issue is evaluated in a subsequent chapter of this dissertation (Chapter 4) by studies designed to improve mean hatch time, and to allow for complete absorption of the yolk material of hatched test animals prior to termination of the test.

Another missing component from this assay was the determination of the absolute amount of test material actually crossing the chorion to the developing embryo. The knowledge of the "permeability factor" of the test substance(s) may have a significant influence on the interpretation of test data from this assay. The evaluation of this permeability factor for four of these chemicals is presented in the following Chapter.

This phase of the research evaluated a number of chemicals over a wide range of doses to define two chemicals of focus for more extensive evaluations employing the MELA procedure. The results indicate that those substances previously shown to be acutely toxic to the medaka and other fish embryos (i.e., TBTCl and PCP) were toxic employing these procedures. Those chemicals shown to have little toxic effect (at comparable concentrations) on fish eggs in previous studies (i.e., DENA, BaP) had little effect on medaka eggs using this approach. Other chemicals, such as naphthalene, phenanthrene and lindane, presented surprisingly low toxicity, but did cause significant adverse effects on the eggs, primarily through latent hatching and deformed or dead fry. These results indicate that the medaka generally exhibits toxic

and teratogenic responses similar to other fish species and that, though the exposure route and concentrations are not directly comparable, the MELA protocol produces results similar to those seen employing differing assay techniques, while avoiding complicating treatment factors such as high test concentrations, solubility problems and control mortality.

## CHAPTER SUMMARY

The eggs of the Japanese medaka (*Oryzias latipes*) were used to develop a short-term assay for evaluating potential toxicants and teratogens. Forty-eight hour old eggs were topically treated with nine different toxicants dissolved in DMSO carrier. The toxicants were naphthalene, phenanthrene, benzo[a]pyrene, diethylnitrosamine, dipropylnitrosamine, lindane, 2-nitrofluorene, pentachlorophenol and tributyltin chloride. The eggs were exposed to a wide range of concentrations for each toxicant in order to 1) establish the specific effects of these substances on the developing eggs and 2) to select two chemicals for further intensive studies of salinity-related response.

The toxicants induced a wide range of responses from a low level of adverse impact (primarily unhatched eggs) of the carcinogens benzo[a]pyrene (up to 10$\mu$g/egg) and the nitrosamines (up to 50$\mu$g/egg) to significant developmental anomalies seen at levels as low as 18.7pg/egg for tributyltin chloride. Exposure of 10 day old eggs (following development of the liver) to several of the carcinogens did not appear to significantly increase or decrease the level of effects. This indicates that even after potential liver metabolism had taken place, these components are still not embryotoxic or teratogenic.

The results of these studies suggest that this routine assay is very useful in obtaining large amount of toxicological data (both acute and sublethal) in a short period of time.

# CHAPTER THREE

## Permeability Factor Determination for Four Chemicals

## INTRODUCTION

For the MELA technique to be effectively used in screening of potential toxicants or complex mixtures, the passive topical procedure must be capable of delivering a significant portion of the substance dose across the chorionic membrane and into the developing medaka embryo. Macoubbin and Black (1986) quantitatively evaluated the amount of material penetrating rainbow trout (*Salmo gairdneri*) embryos using radiolabelled $^{3}$H-BaP in DMSO. The eggs were topically treated with a one microliter dose of the DMSO/BaP mixture in a manner similar to that employed in the present study. They determined that one minute after treatment an average of 27.7% of the BaP material was present in the eggs and that the level decreased with time. To date, this is the only published study which has employed DMSO topical treatment and quantitatively ascertained the absolute amount of material entering the egg to elicit a given response.

The limited amount of data pertaining to the DMSO-induced transport of toxicants across embryonic membranes warrants further quantitative evaluations. Therefore, a study was performed with several chemicals to determine the efficiency of the present technique at delivering toxicants into medaka eggs and whether this permeability factor (PF) is consistent or varies from chemical to chemical.

Four chemicals, representing as many chemical classes, were evaluated to determine the PF employing the MELA treatment technique. Two of these chemicals (tributyltin chloride and pentachlorophenol) are further studied in increased salinity and immersion exposure evaluations and, therefore, the results of this PF determination will be applied to the toxicity assay results (see Chapters 5 and 6). This will allow for the determination of the absolute component concentration(s)

eliciting a given response rather than applying the assumed nominal dose concentration. The remaining two chemicals (lindane and diethylnitrosamine) were selected based on frequent detection of these contaminants and other similar compounds (in terms of structure, chemical class, and mode of action) in the environment as well as their distinct chemical structures from the other two representatives.

## MATERIALS AND METHODS

A series of tests were performed to determine the PF of four compounds evaluated in the "freshwater" range-finding study (see Chapter 2). Compounds were selected based on their structures and substitutions as well as their relevance to the subsequent phases of this research. Treatment concentrations were selected based on levels common to the topical MELA assay experiments as well as quantities detectable by available, sensitive instrumentation. Embryos were treated with the DMSO/toxicant mixture, extracted, and analyzed employing gas chromatography techniques to quantitatively determine the PF, as a percentage, for each compound. Each DMSO/toxicant mixture contained a surrogate compound which was chosen based on its similarity to the compound of interest in terms of both extractability and instrument response. The surrogate was included as a compound which would be expected to act similar to the compound of interest but would not otherwise be present in the eggs and, consequently, to provide additional "support" to the findings for the compound of interest.

Lindane

Five replicates of 20 embryos each were topically treated employing the MELA technique discussed in Chapter 2. Each embryo received a 0.1 $\mu$l dose of a DMSO/lindane mixture at a concentration of 12.5ng lindane/egg (or 12.5ng lindane /0.1$\mu$l DMSO). This provided a total dose concentration of 250ng (12.5ng/egg x 20 eggs). The eggs also received an equivalent amount of aldrin (1,2,3,4,10,10-hexachloro-1,4,4a,5,8,8a-hexahydro-1,4:5,8-dimethanonaphthalene) surrogate, a water insoluble insecticide, in each dose aliquot. Analyses were performed employing a gas chromatograph equipped with an electron capture detector (GC/ECD) and quantitation was performed using an external standard method.

Following treatment, the eggs remained undisturbed for one minute to absorb the dose, followed by thorough washing in fresh rearing solution. Each group of 20 eggs was then transferred to a pre-cleaned 15mm x 85mm disposable glass culture tube containing 2ml of UV grade hexane. Prior to use, each tube was treated with silane (5% dimethyldichlorosilane in toluene) to bind all active sites which could cause component loss (i.e., lower extraction efficiency). The mixture was simultaneously homogenized and extracted for 2min using a Tekmar Tissumizer high frequency mechanical homogenizer equipped with a Thyristor speed controller and a 1-10ml volume probe. The samples were homogenized at a Thyristor power rate of 70 which equates to an approximate rate of 7,000rpm internal blade speed. Following homogenization, the probe was thoroughly rinsed into the culture tube with clean hexane. The mixture was transferred to a five milliliter gas-tight syringe with an affixed 0.45$\mu$m pore size Acrodisc (Gelman Sciences) disposable filtration disk. The mixture was filtered through the disk and collected in a class A 1ml volumetric tube

(actual tube volume = 2ml). Filtration was employed to remove superfluous, extracted biological material which would interfere with subsequent extraction and analysis procedures. Each extract was concentrated with a gentle stream of purified nitrogen to a final volume of 1ml, transferred to a 1.8ml amber glass vial and sealed with a teflon coated septa and cap. The extracts were stored at 4 $\pm$ 2°C until GC analysis was to be performed. After the homogenization of each replicate, the Tissumizer probe was thoroughly rinsed with hexane, followed by methanol and finally with additional hexane to avoid "carry-over" of material to the following replicate. Two method blanks were staggered throughout the treatment extractions to ensure that the probe rinsing and cleaning procedures were effectively removing any potential residual toxicant material. Additionally, these blanks were employed to detect any lindane or aldrin contamination as a result of laboratory or instrumental introduction.

In addition to the embryo topical treatment replicates, five replicates of spike standards and five replicates of matrix standards were evaluated. The spike standards entailed spiking the 2ml hexane solvent solution directly with 20 x 0.1$\mu$l treatments of the same DMSO/lindane/aldrin mixture used to treat the eggs and were homogenized/extracted in the same manner as the egg assay treatments. Theoretically, this procedure should provide complete recovery of the lindane and aldrin if the extraction procedure was 100% efficient. This will provide an indication of the level of material lost due to inefficiencies in the extraction procedures and/or analytical instrumentation.

The matrix standards served a similar purpose to that of the spike standards, with the inclusion of the egg matrix provided by the medaka eggs used in the assay.

Hence, this standard accounted for that material expected to be lost (or gained) due to the presence of the egg material as well as the factors accounted for in the spike standard. The egg matrix was rendered through homogenization of 20 eggs in the hexane solvent for 10sec to thoroughly macerate the intact eggs. This mixture was spiked with 20 x 0.1$\mu$l treatments of the DMSO/lindane/aldrin mixture used in the egg treatments and in the standard spikes. The mixture was homogenized, filtered and concentrated employing the same procedures used for the egg topical treatment and the standard spike evaluations.

All replicates of the three treatments (egg topical, standard spike, and matrix spike), in addition to procedural blanks, were analyzed employing gas chromatography with electron capture detection (GC/ECD). A Varian 3700 GC equipped with an electron capture detector was utilized for both the lindane and the aldrin evaluations. The instrument response was recorded on a Shimadzu CR-1A integration system. One microliter of each sample was analyzed employing capillary column chromatography and splitless injection. The GC conditions for this analysis are itemized in Table 3.1. A sample chromatogram with peak identifications is presented in Figure 3.1.

Calibration of the GC system was performed employing standards of lindane and aldrin prepared from the same neat standards used to develop the DMSO mixture. A known amount of each standard was dissolved in hexane and dilutions made in order to yield standards which encompassed the anticipated range of component recoveries. Calibrations curves for both lindane and aldrin were constructed with a minimum of five calibration points. The lindane curve exhibited a regression correlation coefficient (r) of 0.9958 and the six point aldrin curve had an r value of

**Table 3.1.** Gas chromatographic conditions for the lindane/aldrin permeability factor analysis.

| | |
|---|---|
| Varian 3700 Gas Chromatograph<br>Shimadzu CR-1A Integrator | Helium Carrier Gas<br>Carrier Flow Rate = 8 ml/min |
| Electron Capture Detector<br>Detector Temperature = 300 °C | Nitrogen Makeup Gas<br>Flow Rate = 30 ml/min |
| Injector Temperature = 290 °C<br>Injection Volume = 1 $\mu$l | Isothermal Analysis Temperature = 210 °C<br>Runtime = 6 min |
| Splitless Analysis | Quantitation Program = External Standard |
| Column = DB-17 (17% phenyl)<br>0.52mm x 15m x 1.0 $\mu$m film | |

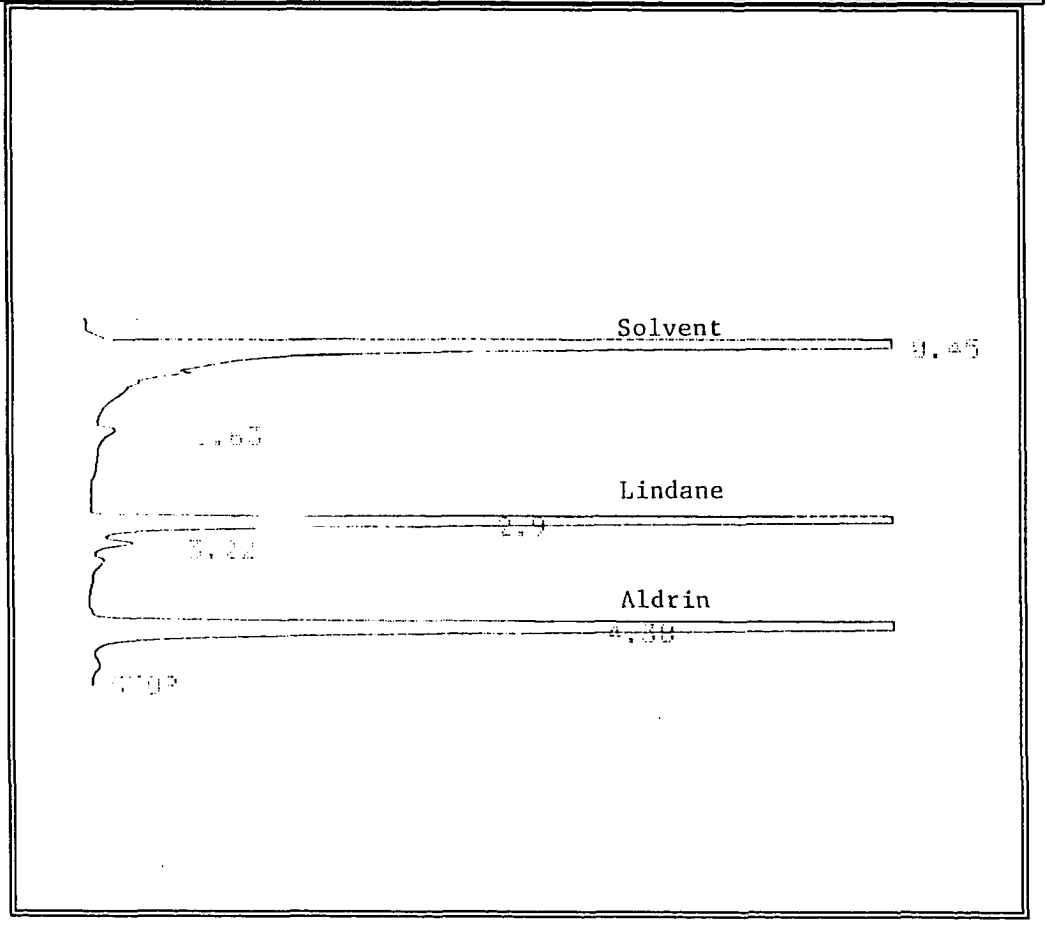



**Figure 3.1.** Sample chromatogram from lindane/aldrin analysis by GC/ECD. Peak identifications are based on retention time matches with known standards.

0.9987. Quantitation was performed employing inverse prediction of the calibration curves with the raw area data provided by the GC integration system.

Diethylnitrosamine

Egg topical treatments, standard spikes and matrix spikes were evaluated for diethylnitrosamine (DENA) in a manner similar to that discussed previously for lindane. Five replicates of 20 eggs each were evaluated to determine the PF for DENA. Each egg received a 0.1$\mu$l DMSO treatment containing 1$\mu$g each of the target chemical, DENA, and diphenylnitrosamine (DPNA) as a surrogate component. Employing the embryo dosing apparatus, each standard and matrix spike replicate received a volume of the DMSO/DENA/DPNA solution identical to that received by the 20 eggs in each topical treatment replicate. The samples were homogenized and filtered as described for lindane, however, it was determined that nearly 100% of the DENA and DPNA was lost from the standard spikes. The loss was determined to be due to binding to the filter disk and, therefore, the experiment was repeated excluding the filtration procedure. An alternative method for the removal of the biological material was employed whereby the tubes and their contents were centrifuged for five minutes at 2200 rpm to pellet the biological material. It was particularly important to remove the residual egg material from the DENA/DPNA treatments as the flame ionization detector employed for this analysis is very sensitive to aliphatic hydrocarbons and fatty acid material. The supernatant of each extract was removed, concentrated to a volume of one milliliter, and stored at 4 $\pm$ 2 °C until GC analysis was executed. Analysis was performed using a Shimadzu GC-9A equipped with a flame ionization detector (GC/FID). One microliter of each extract was analyzed on

this system applying capillary column chromatography and split/splitless injection. The GC conditions for this analysis are listed in Table 3.2. The temperature profile and column flow rate were manipulated to efficiently resolve the peaks of interest from the DMSO "contaminant" peak. A sample chromatogram displaying successful peak resolution of an extract is displayed in Figure 3.2.

Quantitation of the sample extracts for DENA and DPNA was performed employing inverse prediction of the GC data using multi-point external standard calibration curves. Both curves produced a correlation coefficient $\geq 0.9950$ (DENA=0.9976 and DPNA=0.9968) and contained a minimum of five data points surrounding the anticipated component recovery concentrations.

Pentachlorophenol

Pentachlorophenol (PCP) treatments were also performed similar to the detailed methodology provided for the lindane treatment study. Each 0.1$\mu$l dose of the DMSO/PCP solution contained 50.0ng PCP and 12.5ng aldrin as a surrogate. Each egg, standard, and matrix treatment replicate was "dosed", homogenized, filtered and concentrated to a final volume of 1ml. As with the DENA study, the use of the filtration disks was found to interfere with the efficient recovery of the PCP material. Therefore, a centrifugation procedure identical to that utilized for DENA was employed to remove residual egg material from subsequent method steps. One microliter of each final extract was analyzed by gas chromatography employing an ECD/capillary column/splitless injection configuration similar to the system employed in the lindane study. The GC conditions for this analysis are presented in Table 3.3. Treatment data were electronically recorded on a Shimadzu CR-1A integrator. A

**Table 3.2.** Gas chromatographic conditions for the DENA/DPNA permeability factor analysis.

| | |
|---|---|
| Shimadzu GC-9A Gas Chromatograph<br>Shimadzu CR-3A Integrator | Flame Gases:<br>Hydrogen = 5 ml/min<br>Air = 85 ml/min |
| Flame Ionization Detector<br>Detector Temperature = 300 °C | Initial Temperature 1 = 40 °C<br>Initial Time 1 = 8 min |
| Injector Temperature = 310 °C<br>Injection Volume = 1 $\mu$l | Temperature Ramp Rate 1 = 15 °C/min<br>Final Temperature 1 = 120 °C |
| Split Ratio = 1:100<br>Splitless Time = 1 min | Initial Temperature 2 = 120 °C<br>Initial Time 2 = 1 min |
| Column = DB-5 (5% phenyl)<br>0.32mm x 30m x 0.25 $\mu$m film | Temperature Ramp Rate 2 = 20 °C/min<br>Final Temperature 2 = 250 °C<br>Final Hold Time = 5 min |
| Helium Carrier Gas<br>Carrier Flow Rate = 1.2 ml/min | Quantitation Program = External Standard |
| Nitrogen Makeup Gas<br>Flow Rate = 34 ml/min | |

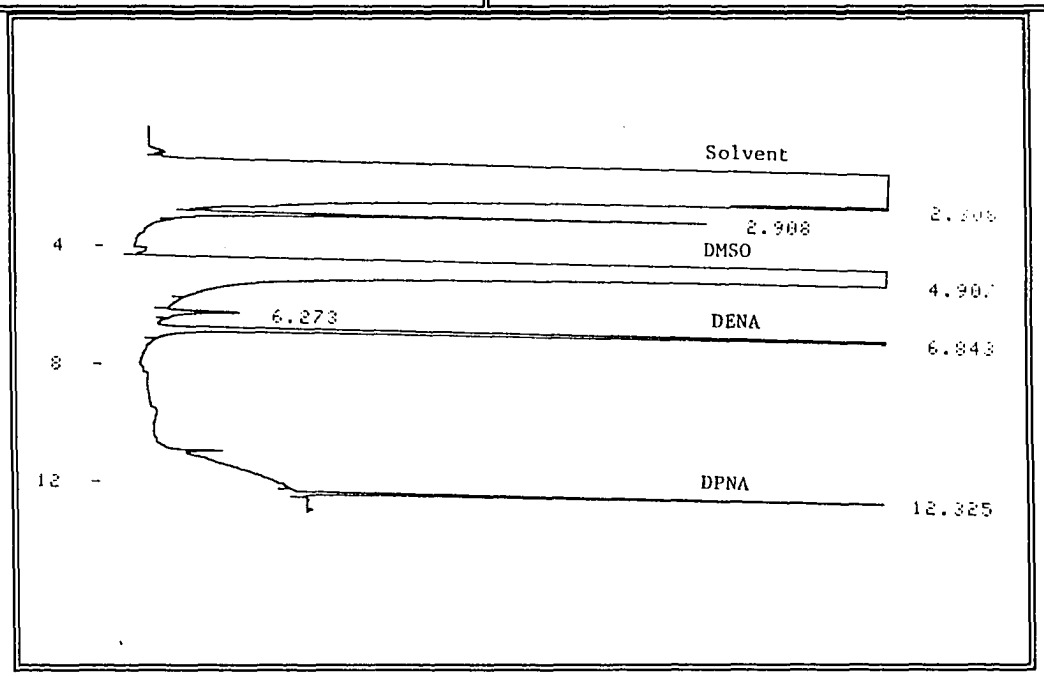



**Figure 3.2.** Sample chromatogram from DENA/DPNA analysis by GC/FID. Peak identifications are based on retention time matches with known standards.

**Table 3.3.** Gas chromatographic conditions for the PCP/aldrin permeability factor analysis.

| | |
|---|---|
| Varian 3700 Gas Chromatograph<br>Shimadzu CR-1A Integrator | Helium Carrier Gas<br>Carrier Flow Rate = 8.0 ml/min |
| Electron Capture Detector<br>Detector Temperature = 290 °C | Nitrogen Makeup Gas<br>Flow Rate = 30 ml/min |
| Injector Temperature = 310 °C<br>Injection Volume = 1 $\mu$l | Isothermal Analysis Temperature = 220 °C<br>Runtime = 5 Minutes |
| Splitless Analysis | Quantitation Program = External Standard |
| Column = DB-17 (17% phenyl)<br>0.52mm x 15m x 1.0 $\mu$m film | |

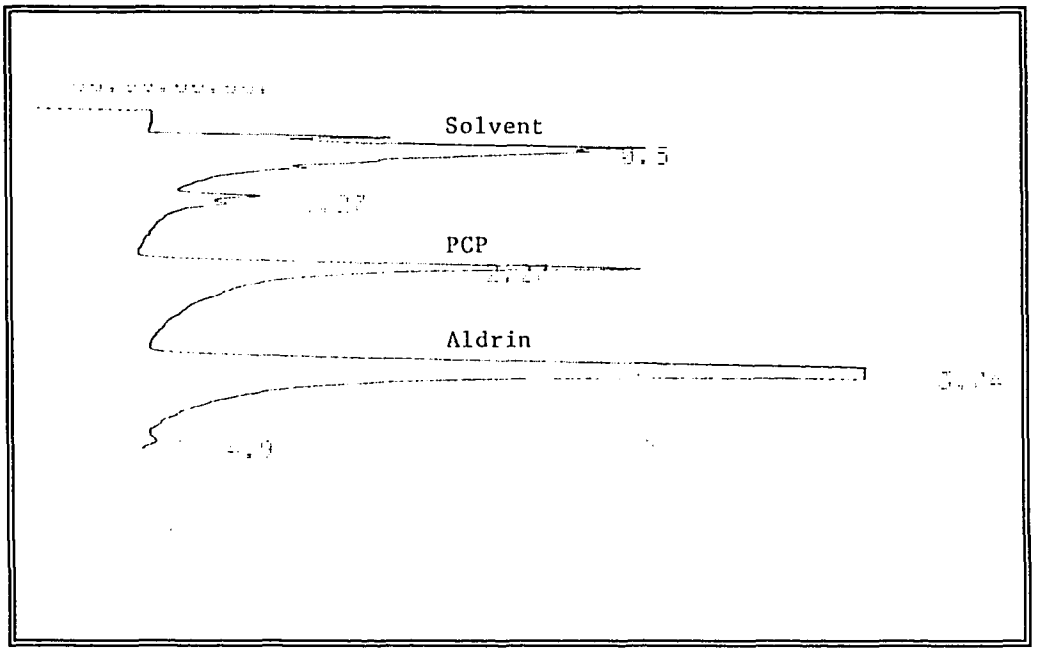



**Figure 3.3.** Sample chromatogram from PCP/aldrin analysis by GC/ECD. Peak identifications are based on retention time matches with known standards.

sample chromatogram with peak identifications is presented in Figure 3.3.

External standard quantitation of the treatment chromatograms for PCP and aldrin were performed using the analysis/integrator generated substance area values and inverse prediction of multi-point calibration curves. The r values were 0.9987 and0.9972 for the PCP and aldrin curves, respectively.

Tributyltin Chloride

Due to difficulties in detecting tributyltin chloride (TBTCl) analytically, the methodologies for this chemical determination differed significantly from the other three toxicants, both in terms of the extraction procedure and in the development of the calibration curves. The treatment groups were, however, identical to the other chemicals with five replicates each of topical treatment, standard spike and matrix spike. The culture tube solvent for the alkyltin homogenization procedure was methanol rather than the hexane employed in the other chemical permeability evaluations. This water-miscible solvent allowed the homogenized "extract" to be introduced into an aqueous medium to allow for subsequent extraction through a modification of the simultaneous derivatization/extraction techniques described by Mathias *et al.* (1986). Due to the extreme glassware binding tendencies of alkyltins, all extraction glassware, in addition to the culture tubes, was treated with silane to complex active sites. Each 0.1$\mu$l DMSO treatment contained 20ng of TBTCl and 20ng of dibutyltin dichloride ($DBTCl_2$) as a surrogate component. The treated egg, standard and matrix spike treatment replicates were then homogenized for two minutes in 2ml of methanol solvent. As with the PCP and DENA evaluations, alkyltins were found to adhere to the membrane of the filtration disc and, therefore,

all tubes were centrifuged for five minutes at 2200 rpm to pellet the biological material.

The supernatant of each tube was quantitatively transferred to 500 ml of bottled, reagent water in a one liter teflon separatory funnel. Each homogenate/water mixture then received an additional surrogate spike (which actually represented an internal standard) containing 100ng of di-n-propyltin dichloride ($DPTCl_2$) in methanol. After agitating the mixture for one minute, 15ml of a 4% (w/v) sodium borohydride ($NaBH_4$) in water solution was added and the mixture was shaken for an additional minute. As a derivatizing agent, the $NaBH_4$ solution promoted substitution of the alkyltin chloride ions with hydrogen ions allowing the alkyltins to be efficiently and sensitively detected utilizing gas chromatographic techniques. Fifteen milliliters of methylene chloride solvent was added to each separatory funnel and the mixture was shaken by hand for 2min to exhaust vapor generation of the volatile methylene chloride. Each separatory funnel was further shaken on a mechanical agitator for an additional 10min. The aqueous and organic phases were allowed to separate for 10min and the dense methylene chloride layer was drained into a silanized 25 ml tapered glass tube. An additional 15ml aliquot of methylene chloride was added to the funnel and the shaking procedure was repeated. The organic layer was combined with the organic phase from the previous extraction. The aqueous layer was properly disposed of and the organic extract was concentrated to less than 1ml under a gentle stream of purified nitrogen. Each extract was transferred to a 1ml tapered sample vial and further concentrated with nitrogen to a final volume of 0.050ml. As the volatile, derivatized alkyltin components are stable for only a short period of time, GC analysis immediately followed the extraction procedure.

A 10$\mu$l injection of each extract was made on a Varian 3700 GC equipped with a glass packed column (3% OV-1) and a flame photometric detector (FPD). The data were recorded on a Shimadzu CR-1A integrator. The GC conditions are listed in Table 3.4. A sample chromatogram with peak identifications for the alkyltin analysis is shown in Figure 3.4.

Both of these organotin compounds are very susceptible to loss during the extraction process. To account for the anticipated variability in recoveries, a relative response factor (RRF) method was employed using $DPTCl_2$ as an internal standard. The RRF values were determined by dividing the TBTCl or $DBTCl_2$ area by the area of the $DPTCl_2$ for each treatment replicate. In order to provide an accurate calibration utilizing this method and, with the known volatility of the alkyltin derivatives, a calibration curve was developed applying a modified internal standard technique. Calibration "samples" were extracted in the same manner as the treatment replicates with the tin compounds added to the 500ml water medium directly at levels of 25, 50, 100, 200, and 400ng/l in addition to the $DPTCl_2$ internal standard (200ng/l). This simultaneous derivatization/extraction calibration procedure is necessary when analyzing for volatile, hydrolyzed alkyltin compounds, as it is neither possible to purchase, nor to produce, stable hydrolyzed alkyltin components for use in typical calibration procedures.

Quantitation of the treatment replicates was performed through inverse prediction of the calibration curves using a relative response factor (RRF)/internal standard technique. Both curves contained five data points encompassing the spike concentration level and anticipated recoveries and exhibited correlation coefficients of 0.9950 or better (TBTCl=0.9992 and $DBTCl_2$=0.9983).

**Table 3.4.** Gas chromatographic conditions for the TBTCl/$DBTCl_2$ permeability factor analysis.

| | |
|---|---|
| Varian 3700 Gas Chromatograph<br>Shimadzu CR-1A Integrator | Nitrogen Makeup Gas<br>Flow Rate = 10 ml/min |
| Flame Photometric Detector<br>Detector Temperature = 200 °C | Flame Gas Flows:<br>Hydrogen = 143 ml/min<br>Air 1 = 80 ml/min<br>Air 2 = 165 ml/min |
| Injector Temperature = 125 °C<br>Injection Volume = 10 $\mu$l | Initial Temperature = 55 °C<br>Initial Hold Time = 1 min |
| Splitless Analysis | Temperature Ramp Rate = 20 °C/min |
| Column = 6 ft/2 mm ID Glass<br>Packed with 3% OV-1 on 100/120 supelcoport | Final Temperature = 175 °C<br>Final Hold Time = 0.1 min |
| Helium Carrier Gas<br>Carrier Flow Rate = 3.5 ml/min | Quantitation Program = Internal Standard |

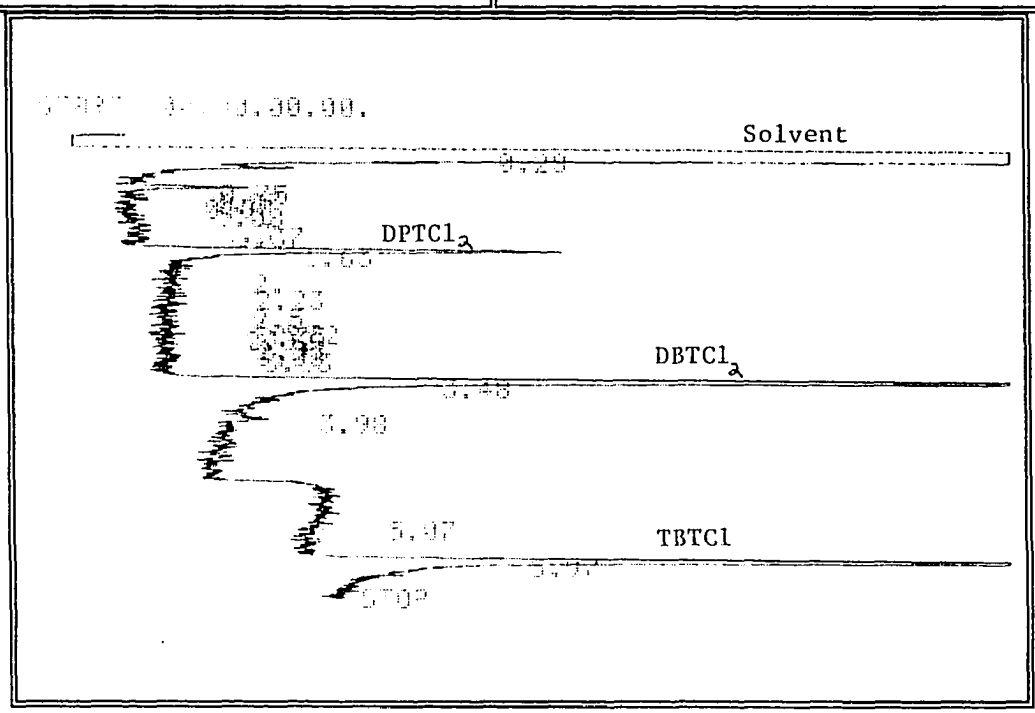
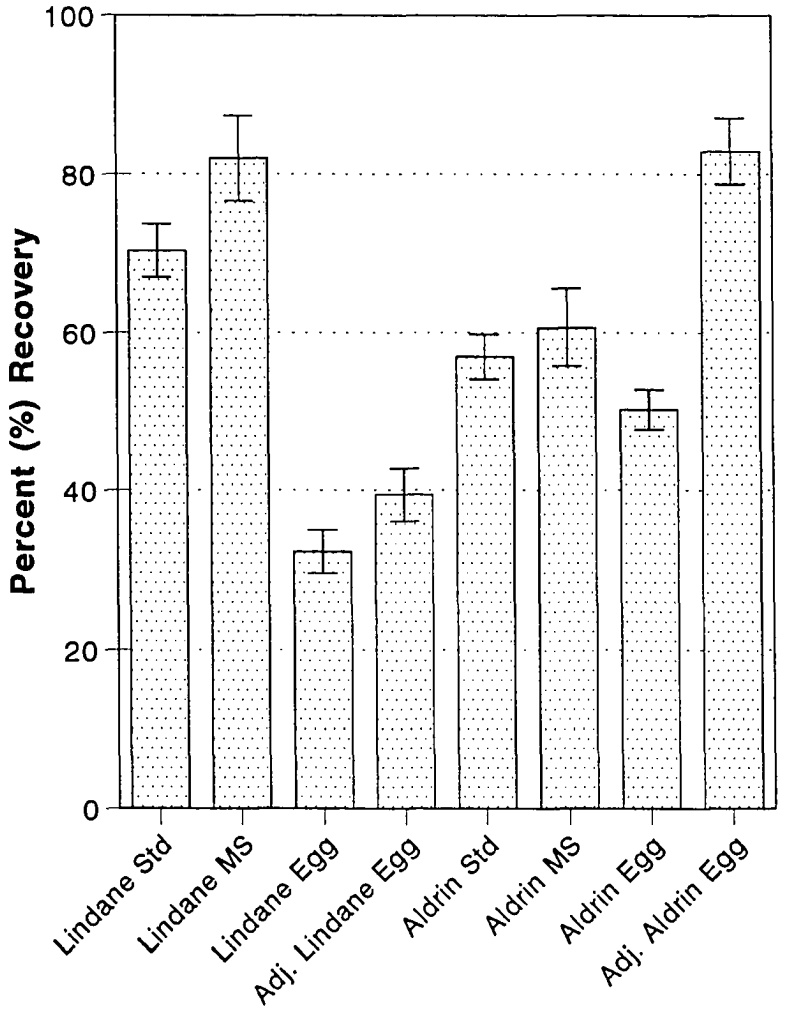



**Figure 3.4.** Sample chromatogram from TBTCl/DBTCl analysis by GC/FPD. Peak identifications are based on retention time matches with known standards.

## RESULTS

### Lindane

The lindane and aldrin data for all treatments are displayed in Figure 3.5. The data are expressed as mean percent recoveries for each treatment type with associated standard errors (variation from the mean as $\frac{Standard\ Deviation}{\sqrt{n}}$) for the five replicates. The results indicate that the matrix recoveries were actually elevated as compared to the standard spikes. The reason for this elevation is not known as one would expect that the egg matrix would be responsible for additional removal of the toxicants over and above that lost to the extraction technique (represented by the standard spike). Nonetheless, the data indicate that though the extraction efficiency was higher for lindane (mean = 82.0%) than for aldrin (mean = 60.6%), the opposite was true for the amount of material entering the egg assisted by the DMSO carrier. The adjusted egg topical "treatment" reflects the egg topical treatment values adjusted by that amount of material lost in the extraction procedure (accounted for by the matrix spike treatment). The amount lost was considered to be attributable to inefficiency of the extraction methodology and due to matrix binding and interference. Accordingly, each egg topical replicate was divided by the mean value for the matrix spike treatment and a mean and standard error for these matrix adjusted replicates determined to yield a mean permeability factor (PF) of 39.6% for lindane and 83.9% for the aldrin surrogate.




| Treatment | Lindane Mean % Recovery | Aldrin Mean % Recovery |
|---|---|---|
| Standard Spike | 70.2 ± 3.35 | 56.9 ± 2.84 |
| Matrix Spike | 82.0 ± 5.40 | 60.6 ± 4.87 |
| Egg Topical | 32.4 ± 2.72 | 50.2 ± 2.54 |
| Adj. Egg Topical | 39.5 ± 3.32 | 82.9 ± 4.20 |

**Figure 3.5.** Summary of permeability factor data for lindane and aldrin. Mean and standard error are for five replicates of each treatment. The adjusted (adj.) egg topical represents the egg topical treatment replicates adjusted for the extraction efficiency represented by the matrix spike mean.

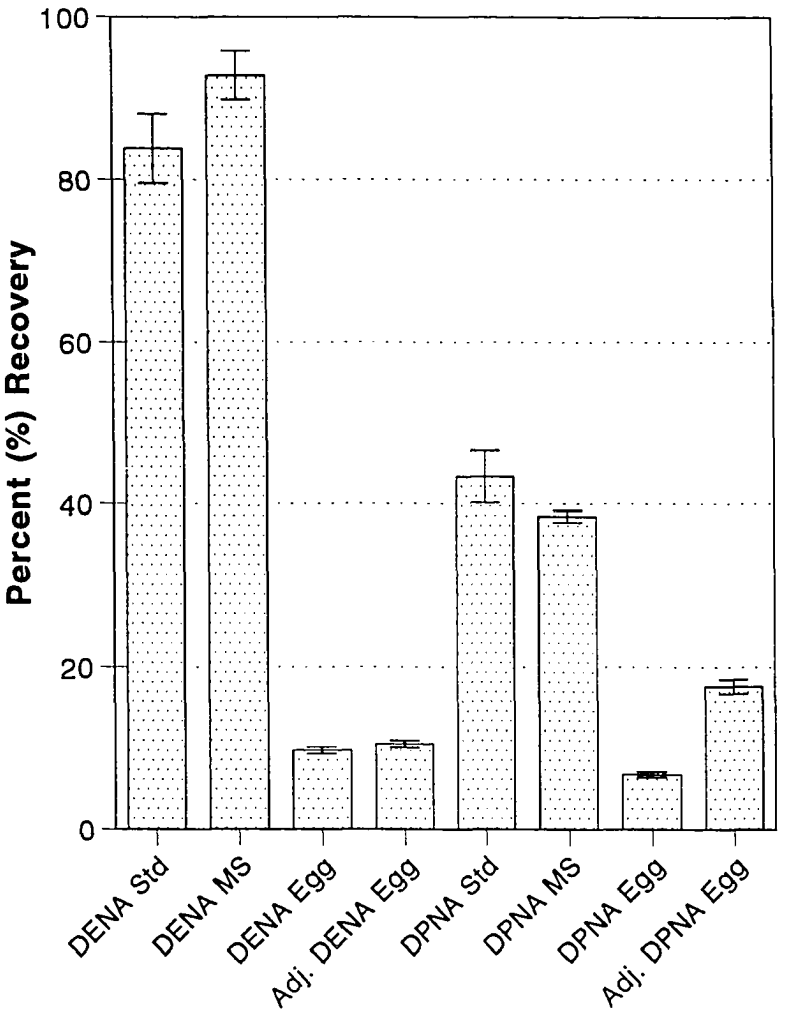
Diethylnitrosamine

The data for DENA and DPNA are presented in Figure 3.6. The results indicate that the extraction procedure was much more efficient for the compound of interest (DENA) than for the surrogate (DPNA). The structural dissimilarities of the alkyl groups between the two compounds apparently have a significant effect on the success of non-polar solvent extraction, suggesting that much of the DPNA may have remained "bound" to the extracted egg material after centrifugation. However, as with the lindane/aldrin study, the adjusted egg topical evaluation demonstrated that the DPNA surrogate was more successful at penetrating the egg, with a mean PF of 17.6%, than the test compound which had a low mean PF of 10.5%.

Pentachlorophenol

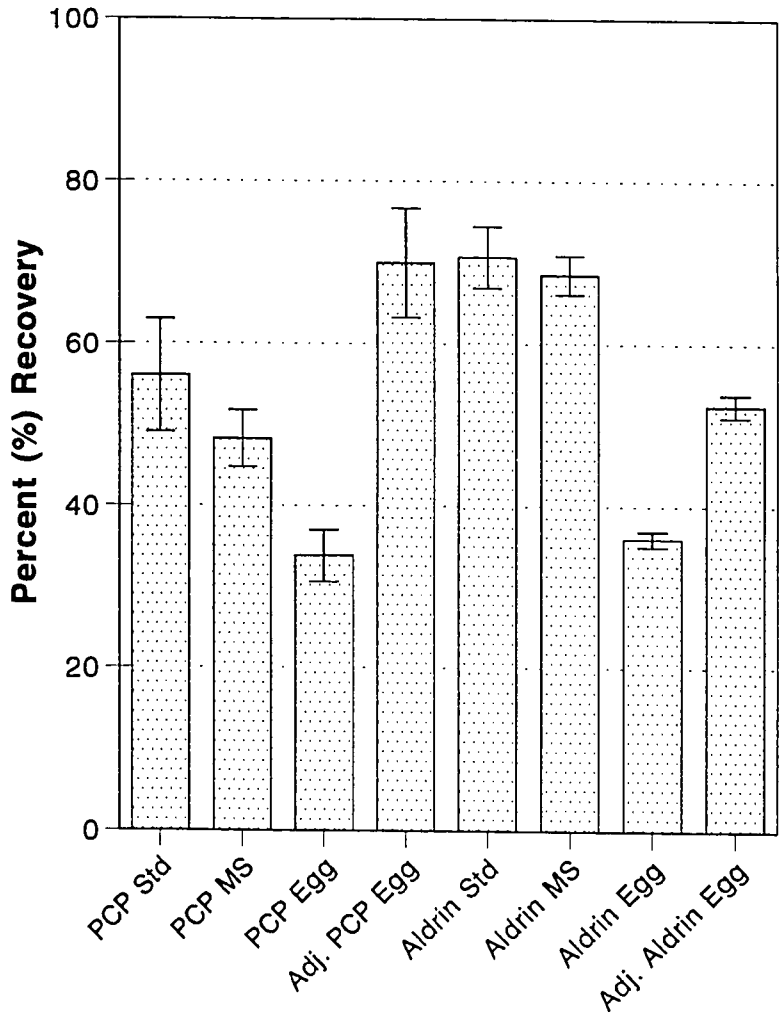
Prior to instrumental evaluation of the PF for all chemicals, a criterion was established whereby the "mid-point" of each compound calibration curve was injected twice prior to evaluation of the treatment replicates. If the response for the toxicants in this mid-point solution varied by more than 10%, it was viewed necessary to inject and analyze each treatment replicate twice and the average of these values was taken to be the raw response. This was the case for the PCP/aldrin evaluations. Therefore, the area and subsequent concentration and recovery data are actually mean values of two injection analyses for each treatment replicate. This procedure was implemented to account for apparent instrument response variability with respect to these compounds.

The summarized results presented in Figure 3.7 indicate that over two-thirds of the PCP applied to the eggs actually entered the eggs with a mean PF of 70.0%.




| Treatment | DENA Mean % Recovery | DPNA Mean % Recovery |
|---|---|---|
| Standard Spike | 83.4 ± 4.30 | 43.3 ± 3.21 |
| Matrix Spike | 92.8 ± 2.98 | 38.4 ± 0.72 |
| Egg Topical | 9.76 ± 0.39 | 6.76 ± 0.34 |
| Adj. Egg Topical | 10.5 ± 0.42 | 17.6 ± 0.90 |

**Figure 3.6.** Summary of permeability factor data for DENA and DPNA. Mean and standard error are for five replicates of each treatment. The adjusted (adj.) egg topical represents the egg topical treatment replicates adjusted for the extraction efficiency represented by the matrix spike mean.




| Treatment | PCP Mean % Recovery | Aldrin Mean % Recovery |
|---|---|---|
| Standard Spike | 56.0 ± 6.98 | 70.7 ± 3.75 |
| Matrix Spike | 48.3 ± 3.50 | 68.6 ± 2.36 |
| Egg Topical | 33.8 ± 3.24 | 35.9 ± 0.95 |
| Adj. Egg Topical | 70.0 ± 6.72 | 52.4 ± 1.39 |

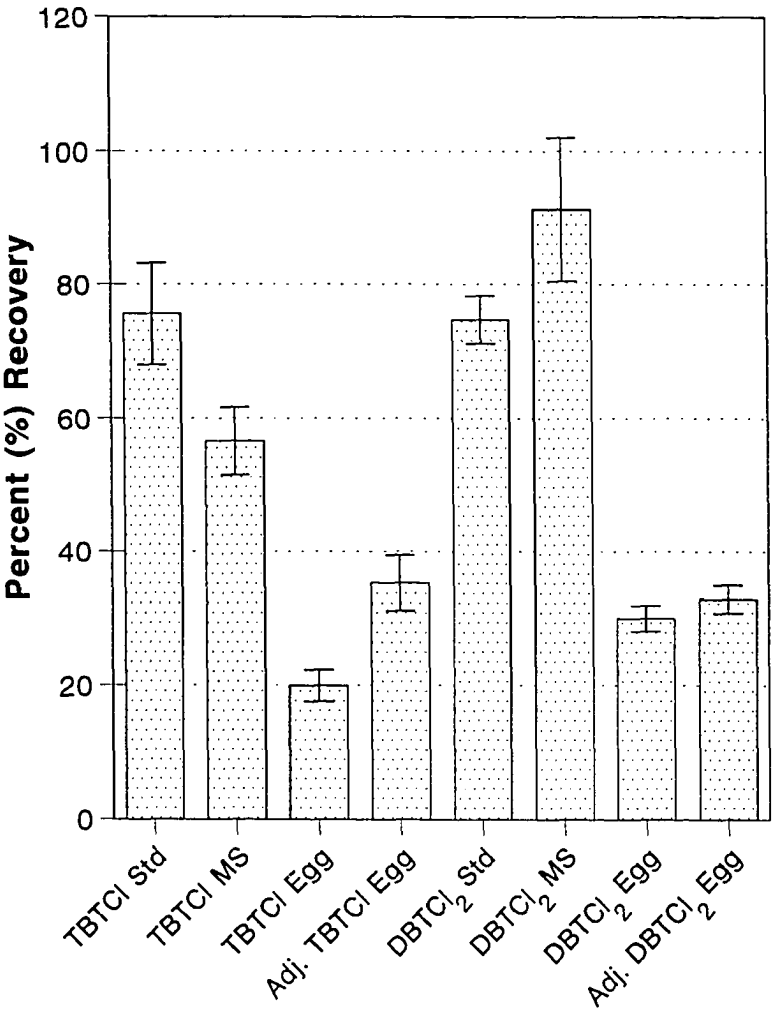
**Figure 3.7.** Summary of permeability factor data for PCP and aldrin. Mean and standard error are for five replicates of each treatment. The adjusted (adj.) egg topical represents the egg topical treatment replicates adjusted for the extraction efficiency represented by the matrix spike mean.

The aldrin surrogate penetration was slightly lower with a mean PF of 52.4%. The cause for this decreased aldrin penetration as compared to the aldrin surrogate used in the lindane evaluation is not known, however, it may be due to procedural differences in the extraction as the lindane/aldrin extracts were filtered to remove extraneous biological material whereas the PCP/aldrin extracts were centrifuged.

Tributyltin Chloride

The data for the two organotin compounds (TBTCl and $DBTCl_2$) are presented as means and standard errors for each treatment in Figure 3.8. As with the PCP/aldrin evaluation, the organotin calibration standard mid-point varied by more than $\pm 10$ %. Hence, the each data value was a mean for two GC injection analyses.

The standard spike recoveries indicate that the unique extraction procedure provides comparable recoveries for both alkyltins, however, the matrix spike treatment data indicate that the egg material "interferes" with the recovery of TBTCl to a greater extent than for $DBTCl_2$. After accounting for the matrix interference, the adjusted topical treatments for TBTCl and $DBTCl_2$ appear to be quite similar with mean PF values of 35.3% and 32.9%, respectively.




| Treatment | TBTCl Mean % Recovery | $DBTCl_2$ Mean % Recovery |
|---|---|---|
| Standard Spike | 75.6 ± 7.62 | 74.7 ± 3.56 |
| Matrix Spike | 56.6 ± 5.06 | 91.3 ± 10.79 |
| Egg Topical | 20.0 ± 2.35 | 30.0 ± 1.91 |
| Adj. Egg Topical | 35.3 ± 4.15 | 32.9 ± 2.09 |

**Figure 3.8.** Summary of permeability factor data for TBTCl and $DBTCl_2$. Mean and standard error are for five replicates of each treatment. The adjusted (adj.) egg topical represents the egg topical treatment replicates adjusted for the extraction efficiency represented by the matrix spike mean.

## DISCUSSION

The toxicant selection was based on their distinct chemical classes which could provide a detailed and broad range of information regarding the DMSO-aided transport of toxicants employing MELA. The variability in permeability between chemicals is apparent in the PF determinations with mean values ranging from 10.5% to 70.0% for the compounds of interest. The PF variability is no doubt related to the differing physico-chemical characteristics of each compound. These many factors include, but are not limited to, compound polarity, steric factors, functional groups, and structural configuration. These characteristics play an active role in the diffusive transport of the toxicant across the chorionic membrane and, therefore, control the PF level. Metabolism and excretion (i.e., the toxicokinetic phase) are not considered factors in this particular PF study as the 48hr old eggs were sacrificed within minutes after treatment. At this early stage in embryonic development, this limited time frame surely restricted the ability of the egg to biotransform the toxicants. It has been documented that given time to "process" toxicants, fish embryos have the ability to modify and eliminate toxicants (Hendricks, 1981; Maccubbin and Black, 1986). However, the focus of this study was simply to determine the amount of substance actually infiltrating the egg using the MELA technique for a diverse group of toxicants.

The main principle that each of the toxicant physico-chemical variables influences is lipid "solubility". Therefore, if it is assumed that the DMSO does not actively alter the lipid solubility characteristics of each toxicant, a hypothesized correlation could be drawn between the bioavailability (represented by lipid affinity) of the toxicant and the PF determined in this study.

The most widely employed and accepted technique for predicting whether a toxicant is hydrophilic or hydrophobic (i.e., lipophilic) is the determination of the *n*-octanol-water partition coefficient ($K_{ow}$). This partition coefficient is based on structure-activity relationships and is calculated by a number of different techniques, each based on the notion that the straight chained *n*-octanol mimics the phospholipid carbon chain of biological tissues. To determine a $K_{ow}$ (most often expressed in the log form), the toxicant is introduced into a *n*-octanol/water biphase system and the resultant distribution is evaluated. The $K_{ow}$ is determined to be the ratio between the concentration in the *n*-octanol to that partitioning into the water. Hence, the higher the $K_{ow}$ the more bioactive the toxicant.

Table 3.5 lists the log $K_{ow}$ values for the toxicants evaluated in this study and their corresponding surrogates. These values indicate that, in addition to representing a wide range of compound classes, these chemicals represent an equally wide range of log $K_{ow}$ values (for example, log $K_{ow}$ values for the 119 U.S. EPA organic priority pollutants range from -0.58 to 8.06; U.S. EPA, 1989). Figure 3.9 displays these log $K_{ow}$ values regressed against the PF values obtained for this study. A valid relationship was established between these two factors, with a regression coefficient for the correlation (r) of 0.9225. The log $K_{ow}$ for $DBTCl_2$ has not previously been published, however, it has been suggested that based on differences in the physical properties between TBTCl and $DBTCl_2$ (e.g., the Hansch Fragment Constant), the dialkyltin would be expected to be slightly less bioavailable (Laughlin *et al.*, 1985), which correlates well with the slight decrease in PF from TBTCl to $DBTCl_2$.

**Table 3.5.** Log *n*-octanol-water partition coefficients ($K_{ow}$) for the PF study toxicants and associated surrogate compounds.

| Toxicant | Log $K_{ow}$ |
|---|---|
| Diethylnitrosamine | 0.00 a |
| Dibutyltin Dichloride | 3.00? b |
| Diphenylnitrosamine | 3.13 c |
| Tributyltin Chloride | 3.20 d |
| Lindane | 3.72 e |
| Pentachlorophenol | 5.00 f |
| Aldrin | 7.40 g |

a - Calculated based on water solubility and $K_{ow}$ values for compounds of similar structure
b - Estimated from physical characteristics; not used in regression
c - Veith *et al.* (1980)
d - Maguire *et al.* (1983)
e - Karickhoff (1981)
f - Veith *et al.* (1979)
g - Briggs (1981)

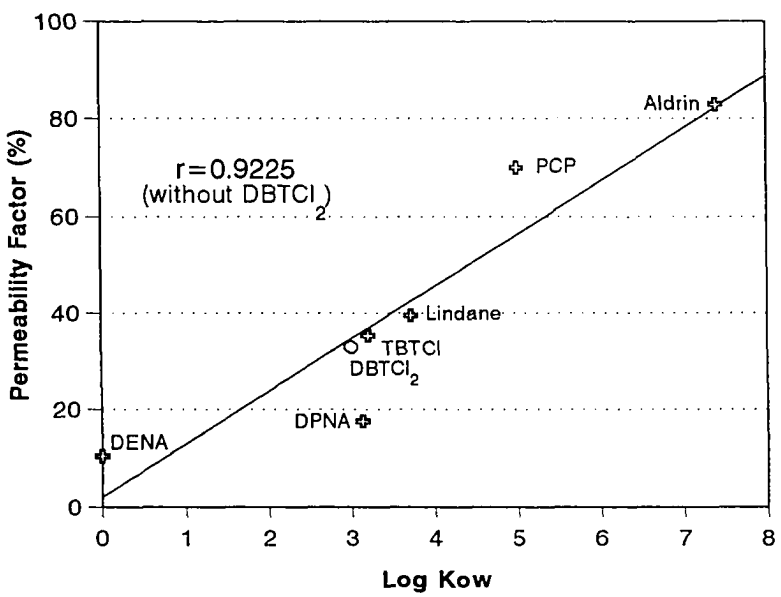



**Figure 3.9.** Correlation of log *n*-octanol-water partition coefficient ($K_{ow}$) and permeability factor (PF) determined employing the MELA technique. Note unique symbol for $DBTCl_2$ due to estimated $K_{ow}$.

This relationship between the PF and log $K_{ow}$ suggests that the transport of the toxicants is truly governed by their physical and chemical properties, even in the presence of a membrane permeable carrier such as DMSO. In other words, relative to one another, the ratio of toxicant permeating the egg for each chemical does not change with the DMSO carrier, however, the carrier may be affecting the actual amount of each toxicant penetrating the egg.

In a typical aqueous immersion embryonic study one can be assured that the characteristics of the toxicant in conjunction with the diffusive abilities of the egg regulate substance transport. This topical treatment study suggests that, even in the presence of a pure solvent "helper" (similar to a protein carrier of a nutrient-like toxicant - i.e., active transport) that passive transport is taking place. Hence, the amount of toxicant penetrating the egg will apparently be controlled by the lipid solubility of the chemical, which is established by the various properties of that chemical, and not by the solubility in, or penetrability of, the DMSO carrier.

This relationship may make it possible to roughly estimate the amount of toxicant which should be applied to the egg, using MELA, to simulate a given environmental situation if the field aqueous concentration, the $K_{ow}$ and MELA PF for that chemical are known. The following equation is one such potential technique to calculate this estimated dose:

$$\textit{Estimated MELA Dose } (\mu g/egg) = \frac{\frac{(K_{OW} \times AWC)}{E_W}}{PF_D} \qquad [3.1]$$

where: $K_{ow}$ = *n*-Octanol-water partition coefficient $\frac{\mu g/ml\ n\text{-octanol}}{\mu g/ml\ water}$

AWC = Ambient water concentration of toxicant in $\mu g/ml$

$E_W$ = Weight of egg

$PF_D$ = Permeability factor expressed in decimal form

The main assumptions made in this calculation are that 1ml of the *n*-octanol represents 1g of lipid filled tissue, that the lipid content of each medaka egg remains constant and that the environmental toxicant equilibrium is at steady state with respect to water and tissue. Another alternative would be to substitute the toxicant bioconcentration factor (BCF) for the $K_{ow}$ in a similar equation. The BCF represents the ratio of the tissue concentration to the water concentration of an exposed organism. BCF and $K_{ow}$ values have been shown to be well correlated for many compounds through numerous equations such as that proposed by Veith *et al.* (1980):

$$\log BCF = 0.76 \log K_{OW} - 0.23 \qquad r = 0.907 \qquad [3.2]$$

This equation was developed for 84 organic chemicals of varying polarity and bioconcentration potential. The BCF should work equally well for the types of components evaluated in this and numerous other studies which propose a similar correlation. In cases where the $K_{ow}$ and BCF do not predict each other, these types of equations should be employed with caution as the $K_{ow}$ may not be a good indicator of the bioconcentration potential.

## CHAPTER SUMMARY

Four chemicals representing different chemical classes were evaluated for their trans-chorionic permeability or permeability factor (PF) using the MELA topical treatment procedure. Eggs were treated employing the standard MELA protocol, but were immediately extracted following treatment. Extraction was carried out through homogenization, and subsequent filtration or centrifugation, with non-polar solvent. Analysis was performed employing gas chromatography techniques with multiple specific detectors.

The PF values for the four chemicals and corresponding surrogate components ranged from 10.5% to 70.0%. These results indicate that even in the presence of the membrane permeable DMSO solvent, passive transport is taking place. The amount of toxicant penetrating the egg apparently is controlled by the lipid solubility of the chemical, which is established by the properties of that chemical, and not by the solubility in, or penetrability of, the DMSO carrier.

The PF data were regressed against the octanol-water partition coefficient ($K_{OW}$) for each chemical to determine if there was a relationship between the PF and this physico-chemical coefficient of lipid affinity. The data correlated well with an r of 0.9225 for the regression line $PF = 10.8\ logK_{ow} + 2.06$. These findings suggest that it may be possible to predict an appropriate MELA topical concentration to represent a given ambient water concentration.

# CHAPTER FOUR

## Optimum Rearing Condition Determination

## INTRODUCTION

One of the primary goals in the development of an efficient pollutant screening assay is production of test results in a rapid fashion. The MELA test length of 21-days is based on an average anticipated hatching time of approximately 14 days at 25°C (Kirchen and West, 1976) and an additional 7 day period for absorption of the yolk sac material (i.e., endogenous feeding). Preliminary studies during the onset of this research (see Chapter 2) indicated that the period to hatch was somewhat longer than the predicted 14 days. Rearing conditions such as temperature, solution contents, number of eggs per ml of solution and culture vessel type vary among previous investigations with medaka. However, even when similar temperature and light conditions are maintained and similar rearing solutions are employed, previous investigations have reported a wide range of control group hatching successes and rates from complete hatch events as early as 9-10 days (Takimoto *et al.*, 1984a) to less than 50% hatch at day 16 (Heisinger and Green, 1975). The culture variables which most often differ between previous medaka embryologic investigations are culture vessel type and the presence or absence of aeration and/or solution changes. A study was conducted to test various combinations of these culture variables on the hatch success and rate for untreated medaka eggs. The aim of the study was to determine which combination of factors provided the shortest mean hatching rate while requiring a minimum amount of labor.

For all possible rearing options, multiple eggs were housed in each culture vessel. Although other investigators have chosen micro-well or small-volume vial containers which accommodate a single egg (see, for example, Cooper *et al.*, 1991), the present study employed multiple specimens in close contact in each container.

This latter approach was employed as the presence of a hatching enzyme is hypothesized to be a significant factor in the synchronous hatching of egg "clusters". This hypothesis is based on the fact that investigators have shown that a hatching enzyme is secreted from glands of the developing embryo into the perivitelline space (Yamamoto *et al.*, 1979; Yasumasu *et al.* 1988). This enzyme facilitates the dissolution of the egg chorion (or envelope) prior to hatching and is subsequently released into the embryo rearing medium where it becomes available to erode the outer chorion of neighboring eggs, expediting concurrent hatching events (Yamamoto and Yamagami, 1975). If this enzyme is part of the natural hatching process for the medaka, and the eggs are completely isolated, hatching rate evaluations may not be appropriate. In other words, the rearing of isolated eggs may result in variability in hatching rate simply due to a lack of essential natural stimulant which is produced by neighboring eggs. Whether or not this theory is valid needs to be supported with extensive future laboratory evaluations. A screw-top scintillation vial (23ml) was employed as the alternate test vessel due to its reduced diameter (2.5cm vs. 9cm), but similar volume as compared to the Petri dish. Therefore, the eggs would be in closer association with each other providing the potential for earlier and more consistent hatching rates within any given group of eggs.

Differences in hatching rates were evaluated for the two test containers (Petri dish and scintillation vial) in the presence and absence of two variables, aeration and solution changes. The intent of this evaluation was to determine which combination provided the most rapid and consistent hatching rate among a given group or replicate of eggs. The results of this investigation were used to develop the experimental methods in the extensive evaluations for pentachlorophenol (PCP) and tributyltin

chloride (TBTCl) discussed in Chapters 5 and 6, respectively.

## MATERIALS AND METHODS

Medaka eggs were collected as specified in Chapter 2 and reared in Petri dishes containing egg rearing solution (see Table 2.3) for 48 hours during which time dead eggs were detected microscopically and removed from the rearing dishes. The eggs were reared at 25 $\pm$ 1°C in a culture incubator. At 48 hours, eggs were randomly selected and separated into five replicates of ten eggs per culture combination. Eggs were reared in multiple combinations of the three variables: vessel type, aeration, and solution change displayed in Table 4.1.

### Petri Dish Groups

Each Petri dish contained 20ml of the embryo rearing solution and ten medaka eggs randomly selected from viable eggs collected 48 hours prior to initiation of the test. The test vessels were maintained in an stable temperature incubator at 25 $\pm$ 1°C. In the *PDC* group, the rearing solution was replaced daily with fresh solution employing vacuum aspiration with a Pasteur pipet which had been modified to reduce the diameter of the tip to avoid damaging eggs. Fresh rearing solution was prepared every three days and stored at 4 $\pm$ 2°C in the dark. The *PDc* group replicates were reared under identical conditions, however, no solution change was performed (i.e., the technique employed in the Chapter 2 range-finding studies). The Petri dish evaluations did not include the aeration variable as it was not possible to effectively and efficiently aerate the solution in a consistent manner from one dish to another.

**Table 4.1.** Hatching rate evaluation treatment combinations.

| Treatment Name | Vessel Type | Aeration | Solution Change |
|---|---|---|---|
| *PDC* | PD | NA | + |
| *PDc* | PD | NA | -- |
| *SVAC* | SV | + | + |
| *SVAc* | SV | + | -- |
| *SVaC* | SV | -- | + |
| *SVac* | SV | -- | -- |

PD - Petri Dish
SV - Scintillation Vial
NA - Variable not applicable with culture vessel type
\+ - Variable was included
-- - Variable was not included

Mortality and hatching occurrences were monitored and recorded daily for each dish. The experiment was terminated at day 30 post-collection of the eggs.

Scintillation Vial Groups

Analogous to the Petri dish evaluation, each scintillation vial contained 20ml of embryo rearing solution and ten viable 48-hour old embryos. Groups *SVAC* and *SVaC* underwent daily solution changes in the same manner as the Petri dish changed series (*PDC*). The unchanged series (*SVAc* and *SVac*) vials were maintained for the duration of the test without changing the vessel solution. A 1in x 1in piece of teflon tape was placed between each vial and lid to provide a reliable seal reducing loss of solution through evaporation.

The scintillation vial evaluation incorporated an additional variable - aeration. The *SVAC* and *SVAc* test groups were aerated with breathing-quality air through use

of 7in Pasteur pipet (see Figure 4.1). A ¼-inch tapered hole was drilled in each vial lid. The pipet tip was inserted through the hole and pierced the teflon tape to provide an adequate seal around the pipet itself. The tapered hole, in conjunction with the teflon tape provided a "sealed system" to avoid unnecessary solution loss. A second smaller hole was made in the seal tape and cap to provide an outlet for the externally introduced air. In the *SVAC* group, the seal tape was replaced after each solution change. Air was introduced into each vial through the pipet at an approximate rate of one bubble per second.

Each vial was monitored daily for egg mortality or hatching events. As with the Petri dish studies, the scintillation vial evaluations were terminated at 30 days post-collection of the test eggs.

Statistical Evaluation

Two-way analysis of variance (ANOVA) was used to evaluate the data for treatment effects and/or effects of solution changes for number of unhatched eggs as well as day-to-hatch. When appropriate, Duncan's Multiple Range Test was performed to determine treatment effects (i.e., as confirmation of the ANOVA procedure). For day-to-hatch data, regression analyses with dummy variables for treatment and change were executed to ascertain results of interaction effects. All statistical evaluations were performed using SAS System software (SAS Institute, Cary, NC).

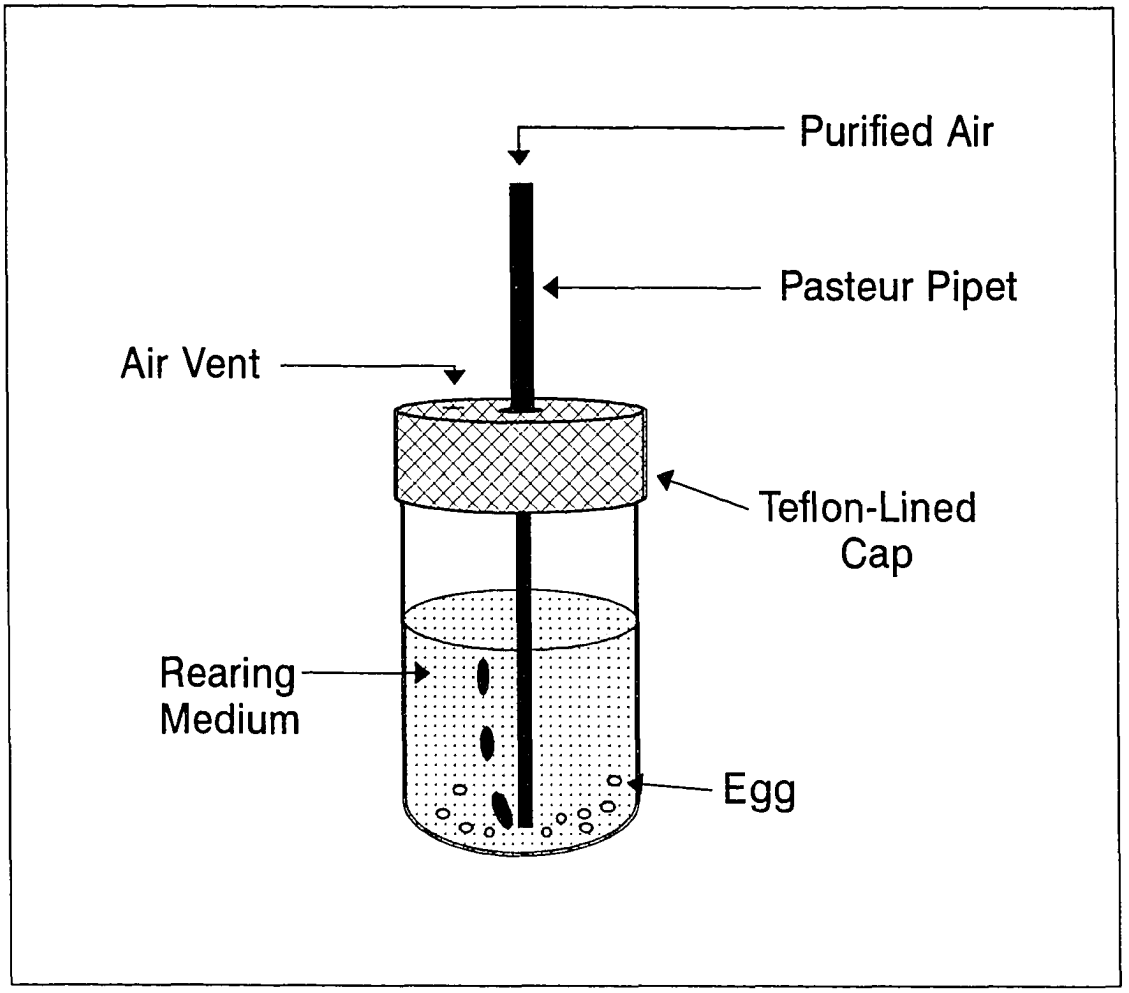


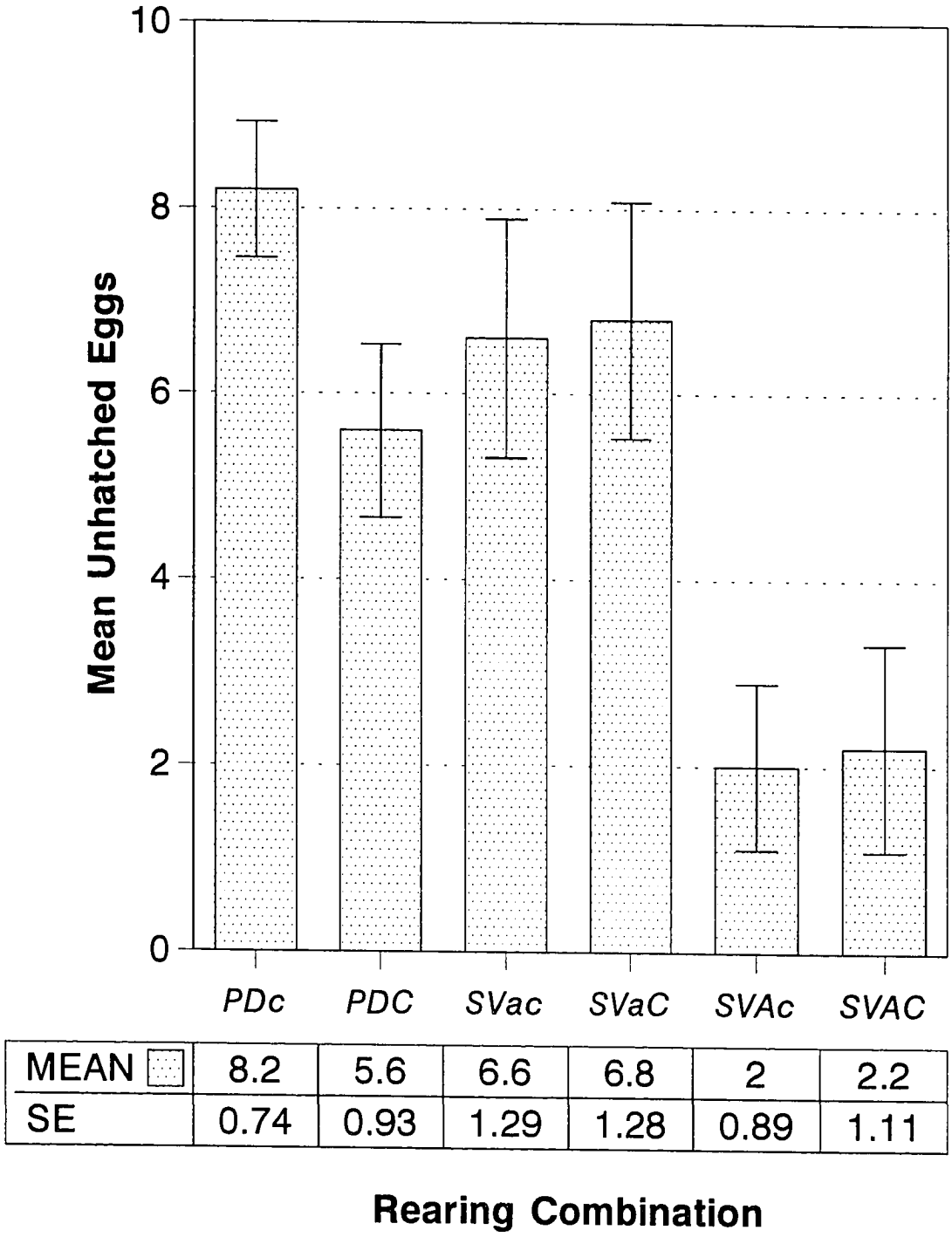

**Figure 4.1.** Schematic of aerated scintillation vial system.

## RESULTS

### Unhatched Eggs

Raw data results of the rearing combination evaluation for unhatched eggs are displayed in Figure 4.2. The data are expressed as mean number of unhatched eggs per replicate $\pm$ standard error of the mean. From these results it is evident that the aerated scintillation vials resulted in fewer unhatched eggs than the unaerated vials and Petri dish treatments. In order to determine if there was a significant treatment effect and/or an effect of changing the rearing medium on unhatched eggs, a two-way ANOVA was performed. The results of this ANOVA procedure are presented in Table 4.2. The data output suggests that there was a significant effect of treatment type (i.e., *PD*, *SVa*, and *SVA*) on unhatched eggs at the 0.0001 level, but no significant effect of changing the solution. Further, it is apparent that there was not a significant interaction between treatment and changing solution testing at the 0.01 level.

Duncan's Multiple Range Test was employed to ascertain treatment effect on unhatched eggs as this was the only main effect detected as being significant in the ANOVA. The Duncan's output for unhatched eggs is presented in Table 4.3. The results indicate that the Petri dish treatments were not significantly different from the unaerated scintillation vial treatments (model predicted 69% and 67% percent unhatched, respectively), however, the aerated scintillation vial treatments were significantly different from these two at the 0.05 level (model predicted 21% unhatched). Hence, the data indicate that the aerated scintillation vials are superior to the other tests in terms of unhatched eggs. Changed and unchanged for




| | PDc | PDC | SVac | SVaC | SVAc | SVAC |
|---|---|---|---|---|---|---|
| MEAN | 8.2 | 5.6 | 6.6 | 6.8 | 2 | 2.2 |
| SE | 0.74 | 0.93 | 1.29 | 1.28 | 0.89 | 1.11 |

**Figure 4.2.** Results of rearing combination treatment for unhatched eggs. Results are replicate means ± standard errors of each mean.

**Table 4.2.** SAS output of ANOVA procedure for dependent variable unhatched eggs.

| Source | Degrees of Freedom | F Value | PR > F |
|---|---|---|---|
| Treatment | 2 | 13.13 | 0.0001 |
| Changed | 1 | 0.72 | 0.4051 |
| Treatment*Changed | 2 | 1.16 | 0.3295 |

Model Predicted Data
Model F = 5.86 PR > F = 0.0011
$R^2$ = 0.5497
Coefficient of Variation = 45.28
Root Mean Standard Error = 2.370
Unhatched Mean = 5.233

**Table 4.3.** Duncan's Multiple Range Test output for dependent variable unhatched eggs.

| Duncan Grouping | Mean Unhatched Eggs Per Replicate | Number of Replicates Tested | Treatment Type |
|---|---|---|---|
| A | 6.900 | 10 | *PD* |
| A | 6.700 | 10 | *SVa* |
| B | 2.100 | 10 | *SVA* |

Model Predicted Data
$\alpha$ = 0.05
Degrees of Freedom = 24
Mean Standard Error = 5.617

**Note:** Means with the same letter for Duncan's Grouping are not significantly different.

each treatment type were lumped for the Duncan's analysis as the ANOVA data presented in Table 4.2 indicated that there was no significant effect of changing or not changing the rearing medium.

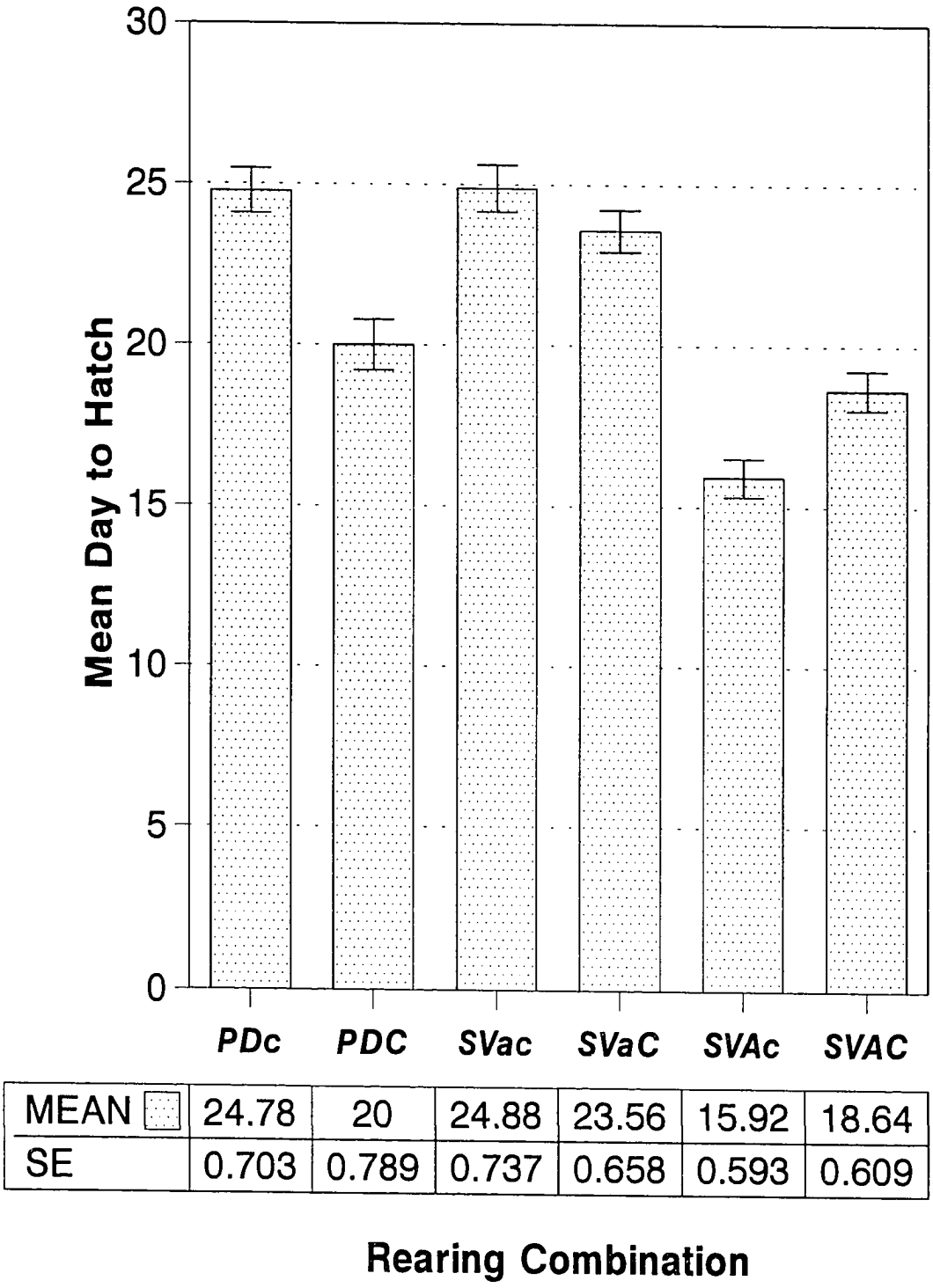
Hatched Eggs

Given the results for unhatched eggs by treatment, the next phase of the data evaluation examined the effect of culture combination on day-to-hatch for those eggs that hatched within the duration of the test. The data are expressed as mean day-to-hatch per ten egg replicate $\pm$ standard error of the mean (Figure 4.3).

The ANOVA indicates that if just the main effects of treatment and change are evaluated, there is a significant effect of treatment, but the effect of change is insignificant (Table 4.4). Further, there is a highly significant interaction between treatment and change (i.e., the effect of changing the medium affects some of the treatments more than others).

One of the limitations of the two-way ANOVA procedure is that when a significant interaction is detected, one cannot look at the main effects to determine which variable is resulting in the variance (i.e., treatment versus change). Therefore, it was necessary to complement and confirm the ANOVA findings with more specific regression analyses using dummy variables to represent treatment effects. The first regression analysis, a stepwise regression, suggested which specific treatment combinations were important (tested at the 0.01 level). Six steps were included in the regression based on the number of significant variables. A condensed listing of these steps is presented in Table 4.5.

Step 1 of the regression indicates that approximately 34 % of the variance is accounted for by the aerated scintillation vial treatments (*SVA*) relative to the intercept which is the default model grand mean for all treatments. Additional variables explain less of the total variance as they are added to the regression analysis. The




| | PDc | PDC | SVac | SVaC | SVAc | SVAC |
|---|---|---|---|---|---|---|
| MEAN | 24.78 | 20 | 24.88 | 23.56 | 15.92 | 18.64 |
| SE | 0.703 | 0.789 | 0.737 | 0.658 | 0.593 | 0.609 |

**Figure 4.3.** Results of rearing combination treatment for variable day-to-hatch. Results are replicate means ± standard errors of each mean.

*SVA* variance is apparent based on the fact that the model predicts these treatment eggs to hatch 5.59 days earlier than the mean for all treatments. Step 2 adds the *SVAc* treatment which only accounts for approximately 4.5% of the variance. This component of the *SVA* treatment group tends to have a shorter predicted hatch date than the overall treatment mean. The third regression step adds the unchanged element for all treatment combinations and accounts for approximately 5.5% of the model variance. In general, it appears that across all treatments, the unchanged component tends to result in a somewhat longer hatch rate ($\approx$3.3 days) except for *SVAc* which is predicted at significantly less than the Intercept ($\approx$6.1 days). This suggests that, in general, the unchanged treatments take longer to hatch, which may be a function of oxygen content and/or agitation.

The unaerated scintillation vial (*SVa*) treatment is incorporated in step 4 of the regression. When testing at the 0.01 level, *SVA* now becomes insignificant. This appears to be due to the fact that the effect of *SVA* on the model is an interaction effect. The unchanged treatment (*SVAc*) appears to demonstrate an interaction resulting in a faster rate of hatching. *SVAC* apparently is not significantly different from the mean so that upon consideration of the *SVAc* treatment group, the effect of the other parameters of the treatment effect become less important and insignificant at the 0.01 level. Very little of the variance is explained in steps 5 and 6 of the regression, therefore, interpretation will stop at the step 4 level.

The stepwise regression was utilized to determine which parameters were important. The next logical step was to put these parameters together in the same regression model to determine if the stepwise model is correct (e.g., is the regression

**Table 4.4.** SAS output of ANOVA procedure for dependent variable day-to-hatch.

| Source | Degrees of Freedom | F Value | PR > F |
|---|---|---|---|
| Treatment | 2 | 50.63 | 0.0001 |
| Changed | 1 | 1.09 | 0.2979 |
| Treatment*Changed | 2 | 11.98 | 0.0001 |

Model Predicted Data
Model F = 25.26 PR > F = 0.0001
$R^2$ = 0.4797
Coefficient of Variation = 17.68
Root Mean Standard Error = 3.494
Mean Day-to-Hatch = 19.77

**Table 4.5.** Stepwise regression procedure for the dependent variable day-to-hatch.

**STEP 1** Action: Variable *SVA* entered $R^2$ = 0.3440

| Variable | B Value | Standard Error | F Value | PR > F |
|---|---|---|---|---|
| Intercept | 22.86 | | | |
| *SVA* | -5.593 | 0.6504 | 73.95 | 0.0001 |

**STEP 2** Action: Variable *SVAc* entered $R^2$ = 0.3893

| Variable | B Value | Standard Error | F Value | PR > F |
|---|---|---|---|---|
| Intercept | 22.86 | | | |
| *SVA* | -4.218 | 0.7608 | 30.75 | 0.0001 |
| *SVAc* | -2.716 | 0.8428 | 10.39 | 0.0016 |

**STEP 3** Action: Variable Unchanged entered $R^2$ = 0.4431

| Variable | B Value | Standard Error | F Value | PR > F |
|---|---|---|---|---|
| Intercept | 21.50 | | | |
| *SVA* | -2.859 | 0.8181 | 12.21 | 0.0006 |
| *SVAc* | -6.062 | 1.219 | 24.72 | 0.0001 |
| Unchanged | 3.346 | 0.913 | 13.42 | 0.0004 |

**Table 4.5.** Continued.

**STEP 4** Action: Variable *SVa* entered $R^2 = 0.4663$

| Variable | B Value | Standard Error | F Value | PR > F |
|---|---|---|---|---|
| Intercept | 20.56 | | | |
| *SVA* | -1.924 | 0.8897 | 4.68 | 0.0323 |
| *SVAc* | -5.545 | 1.216 | 20.78 | 0.0001 |
| Unchanged | 2.829 | 0.9219 | 9.42 | 0.0026 |
| *SVa* | 2.219 | 0.9060 | 6.00 | 0.0156 |

**STEP 5** Action: Variable *PDc* entered $R^2 = 0.4797$

| Variable | B Value | Standard Error | F Value | PR > F |
|---|---|---|---|---|
| Intercept | 20.00 | | | |
| *SVA* | -1.359 | 0.9318 | 2.13 | 0.1470 |
| *SVAc* | -4.036 | 1.449 | 7.76 | 0.0061 |
| Unchanged | 1.320 | 1.217 | 1.18 | 0.2801 |
| *SVa* | 3.562 | 1.148 | 9.63 | 0.0023 |
| *PDc* | 3.458 | 1.842 | 3.52 | 0.0626 |

**STEP 6** Action: Variable Unchanged removed $R^2 = 0.4752$

| Variable | B Value | Standard Error | F Value | PR > F |
|---|---|---|---|---|
| Intercept | 20.00 | | | |
| *SVA* | -1.359 | 0.9323 | 2.12 | 0.1472 |
| *SVAc* | -2.716 | 0.7869 | 11.91 | 0.0007 |
| *SVa* | 4.242 | 0.9624 | 19.43 | 0.0001 |
| *PDc* | 4.778 | 1.384 | 11.92 | 0.0007 |

$R^2$ value similar to the stepwise figure?), to provide parameter estimates (coefficients) for the treatments, and to describe the most parsimonious and best model for the data.

The stepwise procedure indicated that the effect of the *SVA* treatment was most amplified when the rearing medium was not changed. This was reinforced by the first regression when it was placed in the context of all treatment combinations using

the Petri dish treatment (*PD*) as the default (i.e., Intercept; Table 4.6). When compared to the Intercept mean, *SVa* and unchanged for all treatments appeared to produce a longer time to hatch (3.6 and 4.7 days, respectively). The *SVAc* treatment resulted in a significantly shorter hatch date parameter estimate of almost 7 days less than the model default. The *SVa* treatment produced a reduced mean hatching date, however, the result was insignificant at the 0.01 level. Finally, as indicated in the stepwise regression, the *SVA* treatment was insignificant, again suggesting that it is the unchanged parameter of this treatment which is responsible for the effect on hatching date.

A second regression was performed which excluded those variables that were not significant in the stepwise regression model in order to produce the best possible model. Therefore, only those treatments that seemed important (i.e., significant at the 0.01 level) in step 4 of the stepwise regression were included (*SVa*, *SVAc*, and unchanged for all treatments; see Table 4.5). A condensed output for this regression is presented in Table 4.7. The data output is followed by an interpretive table of the findings (Table 4.8) which displays the model predicted mean day-to-hatch values for each treatment combination and the percent change for these treatments relative to the Petri dish changed default.

## DISCUSSION

Investigators have shown that the medaka egg contains hatching glands which form on days three to four of development in the mouth and pharyngeal cavities as well as on the inner surface of the embryo operculum (Leung and Bulkley, 1979).

**Table 4.6.** Initial regression output of all treatment combinations.

| Variable | Degrees of Freedom | Parameter Estimate | Standard Error | PR > \|T\| |
|---|---|---|---|---|
| Intercept | 1 | 20.00 | 0.7450 | 0.0001 |
| *SVA* | 1 | -1.359 | 0.9320 | 0.1470 |
| *SVAc* | 1 | -7.494 | 1.591 | 0.0001 |
| Unchanged | 1 | 4.778 | 1.383 | 0.0007 |
| *SVa* | 1 | 3.562 | 1.148 | 0.0023 |
| *SVac* | 1 | -3.458 | 1.842 | 0.0626 |

Model Predicted Data
Model F = 25.26 PR > F = 0.0001
$R^2$ = 0.4797
Coefficient of Variation = 17.68
Root Mean Standard Error = 3.494
Mean Day-to-Hatch = 19.77

**Table 4.7.** Secondary regression output for significant treatments from the stepwise regression procedure.

| Variable | Degrees of Freedom | Parameter Estimate | Standard Error | PR > \|T\| |
|---|---|---|---|---|
| Intercept | 1 | 19.41 | 0.4421 | 0.0001 |
| *SVAc* | 1 | -6.921 | 1.051 | 0.0001 |
| Unchanged | 1 | 3.430 | 0.8908 | 0.0002 |
| *SVa* | 1 | 3.060 | 0.8293 | 0.0003 |

Model Predicted Data
Model F = 37.64 PR > F = 0.0001
$R^2$ = 0.4482
Coefficient of Variation = 18.07
Root Mean Standard Error = 3.573
Mean Day-to-Hatch = 19.77

**Table 4.8.** Interpretation of the secondary regression output. Absolute values are in days-to-hatch; percent change values are as compared to the Petri dish changed default.

| Treatment | Absolute Predicted Values | Percent Change |
|---|---|---|
| *PDC* | 19.41 | -- |
| *PDc* | 19.41+3.43 = 22.84 | (+)17.7 |
| *SVaC* | 19.41+3.06 = 22.47 | (+)15.8 |
| *SVac* | 19.41+3.06+3.43 = 25.90 | (+)33.4 |
| *SVAC* | 19.41 | 0.0 |
| *SVAc* | 19.41+3.43+(-6.92) = 15.92 | (-)18.0 |

Just prior to a hatching event, the hatching enzyme located in these glands is secreted inside the egg where it immediately begins to dissolve the inner layers of the egg chorion. This dissolution, coupled with increased activity of the fully developed embryo, results in hatching. In natural environments this enzyme then becomes available to the neighboring eggs and may stimulate coincident hatching events through partial degeneration of the outer chorion, coupled with the normal embryonic pre-hatch activities.

Numerous investigators have chosen to isolate embryos in micro-vessels during biological studies with the medaka and other fish species. However, when employing day-to-hatch evaluation, this technique may not be suitable due to the absence of what may be an essential hatching stimulant. If the objectives of the study design are to determine the effects of treatments on laboratory-reared eggs which are to represent natural exposures and a primary endpoint is day-to-hatch, then this factor should be strongly considered.

Operating under the assumption that exposure to neighboring eggs is essential

for synchronous hatch events in control organisms, this evaluation focused on two different types of rearing vessels which provide similar volumes, but dissimilar levels of contact between adjacent eggs. In addition, the variables of rearing solution change and test container aeration were incorporated into the study to determine which combination of container and rearing conditions provided the shortest hatch rate with the fewest unhatched eggs. An added intent was to develop a protocol which would provide these desired conditions, but require a low level of technician effort for routine rearing purposes.

One of the most significant findings from this study was the number of unhatched eggs and the prolonged mean hatch dates for all treatments as compared to other studies conducted in this laboratory under similar conditions (see Chapters 2, 5 and 6). The breeding adults employed for these studies had been subjected to continuous breeding conditions (e.g., altered light-cycle and high temperatures) for over 6 months prior to initiation of this study. The data presented here indicate that these breeding sets appear to produce less viable eggs following extended periods under stressful, simulated breeding conditions. However, all treatment combinations were performed concurrently with the same group of stock breeders during this study and, therefore, the delays in hatching rate and number of unhatched eggs should not have affected the results of the treatments relative to each other. This evidence that the quality of eggs produced by breeding adults diminishes with time further supports the need for regular evaluation of a standard reference toxicant for the MELA assay to detect these changes in egg quality (see Chapter 8).

The results of this study indicate that the use of the scintillation vial alone does not provide significantly better hatching percentages and rates over Petri dish culture

vessels. Therefore, close contact alone may not be the primary stimulus for synchronous hatching events. Incorporation of other "natural" stimulants such as the presence of consistent and sufficient oxygen levels do, however, appear to have a significant effect on coincident egg hatching events within a given "cluster" of eggs.

The data indicate that there was a significant treatment affect (i.e., Petri dish vs. scintillation vial, with and without air), but that solution change and the interaction between this change and treatment type did not have a significant affect on the number of unhatched eggs. Therefore, it is evident that even before evaluating the effects of these variables on the hatching rate of the medaka eggs, the type of container and the presence of externally introduced oxygen can have a significant impact on whether or not the eggs will even hatch. The results indicate that the aerated scintillation vials had significantly fewer unhatched eggs than both the Petri dish treatments and the unaerated scintillation vials.

Similarly, the day-to-hatch evaluations suggested that the aerated scintillation vials provided the shortest mean hatch day for those eggs that hatched within the 30 day test period. The statistical evaluation of these data further determined that, even though unchanged vessels resulted in a longer day to hatch for all treatments, when the solution was changed daily in the aerated vials, the predicted mean hatch date was actually longer than those aerated vials which did not receive fresh solution. In other words, the effect of the aerated scintillation vial seems to be most amplified when the solution is not changed. Therefore, a significant interaction exists between solution changes and treatment type when the endpoint is hatch date. In general, the unchanged treatments resulted in longer hatch times, but it may be a function of oxygen content and/or agitation provided by the aeration. When the vials are aerated

it may stimulate the release of hatching enzyme in the initial hatch, but if the solution is subsequently changed, the hatching enzyme may be washed from the container prior to stimulating concurrent hatching events.

It appears that oxygen stress in the Petri dish treatments and the unaerated scintillation vials may be too great to induce regular, synchronous hatching events. Further, when considering only the aerated scintillation vial treatment, it is apparent that it is beneficial to refrain from changing the rearing solution during the test period.

This study was carried out utilizing the rearing solution recipe employed in the range-finding studies discussed in Chapter 2. When using this solution it is apparent that the *SVAc* culture combination should be used. However, upon increasing the salt content of the rearing solution for high salinity rearing conditions, the aeration of static (i.e., unchanged) rearing solution may be insufficient to maintain a dissolved oxygen concentration in which untreated eggs will thrive. Should this occur in the medium and high salinity studies discussed in Chapters 5 and 6, the *SVAC* option may be required to reduce low oxygen stress through renewal of the solution with fresh, oxygen-saturated medium.

## CHAPTER SUMMARY

A series of experiments was conducted to define the optimum rearing conditions (i.e., those providing the fastest hatch rate while still producing normal, viable fry) for medaka eggs. Three variables (rearing vessel type [scintillation vial or Petri dish], aeration and solution changes) were evaluated for their effects on the endpoints day-to-hatch and number of unhatched eggs. For all treatment options, multiple eggs were housed in each culture vessel, as it was hypothesized that the presence of a hatching enzyme is necessary for normal, synchronous hatching events. The data were statistically evaluated using an approach which included ANOVA, Duncan's Multiple Range Test, and regression models.

The results indicated that aerated scintillation vials produced the fastest hatch rates and the lowest number of unhatched eggs. However, the data indicated that if the solution in the aerated vials was changed daily, the rate was significantly prolonged and the number of unhatched eggs increased. The aeration process appears to stimulate the first hatching events either through increased oxygen content of the solution or through the agitation provided by the aeration. If the solution is subsequently changed, it may be that the hatching enzyme is washed from the vial and, therefore, is not present to initiate subsequent hatching events in neighboring eggs.

# CHAPTER FIVE

## Salinity-Related Response of Topical and Immersion Exposures to Pentachlorophenol

## INTRODUCTION

Pentachlorophenol (PCP) is a broad spectrum biocide first introduced as a wood preservative in 1936 (Lu *et al.*, 1978). Since its introduction into the biocide industry, PCP has also been used as a fungicide, herbicide, insecticide, molluscicide and defoliant (Schimmel *et al.*, 1978). Aside from direct biocidal employment (including use in antifoulant paints), PCP has been used in adhesives, leather, petroleum drilling mud and well completion fluids, cooling tower water, rope and burlap (Borthwick and Schimmel, 1978; Holcombe *et al.*, 1982). The careless application of PCP to crops, and negligent handling of PCP-treated lumber, has resulted in widespread contamination by this substance in riverine and estuarine waters throughout the United States and other countries (Cirelli, 1978). This neglect, in addition to PCP's relative resistance to degradation, has resulted in global distribution of this substance in water, sediment and in the tissue of resident organisms (Lu *et al.*, 1978; Fisher, 1986).

Pentachlorophenol elicits its toxic effects as a metabolic stressor, primarily through the uncoupling of oxidative phosphorylation (the ADP to ATP formation process) - a target site common to all aerobic organisms (Webb and Brett, 1973; Fisher, 1986). However, a negative response has been observed in tests of mutagenicity (e.g., Ames *S. typhimurium* assay, *E. coli*, *D. melanogaster*) and PCP has not been classified for carcinogenicity (U.S. EPA, 1985). The uncoupling of oxidative phosphorylation in fish embryos (i.e., in the absence of exogenous food) results in less efficient use of the yolk energy source, leading to embryo and/or fry mortality (Dave, 1984). The biological half-life of PCP to elicit its toxic effects is

approximately 10 to 20hr; detoxification and elimination are primarily carried out through sulfation or glucuronidation at the phenolic OH or oxidative dechlorination, followed by excretion through renal, branchial and biliary routes (Hamilton *et al.*, 1986).

Pentachlorophenol has previously been shown to be toxic to most life stages of several aquatic species; the median lethal concentration ($LC_{50}$) typically lies between 50 and 500$\mu$g PCP/l and the maximum acceptable toxicant concentration (MATC) tends to be approximately one order of magnitude lower (Borthwick and Schimmel, 1978; Cleveland *et al.*, 1982; Holcombe *et al.*, 1982; Gupta, 1983; Dave, 1984; Johansen *et al.*, 1985; Ozretic and Krajnovic-Ozretic, 1986; Servizi *et al.*, 1988). Further, it appears that early life stages are somewhat more sensitive to this toxicant than the later adult stages of select fish species (Borthwick and Schimmel, 1978; Hodson and Blunt, 1980).

As eluded to in the introduction of this dissertation, little information exists with respect to the variability in effects of chemicals on organisms reared in environments of differing salinities. Pentachlorophenol is one of the few chemicals to receive recent attention with respect to salinity-related differences of uptake and elimination in fish (Tachikawa *et al.*, 1991). These authors found that adults of the medaka accumulated and eliminated PCP at significantly different rates, depending on the salinity of the system. They determined that seawater-acclimated medaka accumulated less PCP and eliminate PCP and its metabolites faster than individuals reared in freshwater. The present study focused on the salinity-related effects of PCP relative to those results discussed in the freshwater experiments presented in Chapter 2. In addition, the bioconcentration factor (BCF) for PCP was applied to the topical

treatment doses to estimate immersion medium concentrations, allowing a side-by-side evaluation of a calculated PCP BCF value of 770 (Veith *et al.*, 1979) as it represents medaka eggs, as well as providing a laboratory validation of the ability of the topical treatments to be extrapolated to medium concentrations. Eggs were exposed over a wide range of concentrations, based on the $ED_{50}$ determined in the freshwater range-finding study, and were reared employing the results of Chapter 4 (i.e., scintillation vial, aerated rearing environment). The results of the permeability factor for PCP presented in Chapter 3 were applied to the nominal topical treatment concentrations to develop toxicity data based on absolute dose concentrations.

## MATERIALS AND METHODS

Adult medaka were reared in accordance with the methods described in Chapter 2 except that the aquarium water was adjusted to 20 parts per thousand (ppt) salinity with HW MARINEMIX brand seasalts (Hawaiian Marine Imports, Inc., Houston Texas). Twenty ppt embryo rearing solution was prepared through enrichment of each 500ml batch of freshwater embryo rearing solution (see Table 2.3) with 10g of seasalts. Salinity was confirmed employing a calibrated, portable refractometer (Bio-Marine Aquafauna, Hawthorne, CA). Following complications during several series of experiments, it was determined that the pH of the seasalt-enriched rearing solution may vary over 3 pH units, resulting in excessive stress and frequent mortality in control eggs. Consequently, the pH was monitored using a calibrated Orion Model 301 pH Meter for each batch of solution and, if necessary, the pH was adjusted to 7.5 $\pm$ 0.1 with hydrochloric acid:water (1:1) or sodium hydroxide:water (1:1). The rearing solution was stored at 4 $\pm$ 2°C in the dark.

In addition to the salinity-related pH problems, parasitic contamination (both protozoan and nematode) was found to occur in eggs reared at high salinity. In order to remove these organisms, eggs were placed on a 250$\mu$m mesh nitex net and washed with 20ppt artificial seawater. Eggs were then separated and placed into Petri dishes containing fresh rearing solution until treatment.

**Topical Treatment**

Topically treated individuals were exposed employing the procedures discussed in Chapter 2. The topical dose concentrations were 12.5, 1.25, 0.625, 0.312, 0.125, 0.062, 0.031, and 0.012$\mu$g PCP/egg. Three replicates of ten eggs each were surveyed for each concentration. Untreated and DMSO (negative) controls were evaluated in conjunction with all treatments at a frequency of $>10\%$ of all treatments and were randomly treated and distributed throughout the rearing incubator. As with the previous studies, any treatment series accompanying a control group which displayed more than 10% adverse effects was halted and re-evaluated with a new group of control replicates.

Following treatment, the eggs were reared in aerated scintillation vials containing 20ml of the 20ppt rearing solution based on the results of the Chapter 4 culture vessel experiments. The volume of the rearing solution was adjusted to 20ml daily, particularly following removal of dead eggs and/or egg cases from hatched fry. Breathing quality air was introduced into each vial at approximately 120 bubbles per minute through a Pasteur pipet. This vigorous aeration was necessary as the higher salinity rearing solution oxygen level was found to be at only 50% saturation ($\approx$ 4mg/l) under the 60 bubble per minute protocol described in Chapter 4. Though this

increased aeration did not appear to significantly affect control mortality, it was of primary importance that those fry hatching in this environment be transferred as soon as possible to the crystallizing dishes as previously described (Chapter 2) to avoid physical harm or stress.

Each egg replicate was evaluated daily for mortalities, hatching events and test developmental anomalies including those listed in Table 5.1. Deformed eggs and hatch fry which died during the course of the test and those alive at day 21 were preserved in 10% phosphate buffered formalin (Mallinckrodt) for 48hrs, followed by an exchange to 70% ethanol (Analytical Grade, Burdick and Jackson) in water (v:v) for further evaluation and photographic records.

**Immersion Treatment**

Immersion concentrations were developed based on a bioconcentration factor (BCF) of 770 for PCP (from Veith *et al.*, 1979) and the following formula:

$$C_I = \frac{C_D \,/\, E_W}{BCF} \qquad [5.1]$$

where: $C_D$ = Concentration of the topical dose per egg ($\mu$g)
$E_W$ = Weight of egg (g) = 0.000187g
$C_I$ = Immersion concentration ($\mu$g/ml)

Neat PCP was dissolved in DMSO and aliquots were taken to develop the top two concentrations in 20ppt rearing medium. The highest equivalent medium concentration was calculated to be 86,800$\mu$g PCP/l, however, this exceeded the solubility of PCP in freshwater ($\approx$ 80,000$\mu$g PCP/l; The Merck Index, 1989) and, therefore, most likely the solubility in 20ppt medium. Nevertheless, the solution was prepared and that fraction remaining in the particulate phase was allowed to remain in

**Table 5.1.** Developmental anomalies evaluated during daily observations of PCP treated eggs.

| | |
|---|---|
| ·Twinning (e.g., Cephalodidymus) | ·Fin stunting |
| ·Microencephaly | ·Stasis of circulation |
| ·Ophthalmic edema | ·Hemorrhages |
| ·Degeneration of eye cup | ·Blood islands |
| ·Incomplete development of bilateral symmetry of eyes | ·Pericardial edema |
| | ·Rudimentary heart/"Tube-heart" |
| ·Unilateral or bilateral microphthalmia and anophthalmia | ·Enlarged ventricles |
| | ·Reduced or increased heart rate |
| ·Cyclopia | ·Retardation of yolk absorption |
| ·Lordosis | ·Chorionic effects |
| ·Kyphosis | ·Abdominal swelling |
| ·Scoliosis | ·Deranged postural equilibrium |
| ·Rigid Coiling | ·Tetanic convulsions |
| ·Retardation of centrum formation | ·Abnormal pigmentation/"Blacktail" |
| ·Centrum damage (e.g., vertebral dislocations and fractures) | ·Sporadic hyperkinesis |
| | ·Activity level |
| | ·Complete developmental arrest |

the exposure solution similar to the approach discussed by Hawkins *et al.* (1990) for benzo[a]pyrene.

The next lower concentration solution (8,680$\mu$g PCP/l) was prepared and each subsequently lower solution was prepared through dilution of this concentration with clean 20ppt rearing medium. It should be noted that a dilution error was made for the 0.125$\mu$g PCP/egg and lower immersion equivalents (i.e., these lower immersion concentrations were not comparable on a one-to-one basis to the topical doses based on equation 5.1). However, this error was detected, and adjusted for, in the data analysis and interpretation of this study. The final nominal immersion concentrations (in $\mu$g PCP/l) were: 86,800, 8,680, 4,340, 2,170, 1,080, 543, 271, and 136. The immersion treatment groups were reared in aerated scintillation vials.

An estimate was made to determine the amount of PCP that would be taken up by all eggs in a given replicate, if the solution was not changed; for example, 20ml of

the 86,800$\mu$g PCP/l concentration would contain 1,736$\mu$g of PCP. If each egg absorbs 12.5$\mu$g (based on the nominal topical dose), 125$\mu$g (or 7.2%) of this material would be removed from solution. Since this is a significant portion of the PCP removed from the solution, its uptake likely alters the absolute medium concentration. Consequently, the solution was changed daily in each vessel to avoid significant depletion of the medium toxicant concentration. The permeability data presented in Chapter 4 were developed subsequent to these experiments, therefore, that information (i.e., 70% of dose actually permeating the egg) could not be used in this estimated depletion calculation. In order to provide comparability between treatments, the topical treatment vessel solution was also changed daily. Scoring procedures for the immersion treatment eggs were identical to those indicated for the topical treatment.

## Statistics

Probit analysis was employed to determine $ED_{50}$ and $LD_{50}$ values for the topical exposure and the median effective and lethal concentrations ($EC_{50}$ and $LC_{50}$) for the immersion treatment. Chi-squared analysis of two-way contingency tables was used to determine significant differences between controls (DMSO and untreated) and between controls and treatment concentrations. In addition, the data from the two treatment types were compared through arcsin transformation and subsequent statistical analysis employing a regression model (i.e., Proc Reg in SAS). The slopes of the two treatment regressions for lethality and all adverse effects were compared through a test for comparing slopes of regression equations (Sokal and Rohlf, 1969). Finally, chi-squared analysis was used to compare the 20ppt data to the 0ppt salinity

pilot experiment conducted with PCP (see Chapter 2) to determine the effects of salinity on PCP toxicity and teratogenicity.

## RESULTS

The range of concentrations employed in this study was designed to provide data which could be compared to the freshwater experiment conducted with PCP (see Chapter 2) and to focus on the concentrations around the calculated 21-day $ED_{50}$ of 0.056$\mu$g PCP/egg. Egg viability data for the topical treatment are presented in Table 5.2. The results are similar to those seen in the pilot study topical treatment where the higher concentrations displayed primarily lethality which dropped off in the lower concentrations and was partially replaced by sublethal effects. Figure 5.1a further segregates the effects into sublethal and lethal responses for each concentration. The patterned bars represent mean values for the three, ten egg replicates and the smaller, solid bars on top of each represent the standard errors for the group. The results from the 0ppt range-finding treatment are presented in Figure 5.1b for comparison. Though the level of effects is similar between the different salinity topical studies, the lower concentrations tend to contain more lethal effects in the 20ppt treatment that the 0ppt treatment. However, the limited number of data provided by the single replicate, log series range-finding study are not as comprehensive as the 20ppt data, allowing for potential gaps in observed effects. Nevertheless, chi-squared analysis on the concentrations common to both exposures indicated no statistically significant effects of salinity ($p > 0.050$) on overall adverse effects of PCP.

Egg viability data for the immersion treatment are presented in Table 5.3. The data are graphically presented in Figure 5.2 along with the 20ppt topical

treatment data for comparison. The patterns of lethal and sublethal effects are similar between the two treatments, indicating that the BCF of 770 used to prepare the immersion media was a good estimate of the bioconcentration of PCP in medaka eggs and that the topical treatment closely represents natural PCP exposure over a three week period. The lethality dropped off slightly earlier in the immersion concentration series than the equivalent topical concentrations, however, this appeared to be compensated for by increased sublethal effects in the middle and lower exposure concentrations. Regardless, both treatments displayed a "classical" toxicological response, with rapid lethality in the higher concentrations which decreases with reduced concentration and is replaced by an increase, followed by a decrease, in sublethal response.

The change in all adverse effects (i.e., lethal+sublethal), with time, is presented in Figure 5.3 for the topical treatment and Figure 5.4 for the immersion exposure. The data are presented as means of the three replicates for each treatment concentration. In general, the higher concentrations were characterized by early effects, primarily due to rapid lethality. The lower concentrations of both treatments tended to exhibit effects later in development, possibly due to the uncoupling of oxidative phosphorylation which should only be apparent after significant development has taken place, accompanied by an increased need for an energy source. Probit analysis of the data indicated that the topical treatment had a 21-day $ED_{50}$ of 0.062$\mu$g PCP/egg (95% confidence limits were 0.046-0.082) and an $LD_{50}$ of 0.113$\mu$g PCP/egg (0.088-0.145). This topical $ED_{50}$ is very similar to the 21-day $ED_{50}$ calculated in the pilot, freshwater study (0.056$\mu$g PCP/egg), indicating that salinity has little apparent effect on the adverse impact of PCP on medaka eggs. A 0ppt $LD_{50}$ could not be calculated

**Table 5.2.** Adverse effects of PCP on medaka eggs and hatch fry topically treated and reared in 20ppt medium.

| Dose (μg PCP/egg) | Percent Adversely Affected[a] |
|---|---|
| 12.5 | 100 (±0.00) |
| 1.25 | 100 (±0.00) |
| 0.625 | 100 (±0.00) |
| 0.312 | 93.3 (±6.67) |
| 0.125 | 50.0 (±5.77) |
| 0.062 | 53.3 (±3.33) |
| 0.031 | 26.7 (±12.0) |
| 0.012 | 13.3 (±3.33) |
| DMSO Control | 4.44 (±1.76) |
| Untreated Control | 7.78 (±1.47) |

[a] Expressed as replicate mean percentage of eggs affected ± standard error of the mean.

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**Table 5.3.** Adverse effects of PCP on medaka eggs and hatch fry treated through immersion exposure in 20ppt medium.

| Concentration (μg PCP/l) | Percent Adversely Affected[a] |
|---|---|
| 86,800 | 100 (±0.00) |
| 8,680 | 100 (±0.00) |
| 4,340 | 100 (±0.00) |
| 2,170 | 93.3 (±3.33) |
| 1,080 | 93.3 (±3.33) |
| 543 | 40.0 (±10.0) |
| 271 | 23.3 (±6.67) |
| 136 | 23.3 (±6.67) |
| DMSO Control | 6.67 (±3.33) |
| Untreated Control | 6.67 (±3.33) |

[a] Expressed as replicate mean percentage of eggs affected ± standard error of the mean.

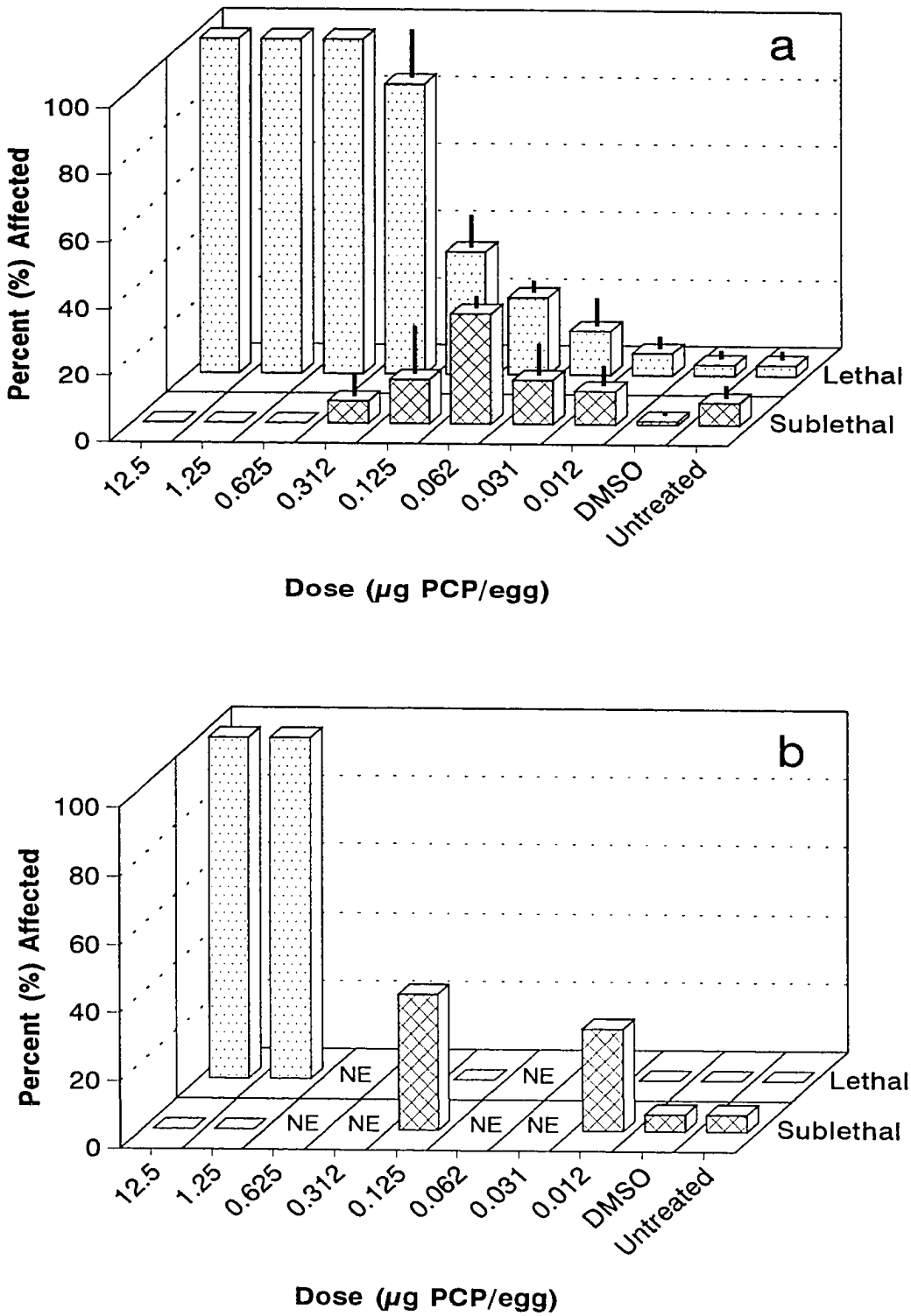
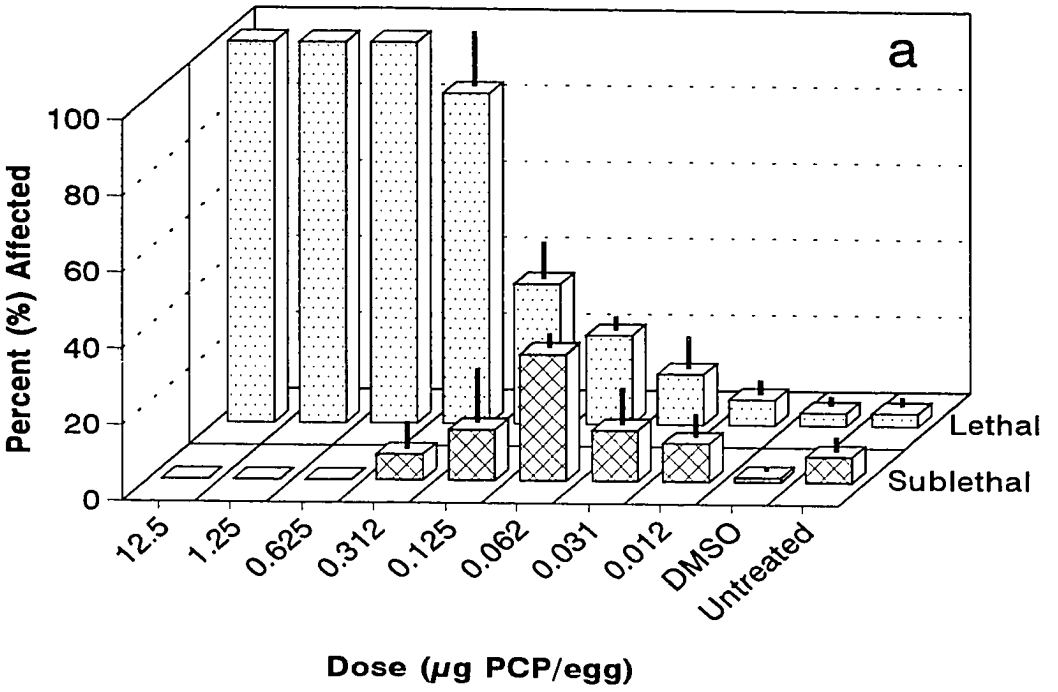



**Figure 5.1.** Breakdown of effects for 20ppt topical treatment results (a) and 0ppt pilot study topical treatment data (b). Values are replicate means ± standard error of each mean. NE indicates that dose was not evaluated.



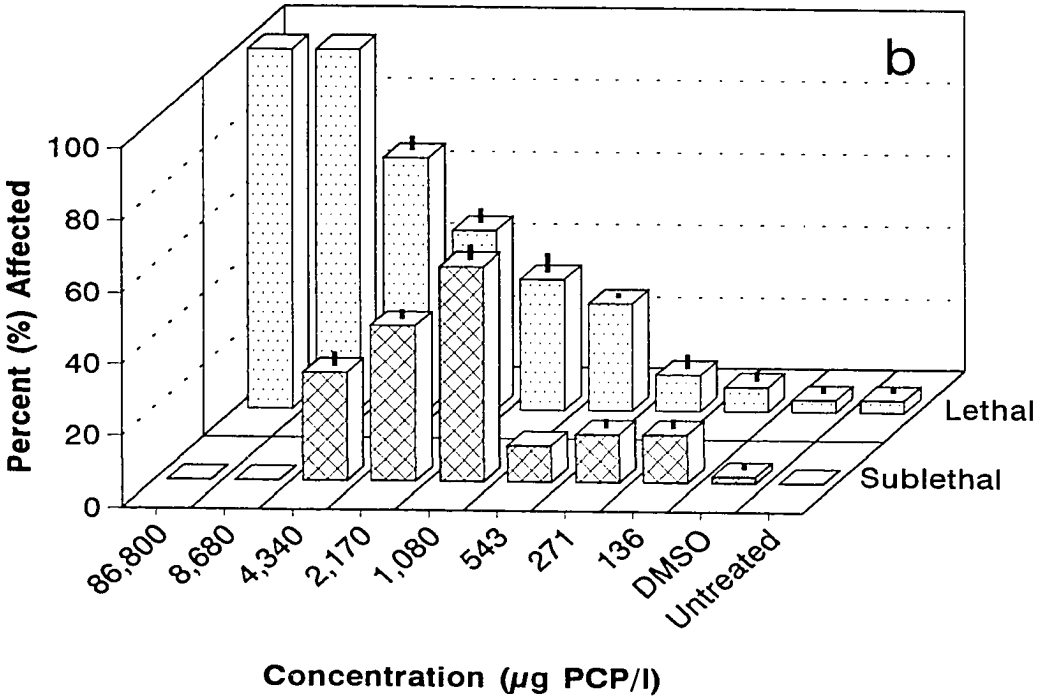




**Figure 5.2.** Breakdown of effects for 20ppt topical treatment results (a) and 20ppt immersion treatment data (b). Values are replicate means ± standard error of each mean.

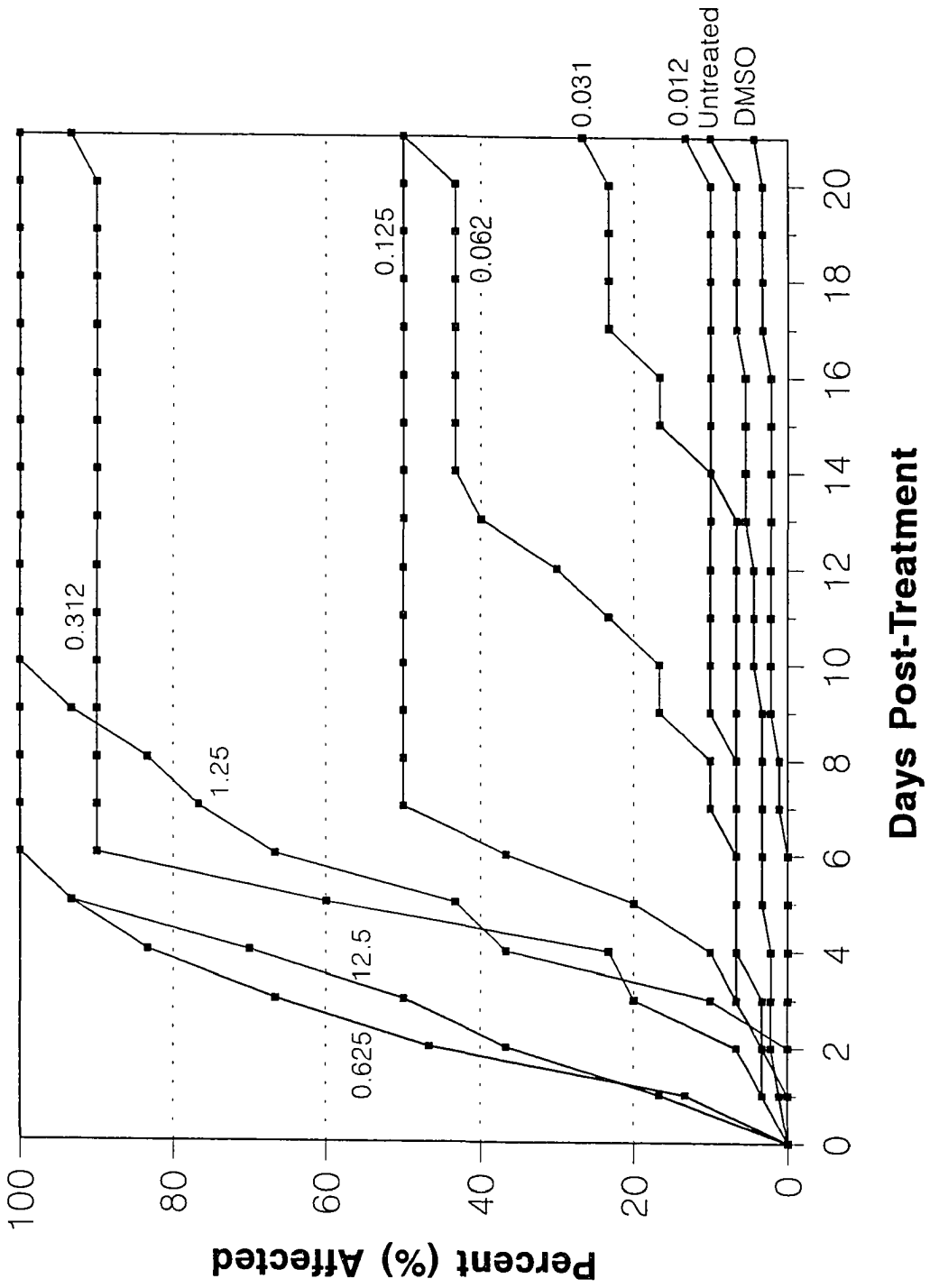



**Figure 5.3.** Time-related changes in all adverse effects on medaka eggs topically treated with PCP. Dose, in $\mu$g PCP/egg, is indicate on each curve. Percent affected values are means of three replicates for each dose.

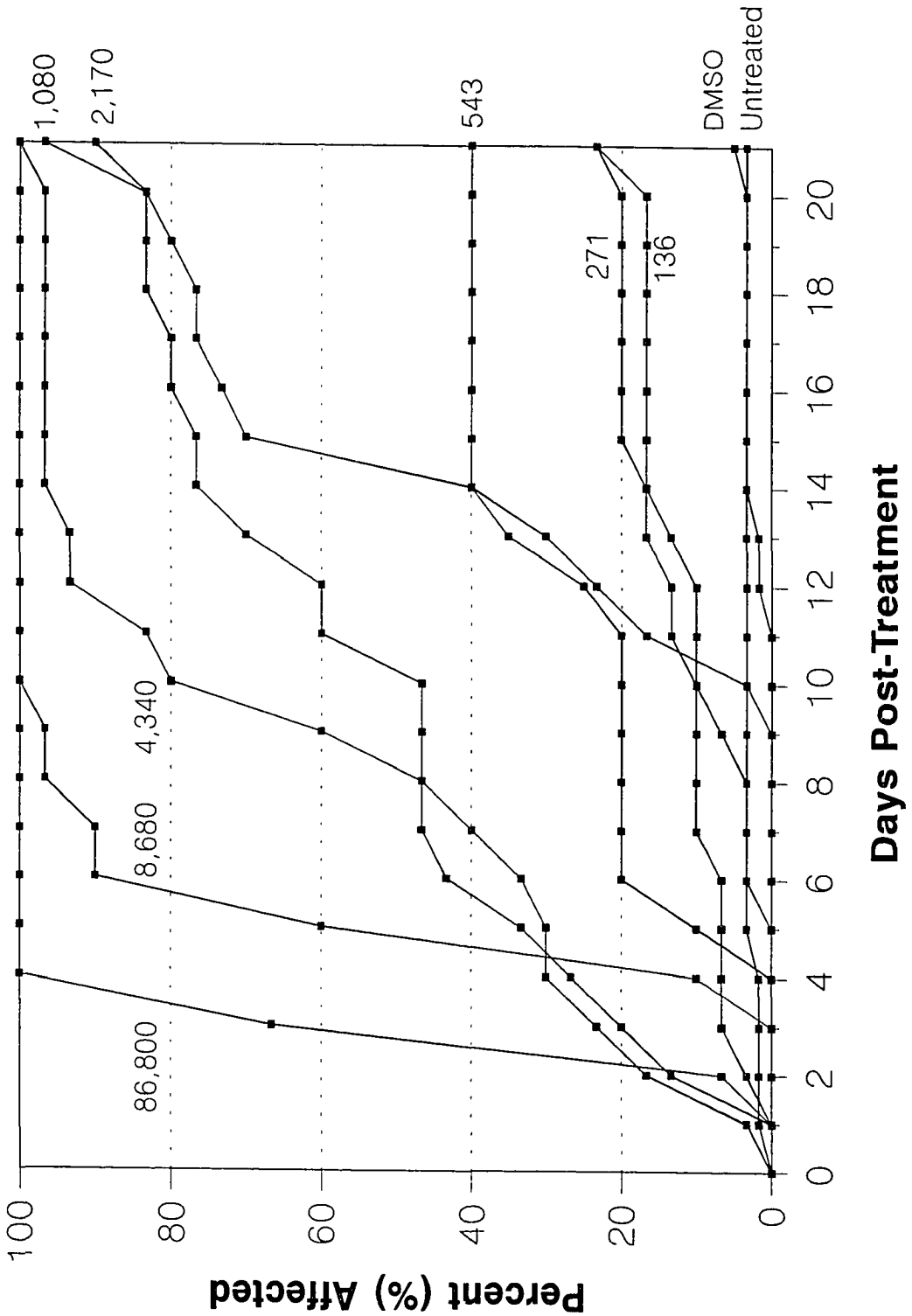



**Figure 5.4.** Time-related changes in all adverse effects on medaka eggs treated through immersion exposure with PCP. Concentration, in $\mu$g PCP/l, is indicate on each curve. Percent affected values are means of three replicates for each concentration.

for comparison (see Chapter 2). Chi-squared analysis indicated no significant difference between the control types ($p>0.05$, $X^2=0.80$) and between the controls and the lowest topical concentration, 0.012$\mu$g PCP/egg ($p>0.050$, DMSO $X^2=3.24$; Untreated $X^2=0.72$), suggesting the maximum acceptable toxicant concentration (MATC), lies between 0.012 and 0.031$\mu$g PCP/egg.

The immersion exposure 21-day $EC_{50}$ was determined to be 409$\mu$g PCP/l (243-643) and the $LC_{50}$ was calculated as 1490$\mu$g PCP/l (1100-2030). Upon applying equation 5.1 to these values (using and egg weight of 0.000187g), approximate topical dose $ED_{50}$ and $LD_{50}$ were calculated to be 0.059$\mu$g PCP/egg and 0.215$\mu$g PCP/egg, respectively. This $ED_{50}$ equivalent is very similar to the 0.056$\mu$g PCP/egg and 0.062$\mu$g PCP/egg $ED_{50}$ values calculated employing the topical procedure in 0ppt and 20ppt media, respectively. As with the topical treatment evaluation, chi-squared analysis determined that their were no significant differences between the control types ($p>0.050$, $X^2=0.0$). The two lowest immersion concentrations, 136 and 271$\mu$g PCP/l, were not statistically significantly different from either control type ($p>0.050$; for 136$\mu$g PCP/l, $X^2=3.74$; for 271$\mu$g PCP/l, $X^2=3.74$), indicating that the MATC for immersion exposure of PCP to medaka eggs is between 271 and 543$\mu$g PCP/l.

To determine how similar the topical response (or the predicted response) and the immersion response (or the nominal response) compared for the 20ppt evaluation, the day 21 data were linearized through arcsin transformation and plotted against dose (expressed in the log form). The immersion concentrations were converted to their topical equivalents and the two data sets were censored to remove 0 and 100% responses, arcsin transformed and regressed against the logarithm of dose for lethal effects (Figure 5.5) and all adverse effects (Figure 5.6). The graphical presentation

indicates that the topical treatment is a good predictor of actual immersion exposure, which represents natural exposure. Regression analysis of these data indicated that the two treatments were not significantly different from each other with respect to lethal effects ($p > 0.0001$) and all adverse effects ($p > 0.0001$). Moreover, neither endpoint indicated a statistically significant interaction between treatment type and dose ($p > 0.0005$). The Sokal and Rohlf (1969) test for comparison of slopes was employed to compare the slopes of the treatment regressions for lethal and all adverse effects. The results indicated that there were no significant differences between the topical and immersion regression slopes for lethality ($p=0.52$, $F=0.47$) or overall negative effects ($p=0.26$, $F=1.54$).

Sublethal effects of the PCP treatments included unhatched eggs, dead fry and developmental deformities. The primary deformities were swollen abdomens, which often resulted in incomplete hatching (similar to those observed in the 0ppt PCP pilot study; Figure 5.7a), developmental arrest of the embryo (Figure 5.7b), slowed heart rate and circulation, pericardial edema and low hatch fry activity level.

## DISCUSSION

The study reported here evaluated a wide range of concentrations of pure pentachlorophenol employing the MELA technique and a typical immersion exposure protocol. The results indicate that PCP has a significant effect on survival of medaka eggs with two apparent modes of action similar to those observed by Dave (1984) in the eggs of *Brachydanio rerio*; one early lethal mode and one slow expressing mode later in development. Based on the cessation of development observed in the higher,

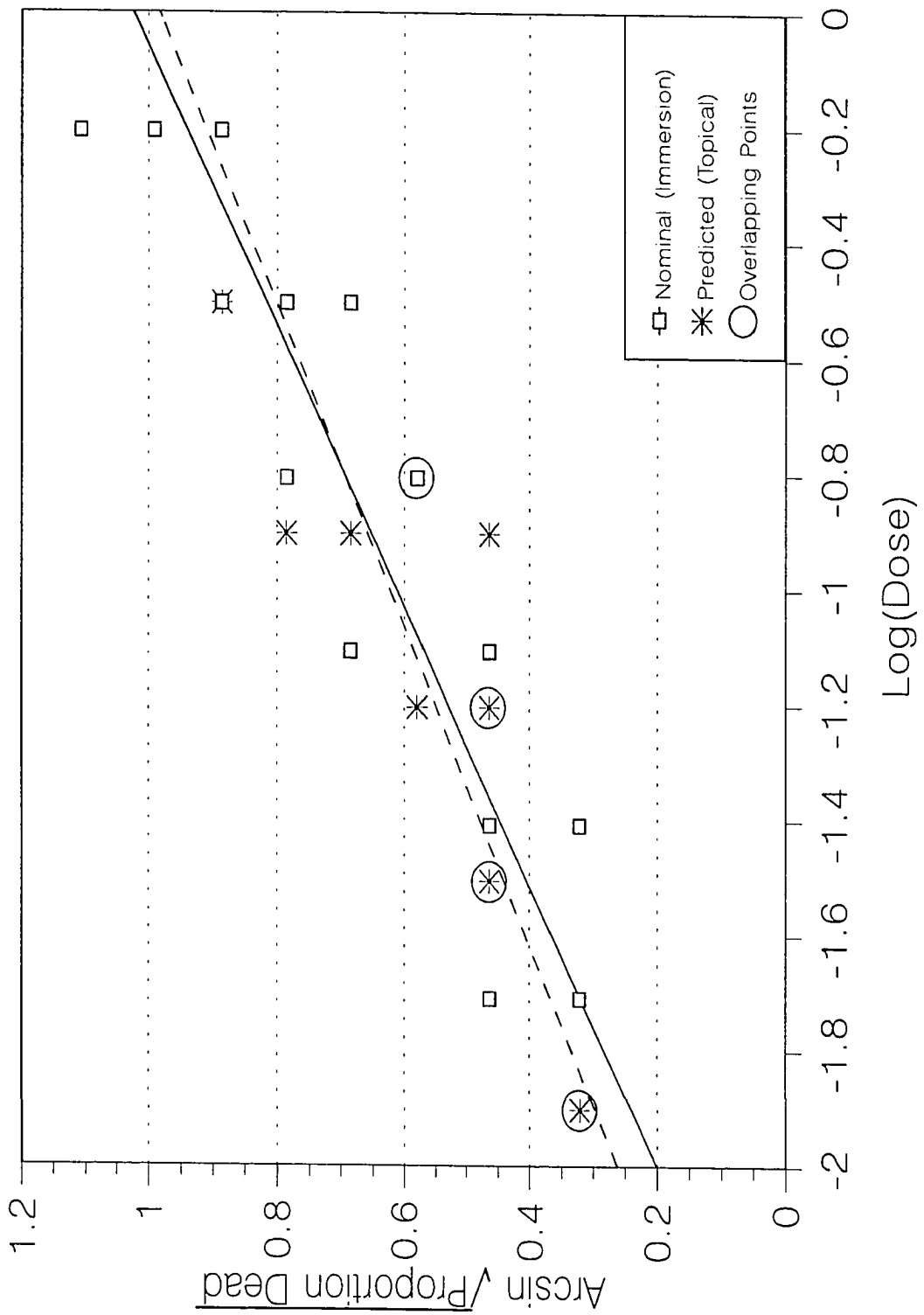



**Figure 5.5.** Regression plots of lethal effects of PCP on topically treated and immersion exposed medaka eggs.

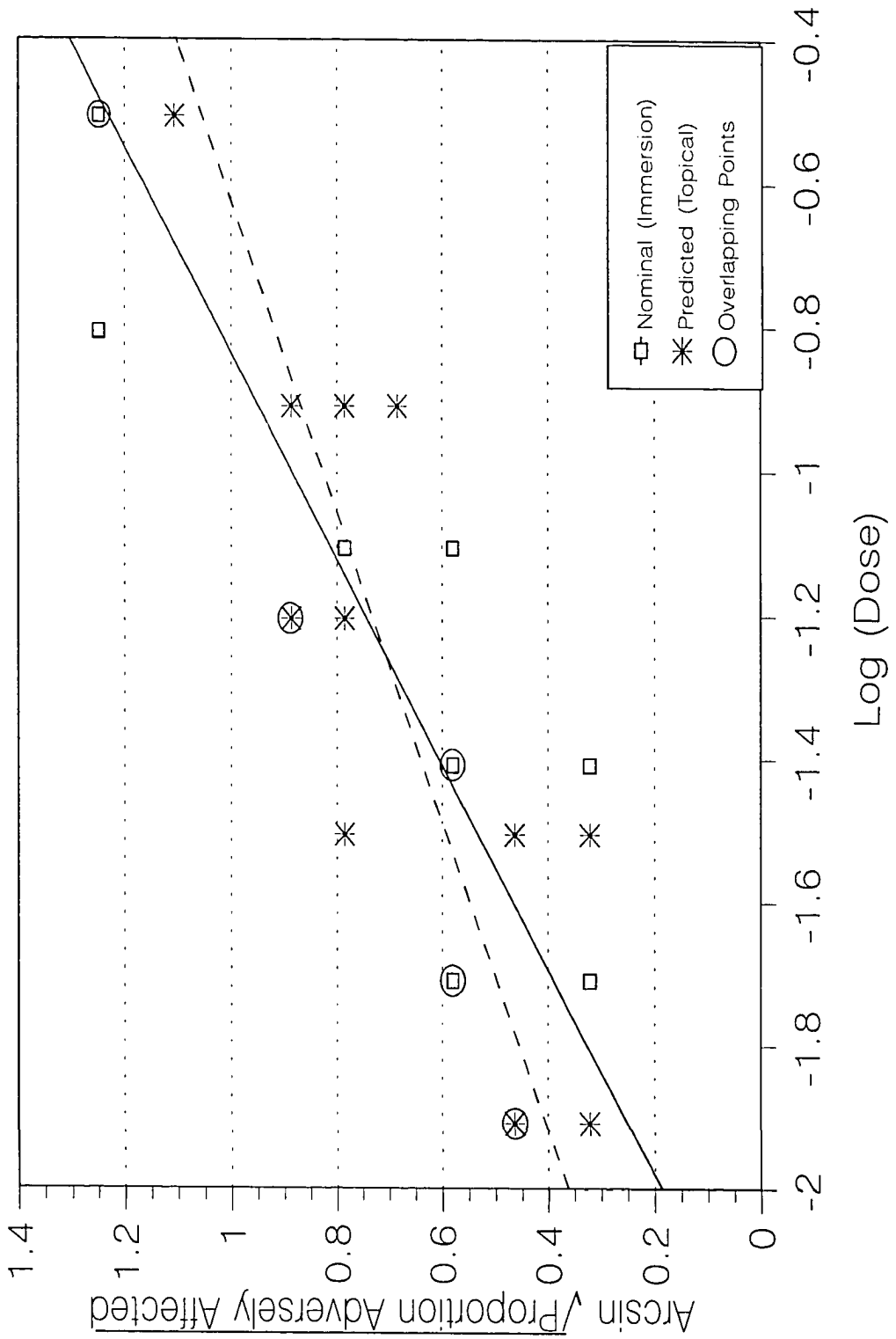



**Figure 5.6.** Regression plots of all adverse effects of PCP on topically treated and immersion exposed medaka eggs.

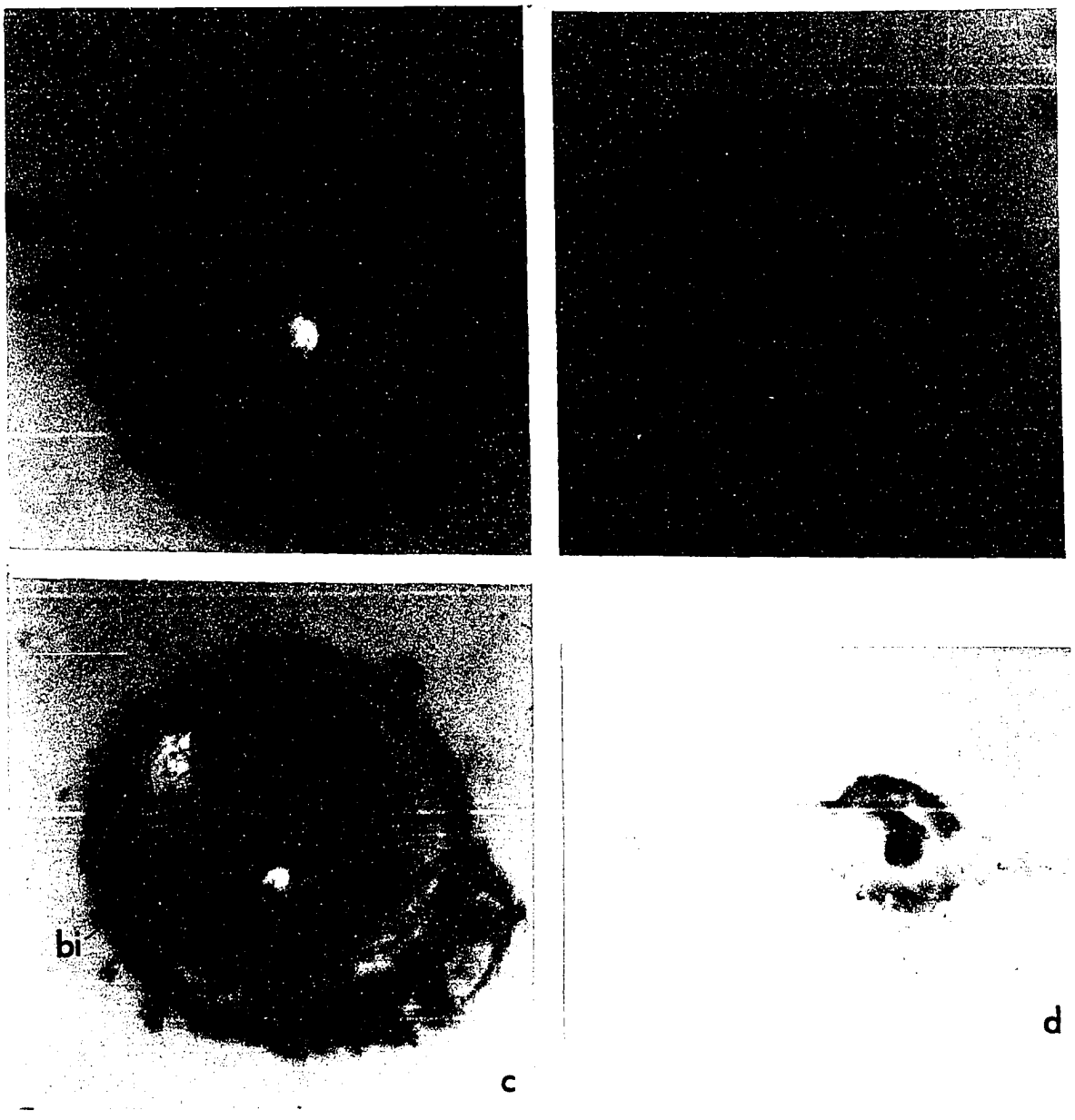



**Figure 5.7.** Developmental deformities common to PCP exposures including: severe developmental arrest and dedifferentiation (a - 8,680$\mu$g/l immersion and b - 12.5$\mu$g/egg topical), underdevelopment and blood islands (bi; c - 1,080$\mu$g/l immersion), and swollen abdomen/yolk sac (d - 543$\mu$g/l immersion).

rapidly lethal concentrations, one might assume that the effect was on overall mechanisms of differentiation and growth. The later expressed effects are most likely due to the documented ability of PCP to uncouple oxidative phosphorylation (Webb and Brett, 1973). Levels of embryotoxicity and fry mortality in the immersion test were significantly lower (considering $LC_{50}$ and $EC_{50}$ confidence limits) than those seen in previous evaluations with fish early life stages with an $LC_{50}$ of 1490$\mu$g PCP/l and an $EC_{50}$ of 409$\mu$g PCP/l. The primary sublethal effects (swollen abdominal region, underdevelopment, pericardial edema, lethargy and delayed hatch) were similar to previous investigations of PCP bioactivity with similar teleost life stages (Holcombe *et al.*, 1982; Ozretic and Krajnovic-Ozretic, 1986). However, others (see, for example, Servizi *et al.*, 1988) observed no abnormal development in fish early life stages exposed to PCP.

These data also indicate that the MELA topical treatment competently represents natural *in situ* exposure under simulated 20ppt salinity conditions over a 21-day period. A 21-day $ED_{50}$, adjusted for the 70.0% mean permeability factor calculated for PCP in Chapter 3, was 0.043$\mu$g PCP/egg and the topical equivalent of the immersion $ED_{50}$ (based on a BCF of 770 and the permeability factor) was 0.041$\mu$g PCP/egg. The $LD_{50}$ equivalent for the immersion exposure was approximately two times greater than the actual topical exposure (0.150 and 0.079$\mu$g PCP/egg, respectively). The regression model employed to determine the statistical significance of the two treatment types on overall adverse effects and lethality indicated no significant difference between the exposure techniques (employing conversions based on equation 5.1 for the immersion treatment) and no significant interaction of treatment type and dose. The comparison of slopes test indicated similar results in

that the regression slopes of the transformed data were not significantly different for the two treatment types whether the model was based on embryolethality or overall effects of the treatments.

Finally, the chi-squared comparison indicated a non-significant effect of salinity on the adverse effects of PCP on medaka eggs, indicating that, for this particular toxicant, changes in osmotic regulation and potentially related stress does not appear to affect medaka in an adverse way.

The topical technique has been shown here to successfully represent natural exposure to waterborne PCP at medium concentration levels ranging from 136 to approximately 86,800$\mu$g PCP/l. Further, this demonstrates that the eggs of the medaka concentrate PCP at a level near the predicted BCF of 770. Insignificant effects of salinity on control embryos and the similarity in adverse effects of PCP toxicity between 0ppt and 20ppt reared eggs, suggests that MELA would be a useful tool in the evaluation of regions of variable salinity within this range, whether employing immersion exposure or topical exposure and saline rearing conditions. These results are very encouraging with respect to the fulfilling of the need for a sensitive euryhaline toxicity test to study estuarine and coastal systems. The evaluation of TBTCl in the following chapter will take this evaluation one step further, through the inclusion of a 35ppt condition to simulate full strength seawater.

## CHAPTER SUMMARY

*O. latipes* eggs were exposed to a wide range of concentrations of pentachlorophenol (PCP) and reared in 20ppt salinity medium. The eggs were exposed using the MELA topical protocol as well as a standard immersion protocol. The immersion concentrations were developed as equivalents to the topical doses using a bioconcentration factor (BCF) of 770 for PCP. The results were compared to the freshwater range finding data presented in Chapter 2 and the two treatment procedures were compared.

The results indicated that the affect of salinity on PCP toxicity in the medaka eggs is minimal with 21-day $ED_{50}$ values of 0.056$\mu$g PCP/egg and 0.062$\mu$g PCP/egg for the freshwater and 20ppt exposures, respectively. In addition, conversion of the immersion concentrations to their topical equivalents, followed by regression analysis and slope comparisons, indicated that using the BCF of 770 results in very similar responses in the two treatment types. The sublethal embryopathies observed were primarily swollen abdominal regions/yolk sacs, pericardial edema and secondary "tube heart" (Marty *et al.*, 1990b), and developmental arrest and associated undifferentiated cells.

These results indicate that there appears to be little stress of salinity itself on the PCP toxicity to medaka eggs. Further, using a published BCF for PCP, it was possible to develop immersion concentrations which resulted in similar responses as the topical treatment. This suggests that, given the appropriate information about the chemical of interest (e.g., a BCF), one may be able to predict an appropriate topical concentration to represent a similar 21-day aqueous exposure.

# CHAPTER SIX

## Salinity-Related Response of Topical and Immersion Exposures to Tributyltin Chloride

## INTRODUCTION

Organotin compounds are defined as $RSnX_3$, $R_2SnX_2$, $R_3SnX$ and $R_4Sn$, where R is an alkyl or aryl group and X is a functional group such as a halide, oxide or hydroxide (van der Kerk, 1976). The only known naturally occurring representatives of this compound class are the methyl-substituted species (Brinckman *et al.*, 1983). Organotins have been used in numerous biocidal applications including applications as fungicides, bactericides, molluscicides and as overall antifoulants in boat paints. Other uses include stabilizers in polyvinyl chloride (PVC), fire proofing agents, additives in water repellents, antioxidants in lubricants, and wood preservation (Goldberg, 1986; Boyer, 1989). The demonstrated ability of organotins, primarily tributyltin (TBT), to effectively eliminate unwanted biological activity, in conjunction with their relatively long lifetimes and low corrosive behavior (Hall *et al.*, 1988), has resulted in significant increases in their use over the last 20 years.

Increases in environmental concentrations of organotins have been demonstrated to be associated with increased and localized use, resulting in adverse effects on both target and non-target organisms. The first reports of association between organotin use and trends in biological anomalies were made in France during the early 1980s (Alzieu, 1986). Oysters harvested from Arcachon Bay on the Atlantic coast showed a distinct shell thickening, often to the point where the shells became ball-shaped. The oysters themselves were very small and cross-sections of the shells revealed numerous chambers between the calcified layers filled with a protein gel not commonly produced during shell formation (Alzieu and Heral, 1984; Champ and Lowenstein, 1987; Cleary and Stebbing, 1987). The level of increased shell thickening was shown in these and other studies to be directly related to the degree of

boat activity and the extent of exchange and dilution of the coastal system, and hence, the concentration of organotins in the immediate area of the oyster beds. Subsequently, investigators have recorded numerous anomalous effects of this compound class on a wide range of invertebrates and vertebrates in field collections and laboratory exposures. Detailed reviews of these investigations are provided by Hall and Pinkney (1985), Champ and Bleil (1988), U.S. EPA (1988b), and Boyer (1989).

Of the organotins, most investigators agree that the tributylated species is the most toxic to both freshwater and marine organisms. Goldberg (1986) states that TBT is probably the most toxic substance ever deliberately introduced by man into the environment. This substance exists in a number of forms depending on the functional group substitution (e.g., O, Cl, OAc, etc.). However, researchers indicate that the metabolic fate and the adverse effects of TBT are independent of the functional group (Evans and Karpel, 1985; Bryan *et al.*, 1988; Sasaki *et al.*, 1988). Tributyltin most commonly exists in freshwater in the dissociated form of $TBT^+$ (Maguire and Tkacz, 1985; Tsuda *et al.*, 1990) and as TBTCl, $TBT_2CO_3$, TBTOH and $TBTH_2$- in seawater, with TBTCl being the major speciation product (Laughlin and Linden, 1985; Laughlin *et al.*, 1986). Following microbial and photolytic degradation, tributyltin undergoes a stepwise debutylation to the less toxic di- and monobutylated species and finally to non-toxic inorganic tin (Maguire *et al.*, 1983; Evans and Karpel, 1985). The half-life of TBT in water is a highly disputed subject with values reported as low as 6 days and as high as 238 days (Maguire *et al.*, 1983; Stebbing, 1985; Seligman *et al.*, 1988; U.S. EPA, 1988b). In addition to differences in

temperature and salinity, this range is most likely due to the level of microbial activity in the test systems as this process has previously been shown to be responsible for the majority of TBT degradation (Seligman *et al.*, 1988). Further, TBT has been shown to concentrate in estuarine surface microlayers (Cleary and Stebbing, 1987; Hall, 1988; Hall *et al.*, 1988; and others) and sediments (Salazar, 1986; Harris and Cleary, 1987; Unger *et al.*, 1987) resulting in potentially increased residence times and, therefore, elevated exposure to planktonic and demersal organisms (e.g., many early life stages).

TBT has been shown to be an effective developmental toxicant in a number of organisms including the fiddler crab (*Uca pugilator* - Weis *et al.*, 1987; Weis and Kim, 1988), the Axolotl (*Ambystoma mexicanum* - Scadding, 1990), and the dog-whelk (*Nucella lapillus* - Bryan *et al.*, 1988; Gibbs *et al.*, 1988) through the alteration of differentiation and growth. However, Davis *et al.* (1987) report that in only one out of six strains of *Salmonella typhimurium* was TBTO mutagenic and this was only in the presence of a toxicant-induced rat liver S9 activation system. TBTO also gave negative mutagenic results in the yeast *Schizosaccharomyces pombe* and the fruit fly *Drosophila melanogaster* using various genetic endpoints. The combination of these results suggest that TBT is a developmental toxicant, most likely at the cytological level, but not at the genome stage. TBT has not been classified with respect to carcinogenic effects, however, no significant accounts of carcinogenicity have been reported (Davis *et al.*, 1987; Walker *et al.*, 1989).

The mode of action of triorganotin toxicity has been linked to the swelling and disruption of mitochondrial membranes, disruption of ionic transport across lipid

membranes and the inhibition of ATP synthesis from ADP (Stockdale *et al.*, 1970; Evans and Karpel, 1985; Boyer, 1989). Moreover, these effects appear to be concentration dependent, with low concentrations of TBT appearing to act as a neurotoxin in nerve tissues and as a metabolic inhibitor of energy metabolism. At higher concentrations, TBT appears to alter and damage sensitive epithelial membranes and tissues (Goldberg, 1986; Laughlin, 1987; Lawler and Aldrich, 1987; Boyer, 1989). Detoxification is typically carried out through dealkylation by the mixed function oxidase (MFO) system and/or hydroxylation of the organic ligand by the MFO system (Laughlin and Linden, 1985).

The moderately lipophilic and hydrophobic nature of TBT enables it to dissolve in fats and lipids and to sequester itself into membranes (Gucinski, 1986). Laughlin *et al.* (1985) support this idea in that the natural log of the $LC_{50}$ for TBT is well correlated to physical parameters and constants which provide evidence that TBT adverse effects are controlled by partitioning behavior (i.e., effects are predictable based on the hydrophobic nature of TBT). TBT has an octanol-water partition coefficient between 2,100 and 7,000 (Ward *et al.*, 1981; Laughlin and Linden, 1985; Laughlin *et al.*, 1986) which seems to vary significantly with salinity (e.g., Laughlin *et al.* [1986] reported that the highest values occurred in deionized water, decreased in 25ppt seawater and then increased again in 45ppt seawater). The bioconcentration of TBT also varies significantly with species, exposure type and length, water concentration and with the organ(s) selected for evaluation. Ward *et al.* (1981) reported bioconcentration factors (BCF) as high as 52,000 in the liver of sheepshead minnows (*Cyprinodon variegatus*). Similar levels were reported in the blue mussel

(*Mytilus edulis*) with BCF values ranging from 5,000 to 60,000 (Zuolian and Jensen, 1989), however, others have reported values over an order of magnitude lower in a similar species (Tsuda *et al.*, 1990). In addition, bioconcentration has been shown to be typically higher in freshwater organisms than in marine organisms (Goldberg, 1986; Tsuda *et al.*, 1990).

Short-term studies have demonstrated that TBT is acutely toxic to numerous organisms, both invertebrates and vertebrates, however, vertebrates have received much less attention, especially their early life stages. $LC_{50}$ values tend to be in the 1 to 50$\mu$g TBT/l range, depending on the organism studied, the medium concentrations and the duration of exposure. However, long-term studies have also revealed toxic and teratogenic effects at 3 to 4 orders of magnitude lower concentrations (e.g., Gibbs *et al.* [1988] report observable effects at levels as low as 0.005$\mu$g TBT/l). In addition, most aquatic organisms appear to be extremely sensitive to TBT toxicity during the time of development from fertilized eggs through the various larval stages. Developmental anomalies include numerous skeletal defects of limbs and vertebrae (Krowke *et al.*, 1986; Weis *et al.*, 1987; Weis and Kim, 1988; Walker *et al.*, 1989; Scadding, 1990; and others), cardiac and optical abnormalities (Weis *et al.*, 1987; Walker *et al.*, 1989), imposex (Bryan *et al.*, 1988; Gibbs *et al.*, 1988; and others) and reduced development (Roberts, 1987; Laughlin *et al.*, 1988; and others).

Due to the fact that differences exist between freshwater and marine fish in their physiological regulation of osmotic pressure, accumulation and excretion of organotin compounds might also differ. In a single study of effects of salinity on TBT toxicity, Tsuda *et al.* (1990) observed a TBTCl BCF of 460 in freshwater

exposed guppies and approximately half as much bioconcentration in seawater acclimated organisms (BCF=240) after 7-14 days of exposure. On the other hand, these authors found that the rates of TBT excretion at the two salinities were very similar.

The present study evaluated the salinity-related effects of TBTCl on the eggs of the Japanese medaka. The chloride substituted species of TBT was used in these experiments, as TBTCl has previously been shown to be one of the most common found in seawater and among the most toxic of the organotin species (of the six organotins tested by Bryan *et al.* [1988], tributyltin chloride proved to be the most effective in promoting imposex in *Nucella lapillus*). Two exposure conditions were employed: 20ppt and 35ppt salinities, and the results were compared to the freshwater TBTCl study presented in Chapter 2. As described in the previous chapter on PCP toxicity, an estimated BCF for TBT was applied to the topical treatment doses to estimate immersion medium concentrations, allowing a side-by-side evaluation of the BCF value as it represents medaka eggs and the ability of the topical treatment to be applied to medium concentrations through the use of this BCF.

Eggs were exposed over a wide range of concentrations, with a primary focus around the $ED_{50}$ determined in the freshwater range-finding study, and were reared in accordance with those procedures defined in Chapter 4 with modifications discussed in Chapter 5. The results of the permeability factor for TBTCl presented in Chapter 3 were applied to the nominal topical treatment concentrations to develop toxicity data based on absolute dose concentrations.

## MATERIALS AND METHODS

Adult medaka were reared as described in Chapters 2 and 5. Twenty ppt aquarium water and embryo rearing solution were prepared as described in Chapter 5. Thirty-five ppt aquarium water and rearing solution were prepared similarly with the addition of appropriate amounts of artificial seasalts. Salinity was confirmed, and pH was determined and adjusted, as described previously in Chapter 5. Egg collections, washings and rearing procedures were as described previously for PCP.

### Topical Treatment

All topical treatment procedures were identical to those described in Chapter 5 for PCP. The topical dose concentrations, as the chloride, were 12.0, 1.20, 0.120, 0.012, 0.0012, 0.0006, 0.00045, 0.0003, 0.000225, 0.00015, 0.000112, 0.000075, 0.0000562, 0.0000375, and 0.0000187$\mu$g TBTCl/egg.

### Immersion Treatment

All immersion procedures were also as described previously for PCP. A TBTCl BCF has not been determined for vertebrate embryos, therefore, a predicted BCF of 4,000 was used based on the results presented by Waldock *et al.* (1983) for the oyster *Crassostrea gigas* (21-day BCF=4000) and the experimentally-derived mean BCF of 4000 presented by Cardwell and Sheldon (1986). As with the PCP immersion concentrations, the TBTCl immersion equivalents were calculated employing the following equation:

$$C_I = \frac{C_D / E_W}{BCF} \qquad [6.1]$$

where: $C_D$ = Concentration of the topical dose per egg ($\mu$g)
$E_W$ = Weight of egg (g)
$C_I$ = Immersion concentration ($\mu$g/ml)

Neat TBTCl (96% purity) was dissolved in DMSO and aliquots were added to the two different salinity rearing solutions to develop the highest concentration, 16,000$\mu$g TBTCl/l. Each subsequently lower solution was prepared through dilution of this high concentration sample. The final nominal immersion concentrations were 16,000, 1,600, 160, 16.0, 1.60, 0.802, 0.602, 0.401, 0.301, 0.201, 0.150, 0.100, 0.075, 0.050, 0.025, and 0.001$\mu$g TBTCl/l. Each concentration stock solution contained methylene blue mold inhibitor and was stored in an amber glass bottle at 4 $\pm$ 2°C to reduce the potential for microbial and photo-degradation of the TBTCl. Under these conditions, it was assumed that the degradation of the TBTCl would be minimal, therefore, sufficient volume of each solution was prepared at the onset of the experiment and used throughout the study duration.

Similar to the approach used for PCP, the approximate level of TBTCl taken up by all eggs in a given replicate was also determined. For example, 20ml of the 16,000$\mu$g TBTCl/l concentration would contain 320$\mu$g of TBTCl; if each egg absorbs 12.0$\mu$g (based on the nominal topical dose), 120$\mu$g (or 37.5%) of this material would be removed from solution. Since this is a large fraction of the exposure concentration, it is suspected that the actual level that the eggs would be exposed to in a given test vessel over time would significantly decrease. Therefore, the solutions were renewed daily with fresh, TBTCl-enriched rearing solution. Further, in order to provide comparability between the two treatment types, the vessels containing topically treated eggs were also changed daily with fresh, untreated rearing solution.

Two procedures were added to the TBTCl evaluations that were not included in the PCP study. First, all glassware employed in the preparation and storage of exposure standards and solutions was treated ("silanized") with silane (5% dimethyldichlorosilane in toluene), followed by thorough rinsing with anhydrous methanol and heating at 110°C for 4 hours. This procedure binds the active sites in the glassware to reduce the potential for TBTCl loss to the walls of these materials. All aeration pipets and rearing vials were also silanized prior to use. The second procedure was the confirmation of select immersion media concentrations to determine the accuracy of the nominal TBTCl concentrations. This was not possible for the topical standards as they were prepared in 100% DMSO and, therefore, could not be analyzed employing the available gas chromatography equipment. The procedure for evaluating the immersion media was a modification of the methods developed by Mathias *et al.* (1986). Briefly, at the onset of the exposure study, 500ml of select test solutions were placed in 1000ml teflon separatory funnels and the TBTCl was derivatized with 15ml of 4% sodium borohydride. Each sample was then extracted twice with methylene chloride, the extracts combined, concentrated and the final concentrate analyzed employing capillary column gas chromatography/flame photometric detection (GC/FPD) techniques. The 0.802 and 0.201$\mu$g TBTCl/l concentrations were evaluated for both salinity levels. The embryological scoring procedures for both the topical and the immersion treatments were as described previously in Chapter 5 for PCP.

## Statistics

The statistical analyses employed included probit analysis, chi-squared analysis, linear regression model analysis and the Sokal and Rohlf (1969) test for comparing slopes of regression equations. Refer to the Chapter 5 section on statistics for more information regarding these procedures.

## RESULTS

The absolute TBTCl determinations resulted in concentrations very similar to the nominal concentrations; 0.201$\mu$g TBTCl/l nominal was 0.197 and 0.195$\mu$g TBTCl/l absolute for 20ppt and 35ppt, respectively, and the 0.802 nominal concentration was 0.789 and 0.785$\mu$g TBTCl/l for the two salinities. The deviation in these values from nominal is well within the typical method and instrument precision and, therefore, the nominal concentrations were assumed to be absolute.

Egg viability data for the topical treatment and immersion exposure are presented in Tables 6.1 and 6.2, respectively. The results of these treatments are further segregated into lethal and sublethal responses and are presented in Figures 6.1 and 6.2. The results of the 0ppt topical treatment pilot study presented in Chapter 2 are shown in Figure 6.3 for comparison to the higher salinity topical treatments. The patterned bars in Figures 6.1 and 6.2 represent means for three treatments in the 20 and 35 ppt exposures while those in Figure 6.3 represent the total for each endpoint (i.e., lethal and sublethal) for the single replicate, pilot data. Error bars on the higher salinity treatments represent the standard error of the replicate means. The results indicate that there was a small apparent effect of salinity on the adverse impact of TBTCl on the medaka, however, there was a large difference in the level of impact

**Table 6.1.** Adverse effects of TBTCl on medaka eggs and hatch fry topically treated and reared in 20ppt and 35ppt media.

| Dose (μg TBTCl/egg) | 20ppt Adversely Affected[a] | 35ppt Adversely Affected |
|---|---|---|
| 12.0 | 100 (±0.00) | 100 (±0.00) |
| 1.20 | 100 (±0.00) | 100 (±0.00) |
| 0.120 | 100 (±0.00) | 100 (±0.00) |
| 0.012 | 90.0 (±5.77) | 76.7 (±6.67) |
| 0.0012 | 80.0 (±5.77) | 50.0 (±10.0) |
| 0.0006 | 76.7 (±3.33) | 33.3 (±6.67) |
| 0.00045 | 53.3 (±8.82) | 33.3 (±6.67) |
| 0.0003 | 73.3 (±8.82) | 33.3 (±8.82) |
| 0.000225 | 43.3 (±3.33) | 33.3 (±3.33) |
| 0.00015 | 43.3 (±8.82) | 26.7 (±8.82) |
| 0.000112 | 50.0 (±10.0) | 23.3 (±8.82) |
| 0.000075 | 36.7 (±8.82) | 16.7 (±8.82) |
| 0.0000562 | 26.7 (±8.82) | 10.0 (±10.0) |
| 0.0000375 | 60.0 (±5.77) | 16.7 (±3.33) |
| 0.0000187 | 40.0 (±5.77) | 16.7 (±3.33) |
| DMSO Control | 4.44 (±1.76) | 6.67 (±2.36) |
| Untreated Control | 8.89 (±2.00) | 5.55 (±2.42) |

**Table 6.2.** Adverse effects of TBTCl on medaka eggs and hatch fry immersion treated in 20ppt and 35ppt media.

| Concentration (μg TBTCl/l) | 20ppt Adversely Affected | 35ppt Adversely Affected |
|---|---|---|
| 16,000 | 100 (±0.00) | 100 (±0.00) |
| 1,600 | 100 (±0.00) | 100 (±0.00) |
| 160 | 100 (±0.00) | 100 (±0.00) |
| 16.0 | 100 (±0.00) | 100 (±0.00) |
| 1.60 | 100 (±0.00) | 100 (±0.00) |
| 0.802 | 100 (±0.00) | 100 (±0.00) |
| 0.602 | 100 (±0.00) | 100 (±0.00) |
| 0.401 | 100 (±0.00) | 100 (±0.00) |
| 0.301 | 100 (±0.00) | 100 (±0.00) |
| 0.201 | 100 (±0.00) | 96.7 (±3.33) |
| 0.150 | 100 (±0.00) | 100 (±0.00) |
| 0.100 | 100 (±0.00) | 90.0 (±5.77) |
| 0.075 | 90.0 (±0.00) | 80.0 (±5.77) |
| 0.050 | 96.7 (±3.33) | 26.7 (±8.82) |
| 0.025 | 80.0 (±11.6) | 20.0 (±5.77) |
| 0.001 | 20.0 (±0.00) | 10.0 (±5.77) |
| DMSO Control | 6.67 (±3.33) | 8.89 (±2.00) |
| Untreated Control | 6.67 (±3.33) | 6.67 (±2.35) |

[a] Expressed as replicate mean percentage of eggs affected ± standard error of the mean.

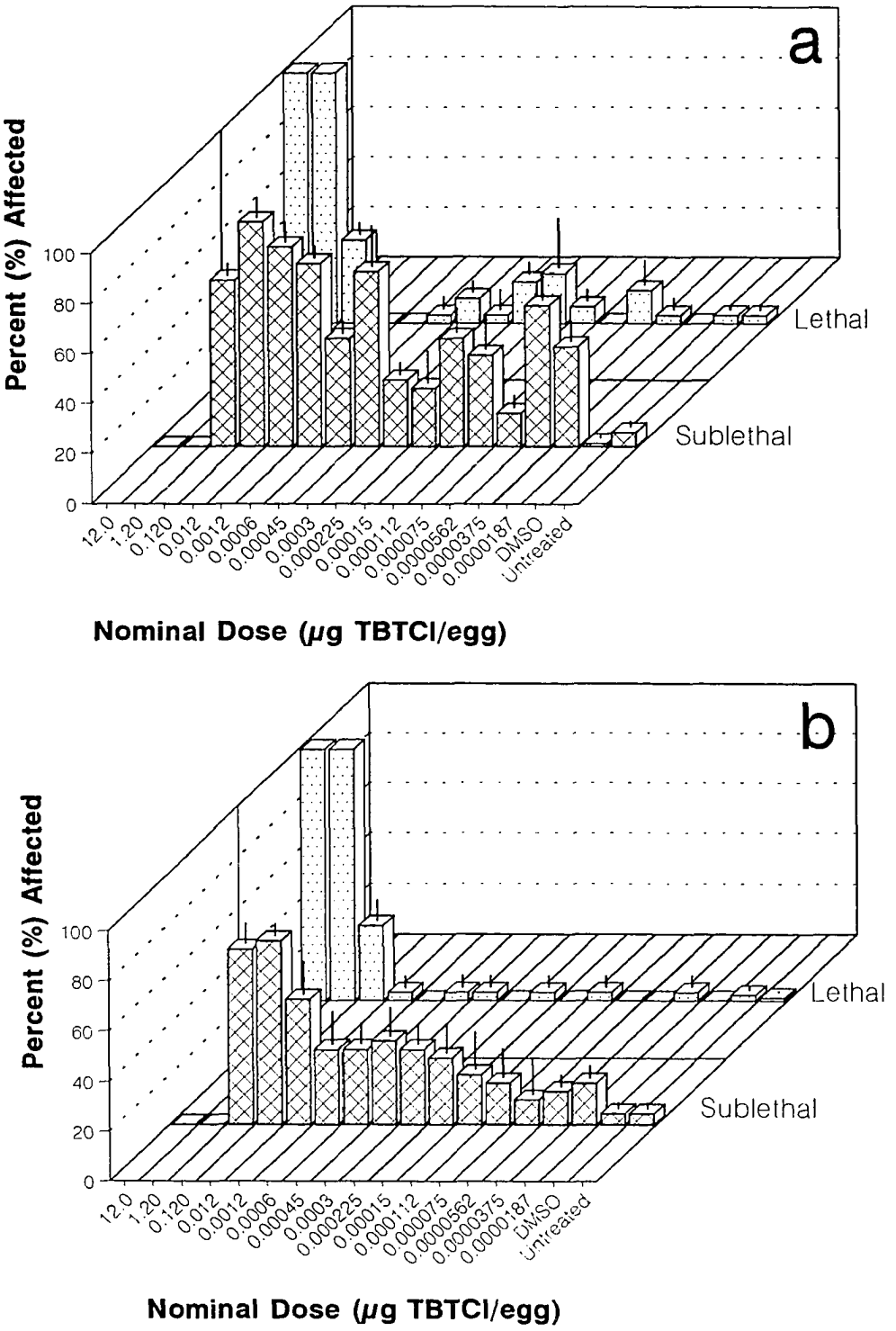



**Figure 6.1.** Breakdown of effects for topical treatment results for 20ppt (a) and 35ppt rearing conditions (b). Values are replicate means ± standard error of each mean.

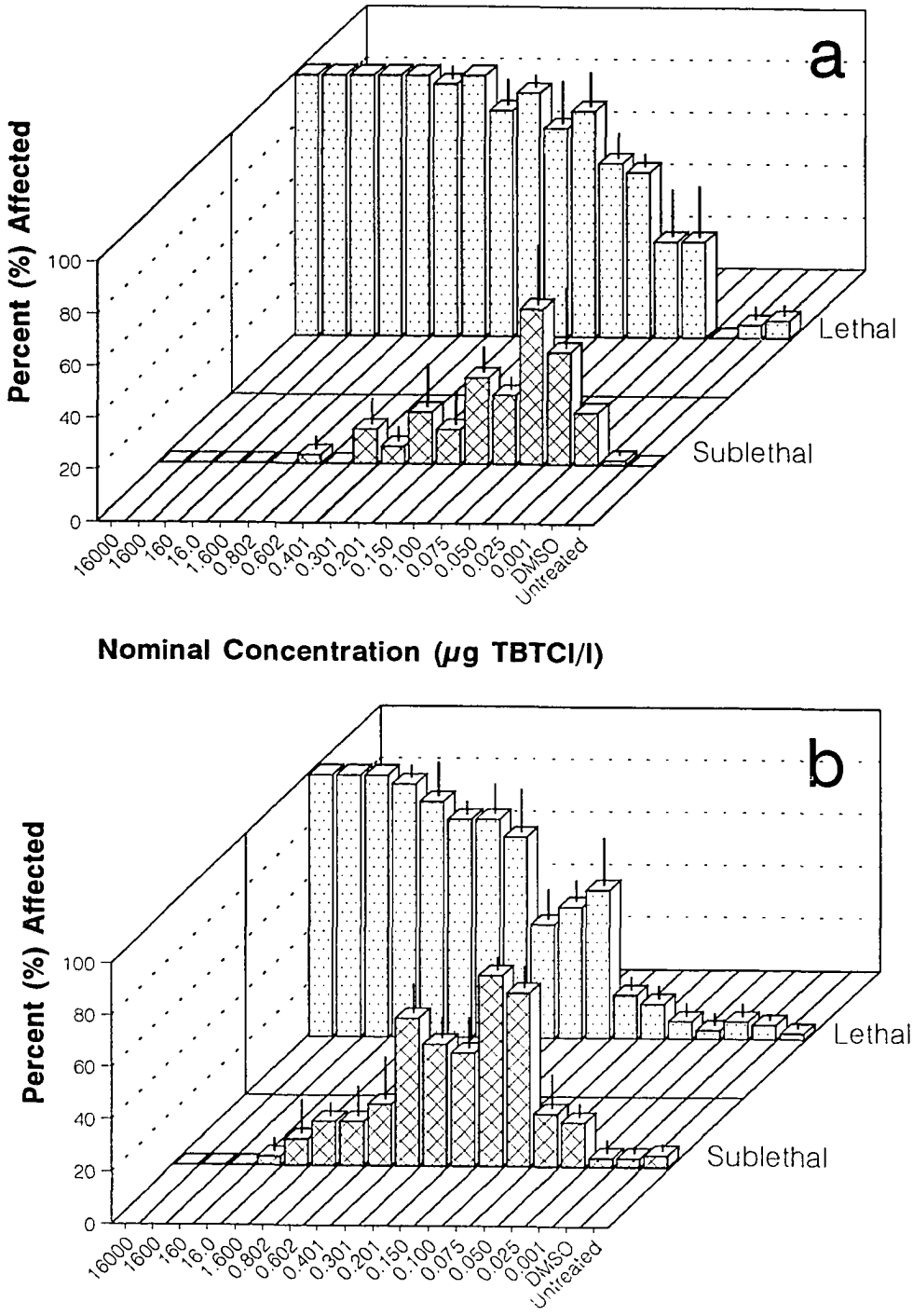



**Figure 6.2.** Breakdown of effects for immersion exposure results for 20ppt (a) and 35ppt rearing conditions (b). Values are replicate means ± standard error of each mean.

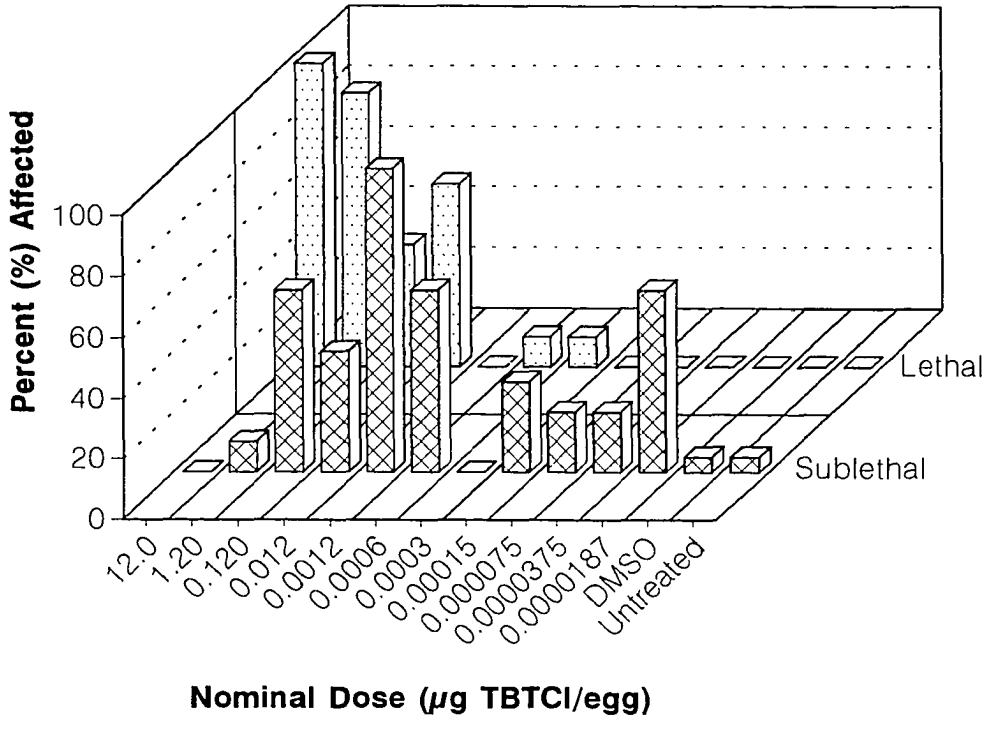



**Figure 6.3.** Breakdown of effects for topical treatment results for 0ppt pilot study data. Values are total percentage affected for a single 10 egg treatment.

between the two treatment types. This suggests that the BCF of 4,000 used to develop the immersion concentration equivalents was a significant underestimate of the bioconcentratable nature of TBTCl in medaka eggs. The data for both treatment types indicate that the 20ppt exposure resulted in slightly more extensive effects than the 35ppt treatment in the immersion exposure and the 0ppt and 35ppt exposures in the topical treatment. Another interesting observation is a slight increase in effects in the lowest concentrations of all three topical treatment exposures. A similar pattern was not observed in the immersion exposure concentration series. All exposures showed a classic toxicity response, with varying degrees of concentration-based severity, with lethality dominating the higher

concentrations, followed by a drop in acute response and a corresponding increase in sublethal impact which eventually dropped off to a level near the control treatments (except for the observed rise in effects seen with the topical treatment lower concentration).

The changes in all adverse effects (i.e., lethal+sublethal) with time were evaluated. In general, the higher concentrations exhibited rapid effects whereas the lower concentrations elicited their effects later during the development process, indicating a potential dual mode of toxic action as discussed previously. The patterns also appear significantly different between the topical exposures and the immersion exposures. The topical treatment time series are relatively well distributed over the test duration as a function of concentration, except for a slight increase in the level of effects in the lowest concentrations. In contrast, the immersion treatments tend to fall into a three phase time-effect pattern. The higher concentrations are dominated by high levels of rapid effects and the lower concentrations appear to be similar to the controls with moderate effects expressed throughout development. However, the intermediate concentrations (approximately 0.025 to 0.802$\mu$g TBTCl/l) seem to be similar and clustered with respect to time related response. The effects of these concentrations appear at, or near, hatching events and are comprised primarily of gross developmental deformities. Figure 6.4 displays a general plot for type of pattern seen for the topical treatment and Figure 6.5 shows the general pattern for the immersion exposure with the overlapping intermediate treatments (0.025 and 0.802$\mu$g TBTCl/l). To illustrate the differences in levels of effects, only select concentrations are shown in these Figures. The complete data sets for all treatment concentrations are presented in Appendix A of this Chapter.

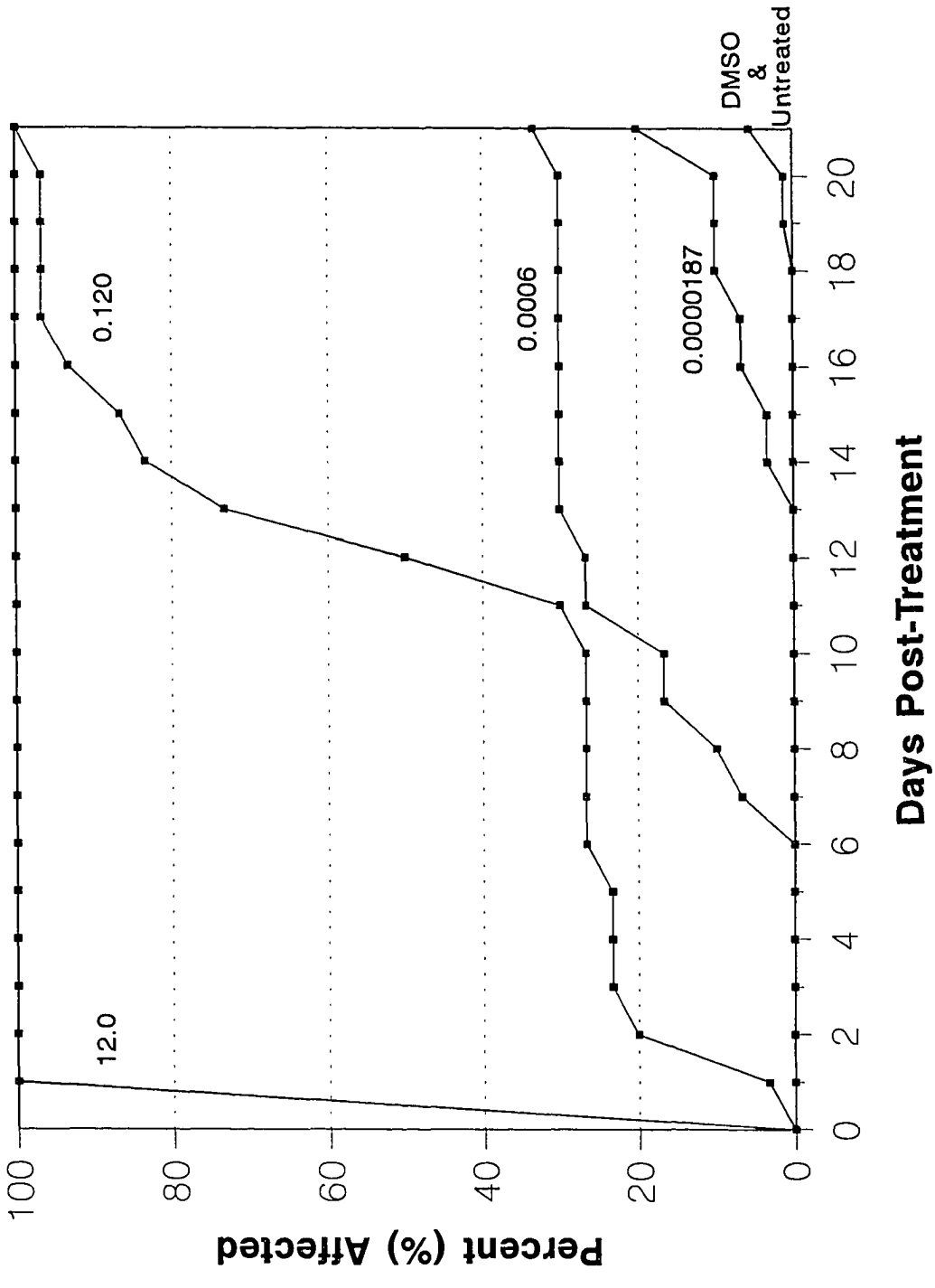



**Figure 6.4.** General pattern for time-related changes in all adverse effects on medaka eggs topically treated with TBTCl. Doses are in $\mu$g TBTCl/egg. Only select treatment doses are shown. See Appendix A for all exposure levels.

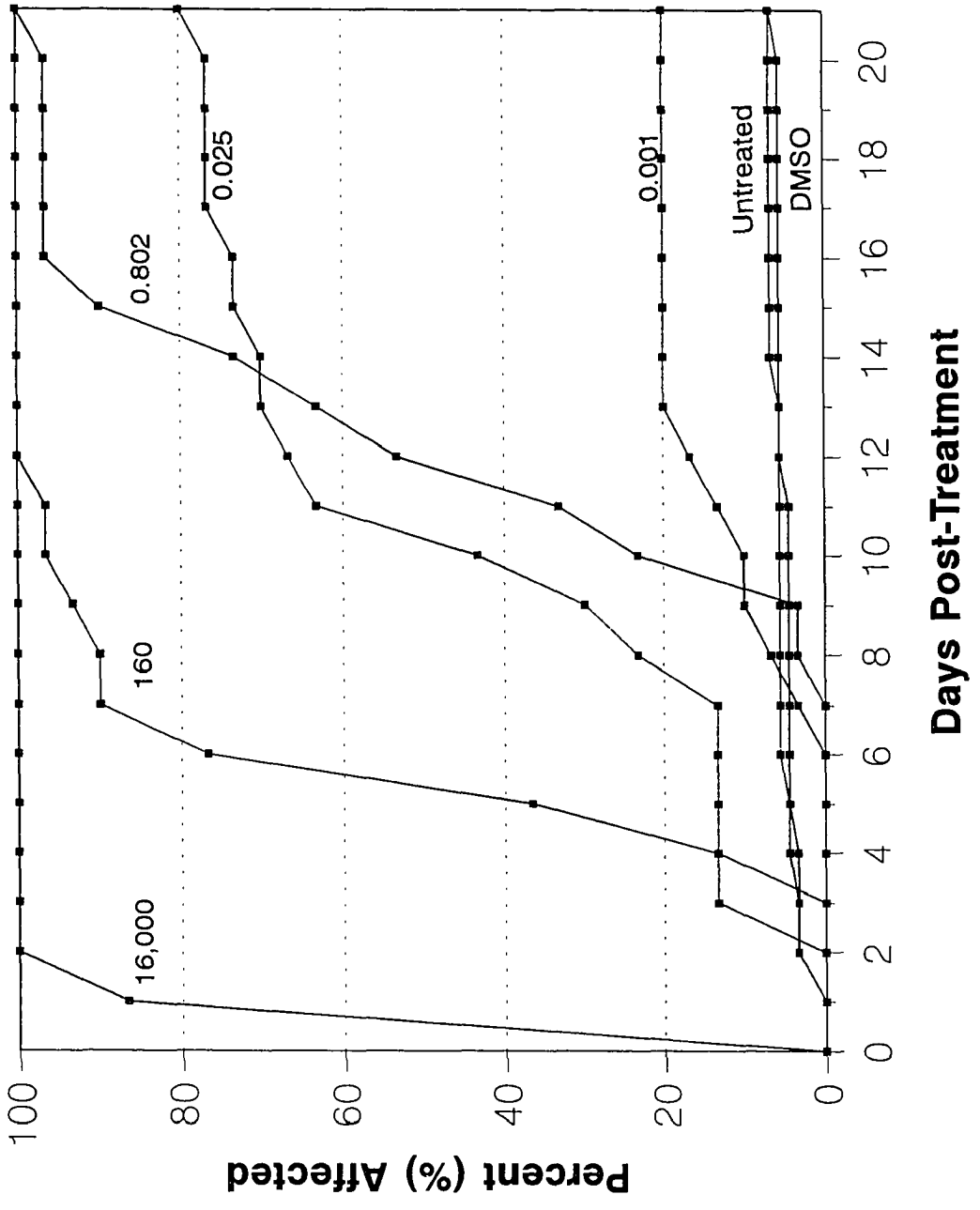



**Figure 6.5.** General pattern for time-related changes in all adverse effects on medaka eggs immersion treated with TBTCl. Concentrations, in $\mu$g TBTCl/l, are immersion equivalents to those doses displayed in Figure 6.4 (except no topical dose equivalent exists for the 0.001$\mu$g TBTCl/l concentration). Only select treatment concentrations are shown. See Appendix A for all exposure levels.

Probit analysis of the data indicated that the 20ppt topical treatment had a 21-day $ED_{50}$ of 0.0001$\mu$g TBTCl/egg (95% confidence limits were 0.00005-0.0002) and a 21-day $LD_{50}$ of 0.072$\mu$g TBTCl/egg (0.001-1.58). The 35ppt topical treatment resulted in an $ED_{50}$ and $LD_{50}$ approximately one order of magnitude higher at 0.001$\mu$g TBTCl/egg (0.0007-0.002) and 0.112$\mu$g TBTCl/egg (0.024-1.10), respectively. These data indicate that the 20ppt topical exposure effects were very similar to the 0ppt data which had a 21-day $ED_{50}$ of 0.0002$\mu$g TBTCl/egg and a 21-day $LD_{50}$ of 0.047$\mu$g TBTCl/egg. However, the 35ppt effects appear to be somewhat less profound as compared to the two lower salinity exposures, possibly due to reduced osmoregulation stress at the higher salinity as isotonic conditions are approached.

Chi-squared ($X^2$) analysis of a 2x2 contingency table indicated no significant differences between any control treatments (i.e., untreated vs. DMSO and between salinities; $p>0.050$, $X^2$ ranged between 0.00 and 1.32). Chi-squared analysis was also used to evaluated differences between these controls and the lower TBTCl treatment doses. As there were no significant differences between the controls types, the untreated and DMSO data for each exposure type (e.g., 20ppt topical exposure) were grouped for further statistical evaluation. In the 0ppt pilot data, the lowest concentration, 0.0000187$\mu$g TBTCl/egg, was found to be significantly different from the controls ($p>0.050$, $X^2=14.1$), however, the next two highest doses (0.0000375 and 0.000075) were not significantly different from controls ($p>0.050$, $X^2=0.832$ for both doses). The remainder of the higher concentrations had significantly more effects than the controls. The 20ppt concentrations were all found to be significantly different from the control groups ($p>0.050$), however, a similar pattern of increasing effects in the lowest concentrations was also observed (for the 0.000075 treatment,

$X^2=19.2$; for 0.0000562, $X^2=8.75$; for 0.0000375, $X^2=52.8$; for 0.0000187, $X^2=23.31$). This pattern was also seen in the 35ppt topical treatment, however, the effects appeared to be somewhat less than those observed in the 20ppt exposure. The $X^2$ values for the 35ppt lower concentrations were as follows: for 0.00015, $X^2=10.8$; for 0.000112, $X^2=7.66$; for 0.000075, $X^2=2.71$; for 0.0000562, $X^2=0.156$; for 0.0000375, $X^2=2.71$; for 0.0000187, $X^2=4.93$. Only the 0.0000375-0.000075 exposures were not significantly different from the controls, however, the 0.0000187 exposure was only slightly significant at $p>0.050$ and was not significant at $p>0.025$.

Probit analysis of the immersion treatments revealed very low $EC_{50}$ and $LC_{50}$ values for both salinity exposures. The 20ppt 21-day $EC_{50}$ was 0.004$\mu$g TBTCl/l (0.002-0.007) and a 21-day $LC_{50}$ of 0.052$\mu$g TBTCl/l (0.037-0.068). Similar to the topical treatment data, the 35ppt exposure condition produced median lethal and effective concentrations approximately one order of magnitude higher than the 20ppt condition with an $EC_{50}$ of 0.031$\mu$g TBTCl/l (0.004-0.067) and an $LC_{50}$ of 0.225$\mu$g TBTCl/l. (0.061-0.931). No 0ppt immersion treatment data were generated for comparison.

Chi-squared analysis of the 20ppt immersion exposure indicated that all treatment concentrations were significantly different from controls, however, the lowest concentration (0.001$\mu$g TBTCl/l) was barely significant at $p>0.050$ ($X^2=3.59$) and was insignificant at $p>0.025$. All other concentrations were highly significant at $p>0.005$ ($X^2\geq 72.6$). As indicated in the topical exposure $X^2$ analysis, the 35ppt salinity condition elicited reduced effects as compared to the 20ppt exposure condition. The 0.001$\mu$g TBTCl/l concentration was not significantly different from

controls ($p>0.050$, $X^2=0.02$), the 0.025$\mu$g TBTCl/l concentration was barely significant at alpha level of 0.050 ($X^2=3.67$) and was not significantly different at $p>0.025$, and the third lowest concentration, 0.050$\mu$g TBTCl/l, was somewhat more significantly different from controls at $p>0.05$ ($X^2=8.75$). All other concentrations had $X^2$ values $\geq$ 88.9 and were therefore highly significantly different from the control groups.

Following conversion of the immersion concentrations to their topical dose equivalents, the topical and immersion data were censored to remove 0% and 100% responses, arcsin transformed and regressed against the logarithm of dose to compare the topical response (or the predicted response) and the immersion response (or the nominal response). The data were regressed for lethal and all adverse effects and the resulting plots are presented in Figures 6.6 and 6.7 for lethal and adverse response, respectively. This graphical presentation indicates that there appears to be a significant difference between the predicted (topical) response and the nominal (immersion) response, however, the effect of salinity appears to be minimal. Regression analysis of the transformed data confirmed these graphical predictions. The regression output for lethality indicated that there was a significant effect of treatment type (alpha level of 0.01) and a significant interaction between treatment and dose as well as treatment and salinity, however, the effect of salinity alone was insignificant. The results for all adverse effects were similar except salinity becomes significant upon inclusion of the sublethal data and the interaction effect between salinity and treatment type becomes insignificant.

The censored, arcsin transformed data were then employed in a test to compare the slopes of these regressions (Sokal and Rohlf, 1969) for lethal and all

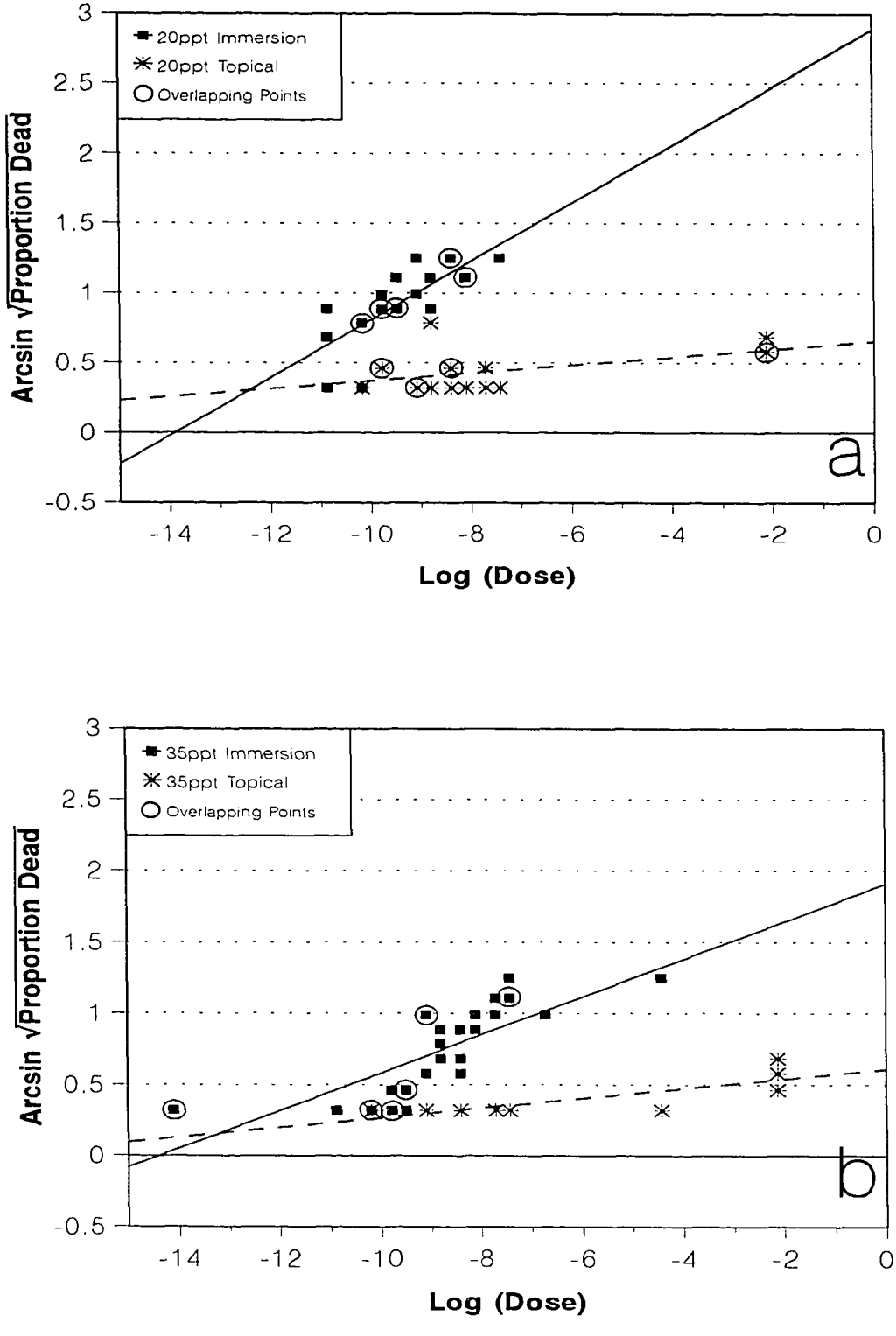



**Figure 6.6.** Regression plots of lethal effects of TBTCl on medaka eggs reared in 20ppt (a) and 35ppt (b) salinity medium.

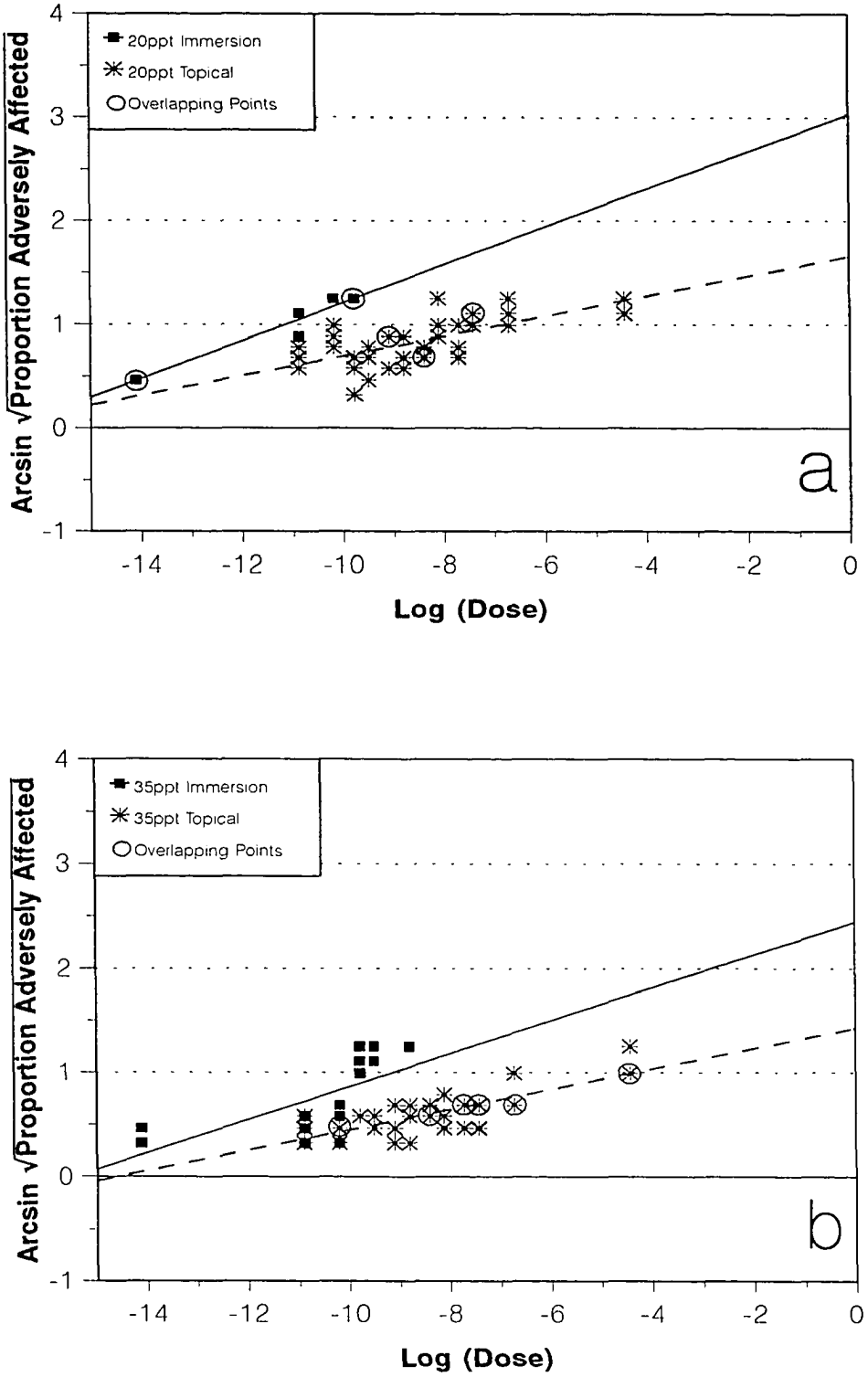



**Figure 6.7.** Regression plots of adverse effects of TBTCl on medaka eggs reared in 20ppt (a) and 35ppt (b) salinity medium.

adverse effects. The topical data were also compared to the 0ppt pilot study data slopes for lethality and all adverse effects. The results of all slope comparisons are presented in Table 6.3. The various salinity topical treatments did not significantly differ from each other at $p>0.01$, nor did the immersion treatments at 20ppt and 35ppt. However, upon comparing the two exposure methods (topical & immersion), a significant difference for lethality is detected in the 20ppt medium and the 35ppt salinity is only barely insignificant at $p>0.01$. A similar pattern was seen for all adverse effects data, however, all slope comparisons were found not to be significantly different at $p>0.01$. This indicates that the effect of salinity on the slope of these lines is minimal and that, though the slopes are somewhat different between the two treatment types, these differences are only slightly statistically significant for lethality and not significant for all adverse effects.

Numerous developmental anomalies were detected in all TBTCl exposures, the most prevalent of which were skeletal deformities (lordosis, kyphosis, pectoral stumping, etc.), cardiac malformations and gross underdevelopment. The appearance and extent of these anomalies was strongly concentration dependent, with the higher concentrations resulting in full developmental arrest (subsequently followed by death) and the lower concentrations exhibiting slight spinal abnormalities and associated swimming problems as well as reduced hatching events. These developmental anomalies were common to all treatment and salinity exposures. Examples of the most common anomalies are presented in Figure 6.8.

**Table 6.3.** Model predicted data for test for comparison of slopes between all TBTCl treatment and salinity pairs.

| TREATMENT COMPARISONS | MODEL DATA | |
|---|---|---|
| **Lethal Effects** | **F(SLOPE)** | **P>F** |
| 0ppt Topical & 20ppt Topical | 6.12 | 0.035 |
| 0ppt Topical & 35ppt Topical | 5.74 | 0.048 |
| 20ppt Topical & 35ppt Topical | 0.168 | 0.689 |
| 20ppt Immersion & 35ppt Immersion | 1.44 | 0.245 |
| 20ppt Topical & 20ppt Immersion | 27.6 | 0.0001 |
| 35ppt Topical & 35ppt Immersion | 7.22 | 0.016 |
| | | |
| **All Adverse Effects** | **F(SLOPE)** | **P>F** |
| 0ppt Topical & 20ppt Topical | 0.342 | 0.568 |
| 0ppt Topical & 35ppt Topical | 0.969 | 0.343 |
| 20ppt Topical & 35ppt Topical | 0.005 | 0.946 |
| 20ppt Immersion & 35ppt Immersion | 0.067 | 0.804 |
| 20ppt Topical & 20ppt Immersion | 3.67 | 0.079 |
| 35ppt Topical & 35ppt Immersion | 1.31 | 0.271 |

## DISCUSSION

The research presented in this Chapter focused on the effects of tributyltin chloride (TBTCl) on the eggs of the Japanese medaka (*Oryzias latipes*) over a concentration range spanning six orders of magnitude. The effects of salinity were evaluated in conjunction with an exposure technique comparison between the MELA topical treatment and a static-renewal immersion procedure. The results indicate that under these exposure conditions, the early life stages of the medaka may be among the most sensitive to this chemical. The no observable effects concentration (NOEC)

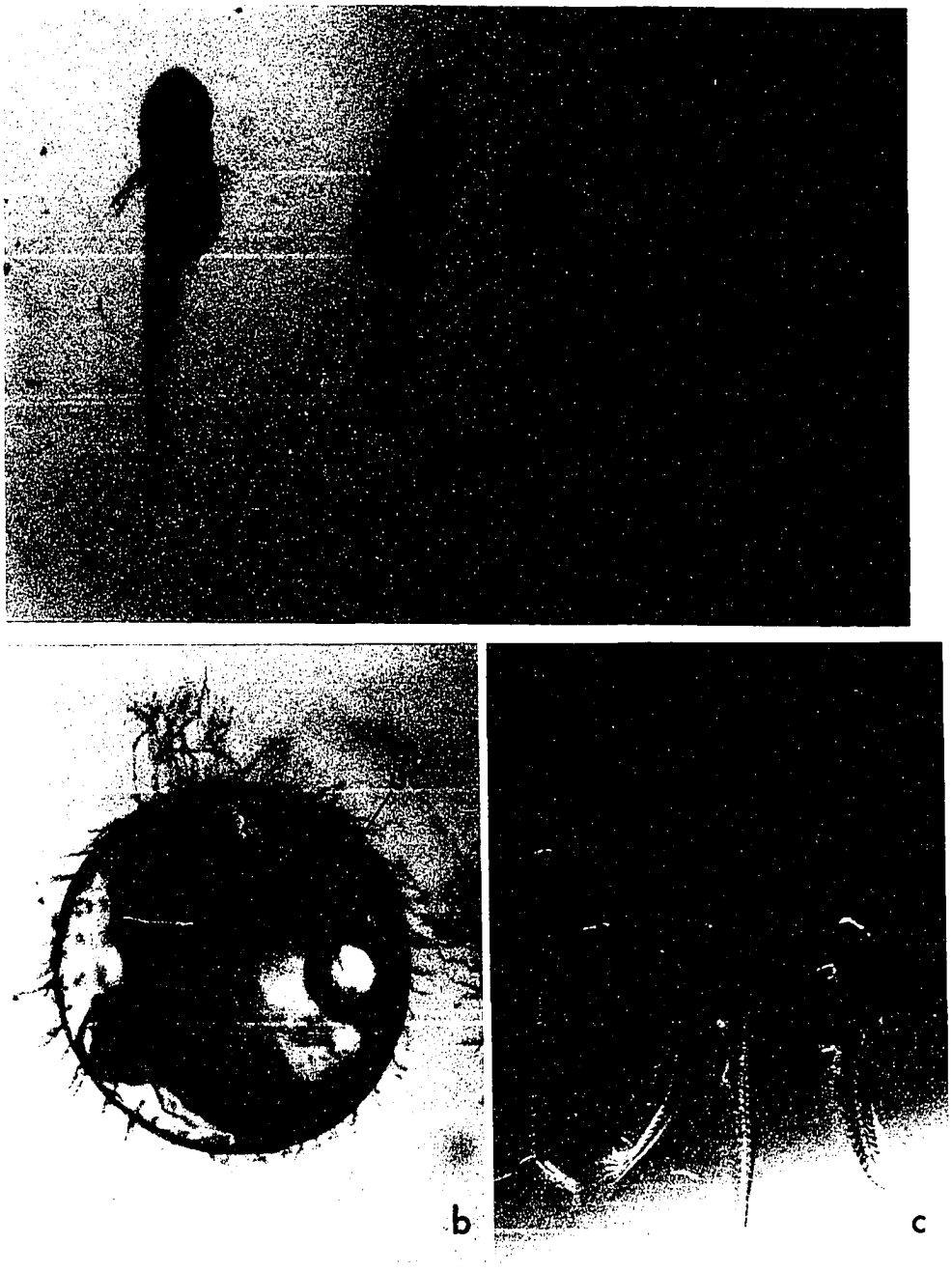
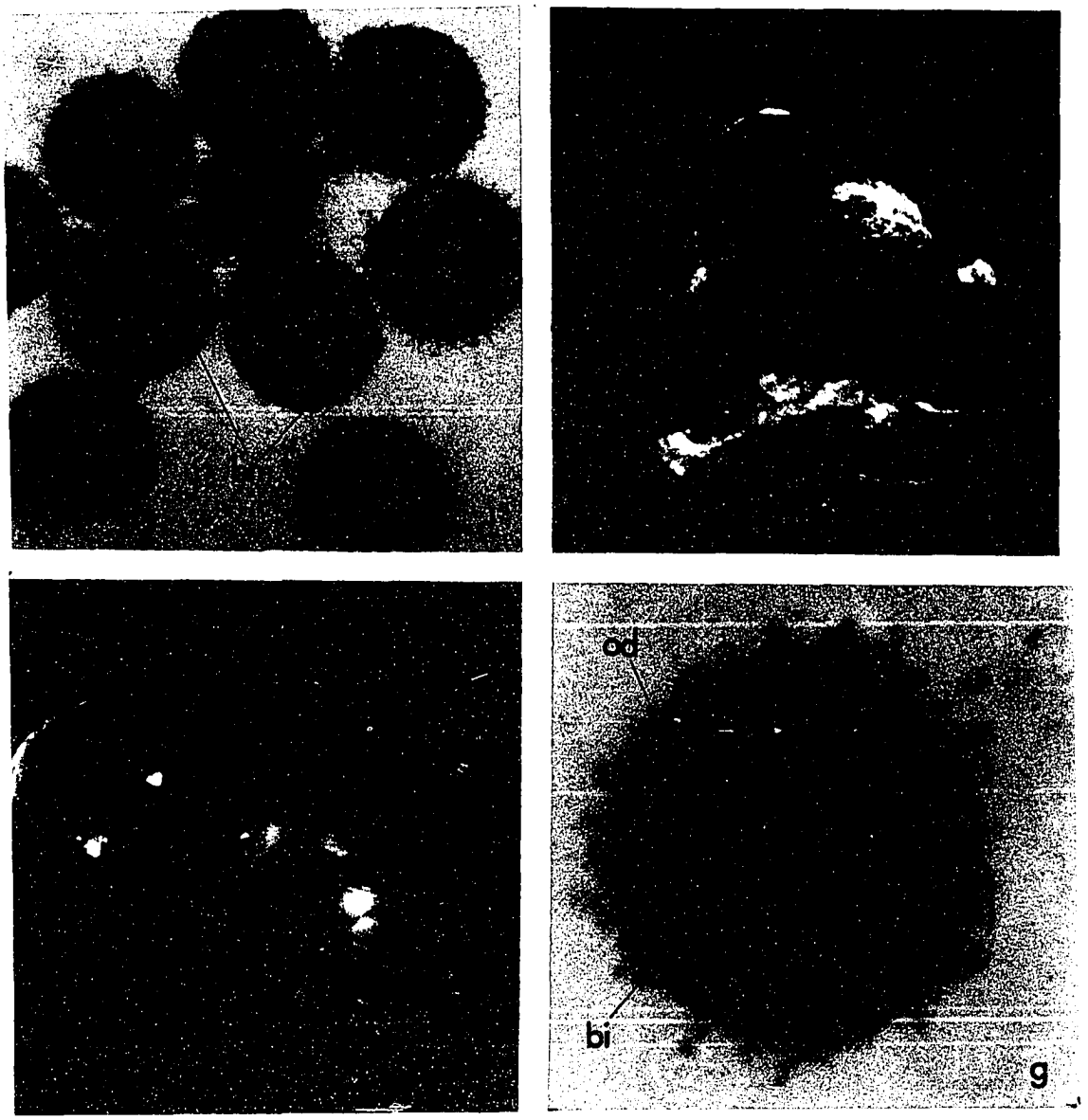



**Figure 6.8.** Developmental deformities common to all TBTCl exposure conditions. See legend for exposure concentrations and descriptions.




**a** - Varying intensity vertebral damage and curvature (0.12$\mu$g/egg topical - 35ppt)

**b** - Severe skeletal and optical deformation. Large masses of undifferentiated cells (uc; 0.00015$\mu$g/egg topical - 35ppt)

**c** - Severe kyphosis and lordosis of spinal column (0.0000187$\mu$g/egg topical - 20ppt)

**d** - Severe deformation of all major organs and of skeletal structure. Large blood masses (bm) seen at end of stunted tail (0.150$\mu$g/l immersion - 20ppt)

**e** - Stunting of notochord accompanied by twinning (0.050$\mu$g/l immersion - 20ppt)

**f** - Stunting of pectoral fin (ps; 0.025$\mu$g/l immersion - 35ppt)

**g** - Unilateral ophthalmic deformity (od) and blood islands (bi; 0.001$\mu$g/l immersion - 20ppt)

**Figure 6.8 (Cont'd).**

in the immersion exposures was determined to be less than 0.025$\mu$g TBTCl/l for both salinities and appears to be less than 0.001$\mu$g TBTCl/l in the 20ppt treatment. These results further suggest that the maximum acceptable toxicant concentration (MATC) lies somewhere below 0.025$\mu$g TBTCl/l and may be as low or lower than 0.001$\mu$g TBTCl/l, particularly under 20ppt conditions. Calculating precise NOEC and MATC values for the topical treatments was not possible due to the appearance of an increase in sublethal effects at the lower concentrations of both salinities and in the 0ppt pilot study data. Though several of the intermediate topical doses were not significantly different from controls, the increase in effects observed in the lower doses suggests that the NOEC and MATC may lie below a nominal exposure of 0.0000187$\mu$g TBTCl/egg and an absolute dose of 0.0000066$\mu$g TBTCl/egg considering the permeability factor of 35.3% determined in Chapter 3. The absence of the escalation in low concentration adverse impact in the immersion treated data may have been due to the fact that the immersion equivalents were not appropriate (due to the underestimated BCF of 4,000). Therefore, the immersion concentrations which elicited this rise in effects may not have been tested in the concentration range employed in this study. Further study should evaluate concentrations below 0.001$\mu$g TBTCl/l to determine if a similar rise would be detected at immersion concentrations more comparable to the predicted (i.e., topical) egg exposure.

The immersion results differ significantly from previous studies employing the medaka and TBT as an exposure toxicant. Walker *et al.* (1989) evaluated the effects of tributyltin oxide (TBTO) on the medaka. As a component of their study, these investigators evaluated the eggs of adult medaka reared in artificial freshwater containing varying concentrations of TBTO for abnormalities, toxicity, percent hatch,

time to hatch as well as fry anomalies and mortalities. Though the eggs themselves were not reared in the TBTO solutions, abnormalities similar to those observed in the present study were observed. However, a lower level of occurrence of abnormalities was observed by Walker *et al.* (1989) as compared to that seen in the present study. Ophthalmic, skeletal, cephalic and circulatory abnormalities were all detected in eggs collected from exposed adults in concentrations ranging from 0.40 to 3.60$\mu$g TBTO/l, however, the level of abnormalities in a 0.09$\mu$g TBTO/l exposure were not statistically significantly different from the control groups.

As a component of their study of the effects of two organotins on two teleost fish species, Wester *et al.* (1990) evaluated the effects of TBTO on the eggs of the medaka in a freshwater environment. The exposures were carried out under static-renewal conditions at concentrations ranging from 0.1 to 32$\mu$g TBTO/l. Complete mortality was observed in the 32$\mu$g TBTO/l concentration, however, the no observable effects level for toxicological parameters (defined as mortality, growth, general appearance and abnormal behavior) was determined to be 3.2$\mu$g TBTO/l. Inclusion of histological evaluation resulted in a reduction of the NOEC to 0.32$\mu$g TBTO/l. None of the anomalies observed in the present study or in the Walker *et al.* (1989) study were observed by Wester *et al.* (1990); the primary abnormal observations were at the organ level.

The elevated level of effects observed in the present immersion exposures as compared to the two previous studies may have been due to the higher salinities employed in this study and the response of the medaka to this saline environment. Though 0ppt was not evaluated for the immersion response, the statistically insignificant differences between the effects of the three salinities on topically treated

eggs suggests that it is not an effect of salinity stress, but could be due to another chemical interaction occurring when the TBT is contained in the rearing solution itself. On the other hand, differences in rearing, exposure and other techniques may have been responsible for the variation in response between the studies. For example, in the present study, silane was used to bind all active sites in any glassware used during the course of the dilution preparations and rearing events. In addition to the reduction in loss of TBTCl through binding of these active sites, the solutions contained methylene blue, were stored in amber bottles in the dark and were refrigerated between renewals which may have significantly reduced the loss of TBT, therefore, allowing more of the substance to be available to elicit a response.

Relative to other sensitive organisms exposed to TBT, the eggs of the medaka appear to be one of the most sensitive species to this substance. The lowest previously observed response was in the dog-whelk (*Nucella lapillus*) where signs of imposex were observed in 0.005$\mu$g TBT/l exposures (Gibbs *et al.*, 1988). Statistically significant ($p > 0.050$) adverse effects on the medaka were observed at 0.001$\mu$g TBTCl/l in 20ppt salinity rearing conditions and the 35ppt exposure at this concentration was significantly different from controls at $p > 0.025$. The approximate 96hr $LC_{50}$ for the medaka eggs was in the same range of most other tested species (invertebrates and vertebrates) at 99.4$\mu$g TBTCl/l and 93.8$\mu$g TBTCl/l for the 20ppt and 35ppt salinities, respectively. However, following extended exposure and the potential impact of a secondary mode of action, these $LC_{50}$ levels drop dramatically to 0.052 and 0.225$\mu$g TBTCl/l for 20ppt and 35ppt, respectively after the 21-day exposure. In addition, though the effect of salinity was not determined to be statistically significant and the initial response (e.g., 96hr) in the two salinities was

very similar, it is apparent that, if a secondary mode of action exists, its impact is more severe under 20ppt exposure conditions. This pattern of increased effects from 0ppt to 20ppt and a decrease in 35ppt is opposite from what one might expect when considering historical data which have indicated that the affinity of TBT for lipids is lowest at 25ppt, higher at 45ppt and yet higher in deionized water (Laughlin *et al.*, 1986). This may be due to the eggs reaching isotonic conditions near or at the 35ppt salinity exposure, thereby reducing the osmoregulation stress on the eggs. Nevertheless, it is evident that none of the control groups, whether untreated or DMSO sham controls, were significantly different from each other, indicating that a salinity up to 35ppt does not have a differential effect on successful and timely development of *O. latipes* eggs.

Another factor supporting the suggestion that TBT may be acting under two different modes of action can be seen in the time plots (Figures 6.4 & 6.5 and Appendix A). Over the duration of the 21 day exposure study there appears to be an initial, rapid, lethal response to TBTCl followed several days later by the appearance of secondary lethal and sublethal responses (these effects are most pronounced at the intermediate concentrations of approximately 0.025 to 0.802$\mu$g TBTCl/l). These secondary responses may be due to the alteration of tissues and the mechanisms for ionic transport and ATP synthesis as described in previous studies of TBT mode of action (Stockdale *et al.*, 1970; Evans and Karpel, 1985; Goldberg, 1986; Laughlin, 1987; Lawler and Aldrich, 1987; Boyer, 1989).

The most significant and consistent adverse effect of TBTCl on the medaka eggs was the skeletal deformation shown in Figure 6.11 and described by Walker *et*

*al.* (1989) and others (see Boyer, 1989). The reason for this apparent cessation in differentiation of the spine is unknown; however, based on the studies by Rice and Weeks (1989, 1990) it is possible that TBT is somehow altering the calcium pump of the developing embryo. Evaluating the effect of TBT on the oyster toadfish (*Opsanus tau*) macrophages, these authors found that a concentration 50$\mu$g TBT/l initiated an influx of calcium and a concentration of 500$\mu$g TBT/l inhibited this influx; in other words, TBT alters membranes, either chemically or physically, and subsequently affects the mobilization of calcium across these membranes in a dose-dependent fashion. Therefore, it is possible that the skeletal effects observed in the medaka eggs may be the result of the alteration of calcium mobilization and that at high concentrations, this may result in rapid mortality and severe skeletal malformations and at lower concentrations the calcium influx may induce developmental anomalies as the spine is forming. Moreover, Champ and Lowenstein (1987) found anomalies in oysters exposed to TBT where chambers were formed between the calcified shell layers and contained a protein gel. Subsequent research with this protein gel revealed that in the presence of $CaCO_3$ solution, the gel slowed or completely prevented the formation of $CaCO_3$ crystals. It may be possible that such a protein is being formed in the developing medaka fry and subsequently inhibits the successful formation of the calcium-based skeletal vertebrae.

A BCF of 4,000 was chosen here based on prior studies of similar length and design. However, as discussed previously, investigators have also observed BCF values over an order of magnitude higher than this value. The statistically significant difference determined between the two exposure types in conjunction with the statistically similar slopes for adverse effects suggests that the mode(s) of action was

approximately the same for both treatments. However, there appears to be a displacement of the regression lines possible due to an underestimate of the TBTCl BCF as it represents medaka eggs. Through modification of equation 6.1 to include the $LD_{50}$ and $LC_{50}$ values determined in these exposure studies, an approximate BCF value for the medaka eggs was determined to be three orders of magnitude higher than the predicted 4,000 value for both salinities. In addition, it is possible that the BCF may change with concentration, resulting in unpredictable responses with respect to dose. Previous investigations have demonstrated that TBT BCF values actually increase with decreasing concentration (Waldock and Thain, 1983; Unsal, 1984; Zuolian and Jensen, 1989) possibly explaining the increase in effects in the lower topical concentrations.

Three primary conclusions can be drawn from this research. First, the eggs of the medaka appear to be an ideal test species for the evaluation of polluted coastal environments of varying salinity, in that the adults and eggs are hardy under conditions from 0ppt to 35ppt, but that the eggs are exceptionally sensitive to toxic and teratogenic substances such as TBTCl. Through refinement and validation of the theoretical equation applied to BCF and $LD_{50}$ data, it may be possible to use the MELA assay to estimate the effects of natural conditions. In addition, if immersion studies are required, the medaka eggs respond well to a standard static-renewal protocol under increased salinity conditions.

Second, the bioconcentration of TBT in the egg of this teleost appears to be very high, possibly higher than any other reported BCF for TBT. If this is representative of other fish early life stages, there is substantial cause for concern as the majority of these early life stages reside either at the seasurface microlayer or at

the sediment surface, where TBT tends to accumulate to very high levels. Finally, in conjunction with the federal government, Virginia has been a leader in establishing regulations on the use and input of TBT into coastal waterways. The sale of TBT to the general public has been limited and application has been restricted to vessels greater than 25 meters (except for aluminum hulled boats). Along with these restrictions, the U.S. Environmental Protection Agency has issued an ambient water quality advisory concentration of 0.010$\mu$g TBT/l (U.S. EPA, 1987). Using this standard as guidance, Virginia set their standard a conservative order of magnitude lower at 0.001$\mu$g TBT/l, the lowest criterion in the country. However, based on the toxicological data presented here, it is possible that this standard may be yet too high to protect sensitive early life stages of fish populations. Further, it is imperative that this, or a reduced standard, be enforced with the strictest penalties as it is apparent that TBT may indeed be the most toxic substance knowingly introduced into natural waterways. Numerous research papers have presented ambient water and sediment concentrations which were well over these limits, especially during high boating activity (see reviews by Hall and Pinkney, 1985; Champ and Bleil, 1988; U.S. EPA, 1988b; and Boyer, 1989). In addition, ten sampling events between September, 1991 and September, 1992 in the Elizabeth River, Virginia revealed the presence of TBT in all ambient samples with concentrations ranging from 0.017 to 0.228$\mu$g TBT/l (Alden and Helmstetter, unpublished data). If the level of impact of these concentrations on the eggs of the medaka is any indication of the potential effects on indigenous species, it is possible that TBT may be causing intense reduction in successful hatchings and potential alterations in finfish species diversity, abundance and recruitment.

# APPENDIX A

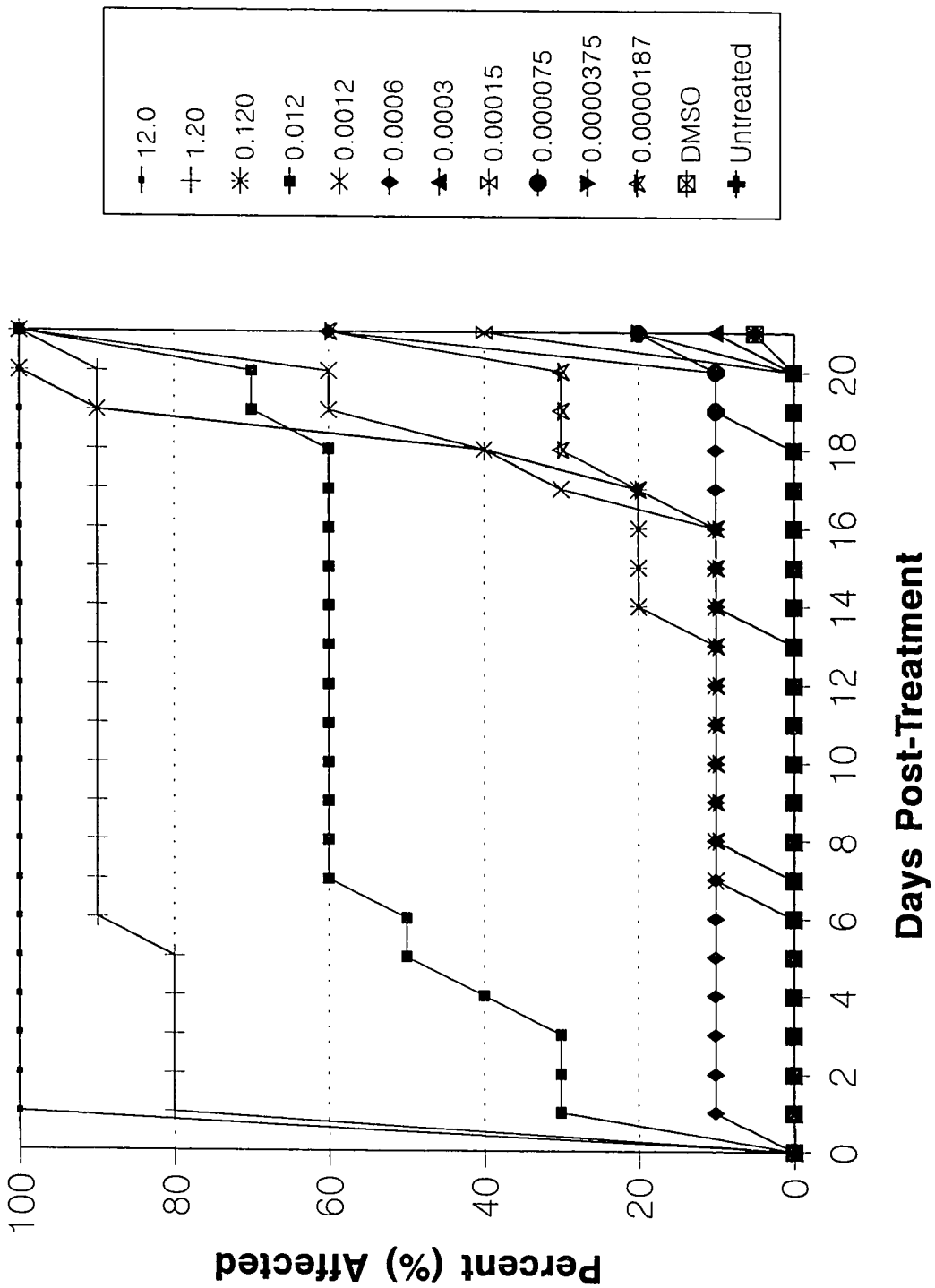



**Figure A.6.1.** Time-related changes in all adverse effects on medaka eggs topically treated with TBTCl and reared in 0ppt medium. Values are data for single, pilot study sample.

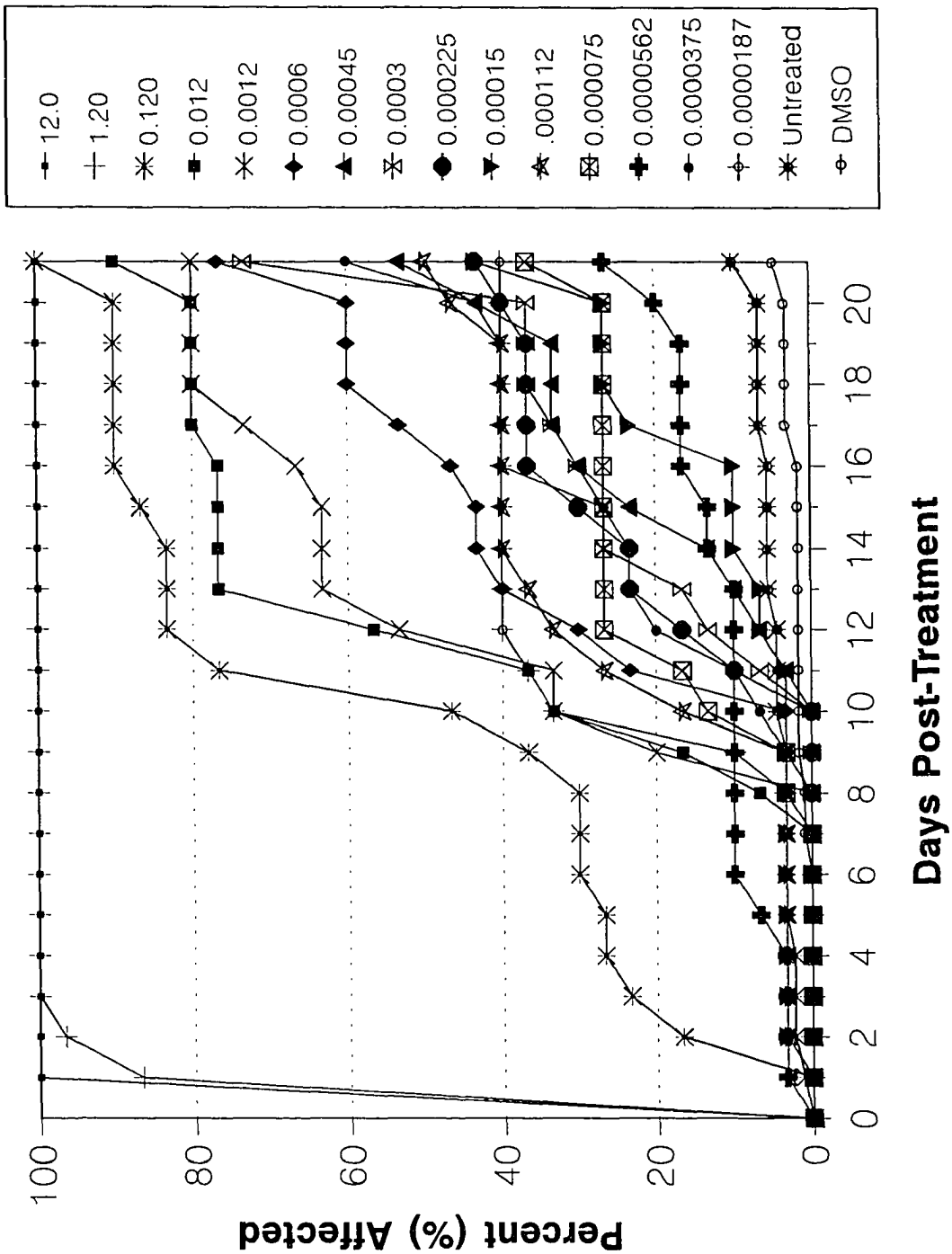



**Figure A.6.2.** Time-related changes in all adverse effects on medaka eggs topically treated with TBTCl and reared in 20ppt medium. Values are means of three replicates for each concentration.

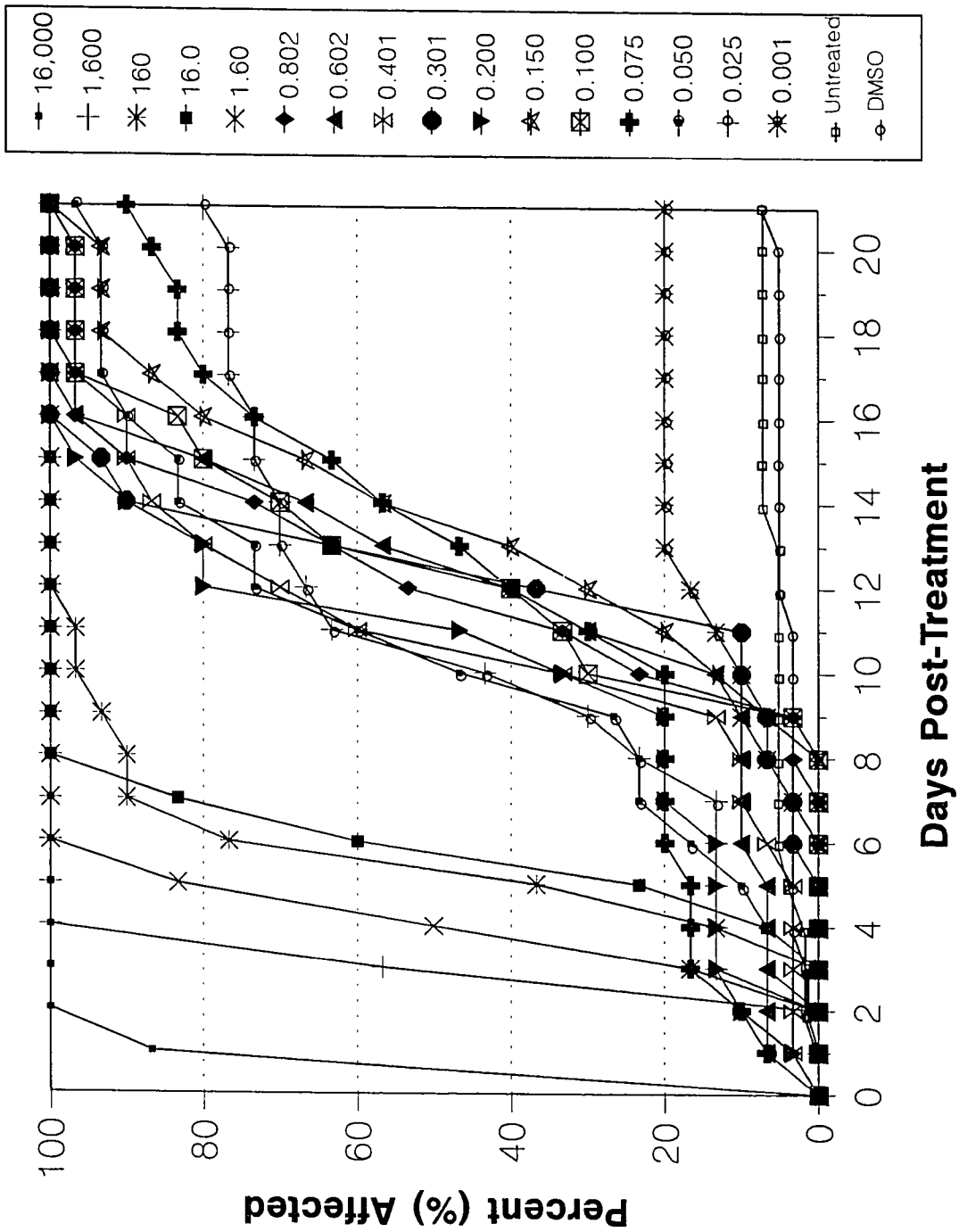



**Figure A.6.3.** Time-related changes in all adverse effects on medaka eggs immersion treated with TBTCl in 20ppt medium. Values are means of three replicates for each concentration.

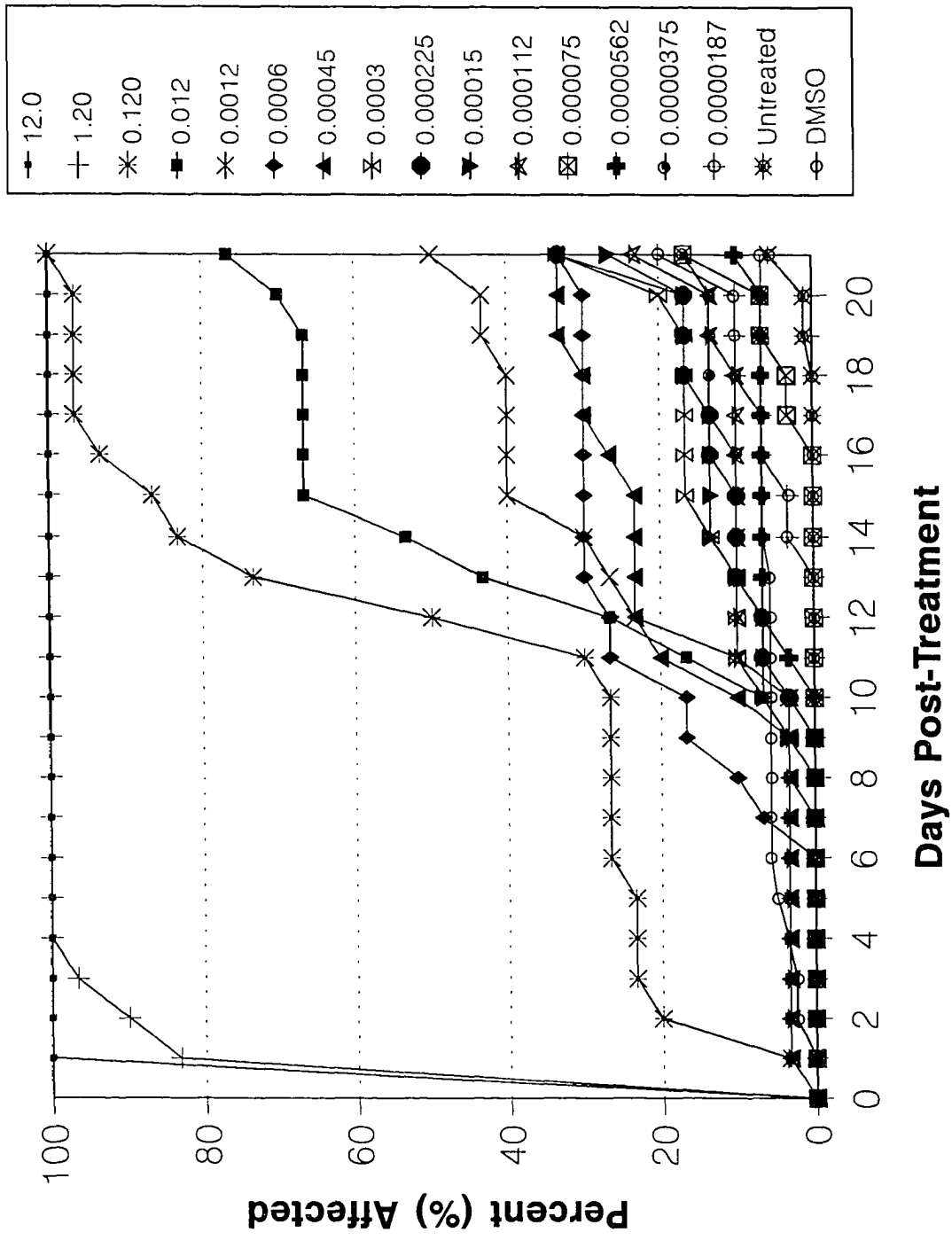



**Figure A.6.4.** Time-related changes in all adverse effects on medaka eggs topically treated with TBTCl and reared in 35ppt medium. Values are means of three replicates for each concentration.

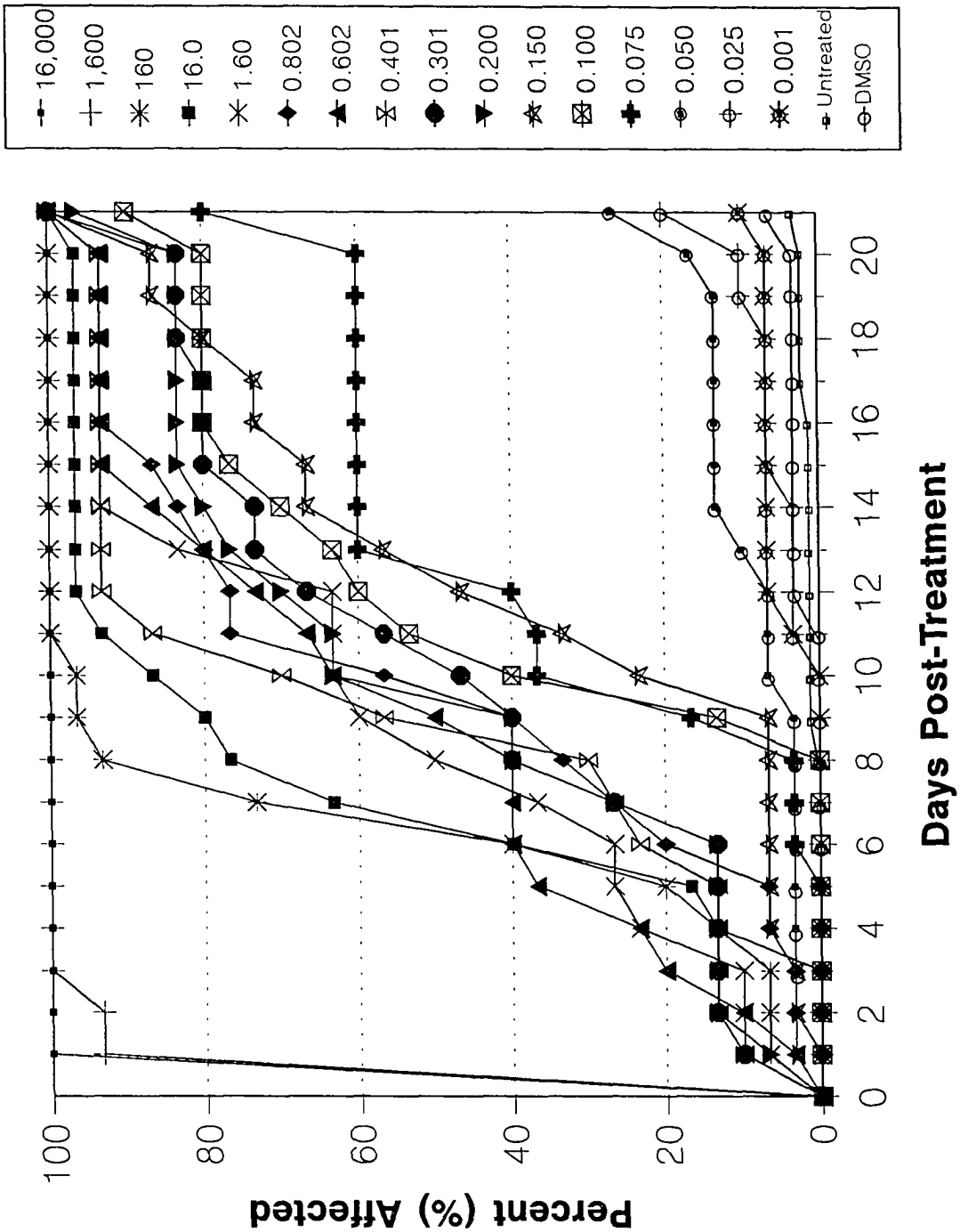



**Figure A.6.5.** Time-related changes in all adverse effects on medaka eggs immersion treated with TBTCl in 35ppt medium. Values are means of three replicates for each concentration.

## CHAPTER SUMMARY

The present study evaluated the salinity-related effects of TBTCl on the eggs of the Japanese medaka. Two exposure conditions were employed: 20ppt and 35ppt salinities, and the results were compared to the freshwater TBTCl study presented in Chapter 2. An estimated BCF for TBT (4000) was applied to the topical treatment doses to estimate immersion medium concentrations, allowing a side-by-side evaluation of the BCF value as it represents medaka eggs and the ability of the topical treatment to be applied to medium concentrations through the use of this BCF.

The results indicate that under these exposure conditions, the early life stages of the medaka may be among the most sensitive reported for this chemical. The no observable effects concentration (NOEC) in the immersion exposures was determined to be less than 0.025$\mu$g TBTCl/l for both salinities and appears to be less than 0.001$\mu$g TBTCl/l in the 20ppt treatment.

Statistical analysis indicated that there was no significant effect of salinity on TBTCl toxicity. However, a statistically significant difference was seen between the two exposure types. This, in conjunction with the statistically similar slopes for adverse effects plots, suggests that the mode(s) of action was approximately the same for both treatments. However, there appears to be a displacement of the regression lines, possibly due to an underestimate of the TBTCl BCF for medaka eggs.

The most significant and consistent sublethal effect of TBTCl on the medaka eggs was skeletal deformation, particularly of the spine and pectoral fins and poor tissue differentiation.

# CHAPTER SEVEN

## Employment of MELA in Field Validation Studies

## INTRODUCTION

The research presented thus far has focused on the utility of the medaka embryo-larval assay (MELA) as a laboratory-based test for toxicity and teratogenicity of individual agents of unknown or suspect adverse affect. This Chapter will focus on the application of MELA to field-collected samples in basin-wide evaluations of both water and sediment samples.

The Port of Hampton Roads, Virginia is one of the largest industrial seaports in the country and is the largest military port in the world. In direct conflict with this industrial aspect of the system, the economy of the region is very dependent on tourism and the numerous fisheries found in this area. The Elizabeth River (Figure 7.1), with a drainage basin in excess of 500km$^2$, is the primary source of industrial activity in the Hampton Roads area. Increased military activity as well as extensive commercial and industrial growth have resulted in the input of substantial pollution into this system which drains into the lower Chesapeake Bay via the James River. In addition, the rate of flushing in this system is very low (Neilson, 1975). This, in addition to the slight topographic relief of the area, results in a poorly flushed system which acts as a sink for these anthropogenic inputs. Previous chemical and toxicological investigations of the Elizabeth River system and its receiving waters (i.e., the lower James River and lower Chesapeake Bay) have indicated that this may be one of the most polluted areas in the country, particularly with respect to polynuclear aromatic hydrocarbons and metals contamination (Alden and Young, 1982; Rule, 1986; Alden and Butt, 1987; Huggett *et al.*, 1988; NOAA, 1990; and others).

MELA was employed in two studies of this region. The first was an extensive

evaluation of the waters adjacent to a number of point and non-point sources of pollution to the system. This study incorporated the chemical characterization and examination of the chemical bioactivity of the three branches of the Elizabeth River (Eastern, Western and Southern) as well as the River's Main Stem, extending out into the mouth of the Chesapeake Bay. The second, was a smaller study of the sediments at numerous sites in the River, and lower Chesapeake Bay, in which a number of additional biological and chemical assays were employed for the evaluation of the system.

Both of these field evaluations took place prior to the refinement and validation of the MELA approach and, therefore, contain methods which were precursory to those presented in Chapters 5 and 6. However, these data indicate the effectiveness of this assay to characterize the relative impact of numerous sites, simultaneously, with respect to toxicity and teratogenicity. In addition, results from other assays employed in these studies indicate that the medaka assay complemented and often correlated well with these other assays of mutagenicity and cytotoxicity.

**Study I: Water Samples**

This study consisted of a series of toxicological and chemical investigations into the distribution of toxicants/mutagens associated with various land use activities (LUAs) in the Elizabeth River Basin. The study was comprised of two phases. Phase I involved the evaluation of 52 LUAs employing innovative sample collection and assay techniques designed to distinguish which areas of the River were most associated with biologically-active contaminants. The 52 sites and their respective LUA type and site designations are presented in Figure 7.1 and Table 7.1. Collection

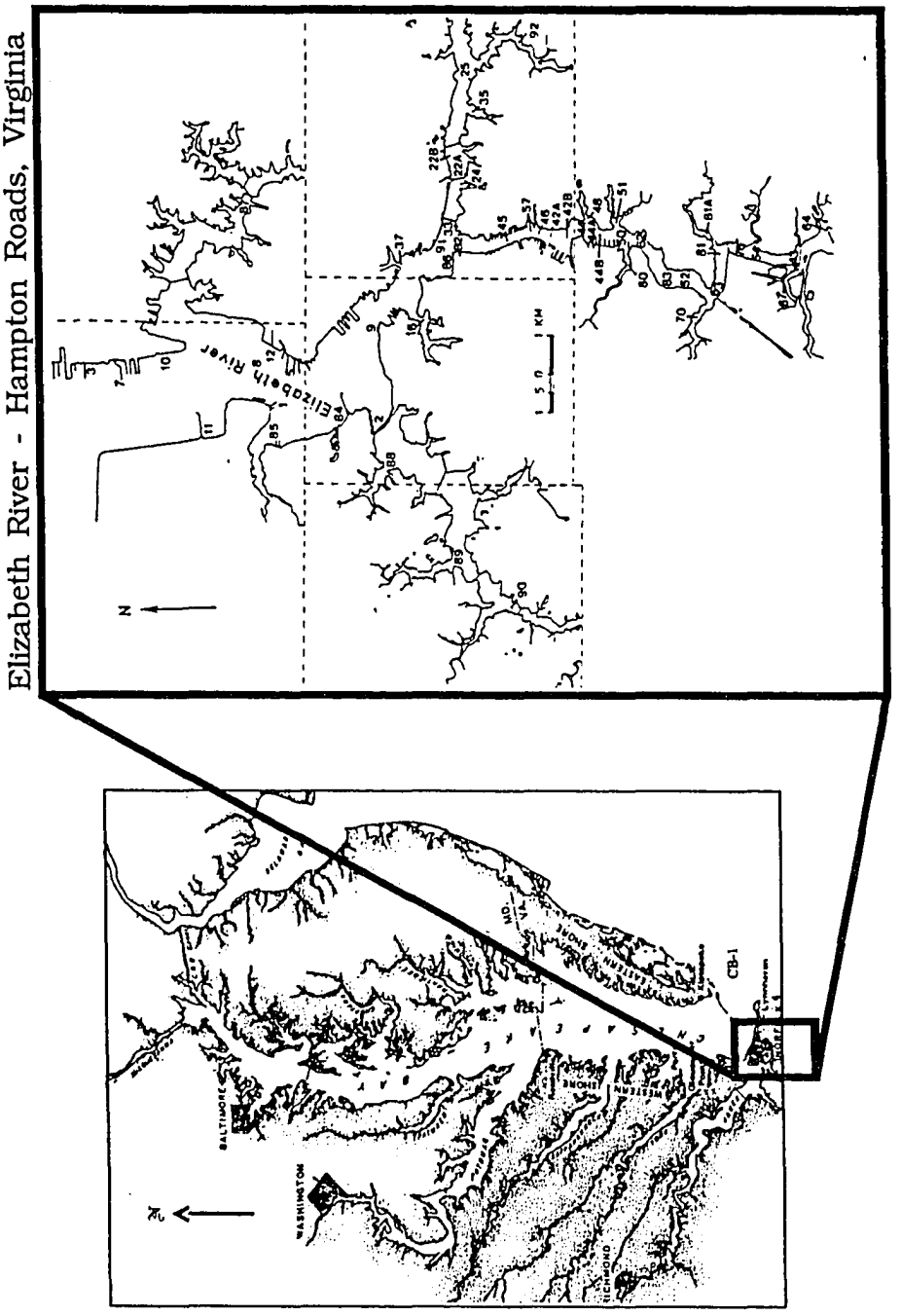



**Figure 7.1.** Map of the Elizabeth River indicating the Phase I study sites. See Table 7.1 for site names and LUA categories.

**Table 7.1.** Sampling site numbers, designations and LUA categories.

| Site No. | Site Name | LUA Category |
|---|---|---|
| 1 | Craney Island Naval Supply | MILIT |
| 2 | Virginia Chemical | CP |
| 3 | Exxon | OT |
| 5 | Lambert's Point Coal Loading | I&C |
| 7 | Army Base STP Outfall | POTW |
| 8 | Lambert's Point STP Outfall | POTW |
| 9 | Pinner's Point STP Outfall | POTW |
| 10 | Norfolk International Terminals (NIT) | I&C |
| 11 | Craney Island Rehandling Basin | LF&DA |
| 12 | Lambert's Point Drainage | LF&DA |
| 16 | Scott Creek | M&D |
| 22A | NORSHIPCO-Brambleton | SY |
| 22B | NORSHIPCO-Brambleton | SY |
| 24 | Colonna's Shipyard | SY |
| 25 | Ford Plant | I&C |
| 33 | Metro Machine | SY |
| 35 | Campostella Landfill | LF&DA |
| 37 | The Hague | UR |
| 42A | Norfolk Naval Shipyard (NNSY) | SY |
| 42B | Norfolk Naval Shipyard (NNSY) | SY |
| 43 | Virginia Power (VEPCO) Dike | I&C |
| 44 | Royster | CP |
| 44A | Atlantic Wood Ditch | CP |
| 44B | Atlantic Wood | CP |
| 45 | NORSHIPCO-Berkley | SY |
| 46 | Tropicana | OT |
| 48 | Amoco | OT |
| 50 | Cargill | CP |
| 51 | Gilligan Creek | UR |
| 52 | Tenneco | OT |
| 54 | Weaver Fertilizer | CP |
| 57 | Seahorse Marine | I&C |
| 62 | Norfolk & Western Railroad | I&C |
| 63 | Swan Oil | OT |
| 64 | Huntsman Chemical | CP |
| 67 | Virginia Power (VEPCO) Canal | I&C |
| 70 | St. Juliens Creek | MILIT |
| 76 | Smith-Douglass | CP |
| 80 | Paradise Creek | UR |
| 81 | Milldam Creek | UR |
| 81A | Milldam Creek | UR |
| 82 | Portside | M&D |
| 83 | Blows Creek | MILIT |

**Table 7.1 (Cont'd).**

| Site No. | Site Name | LUA Category |
|---|---|---|
| 84 | Lake Kingman | CP |
| 95 | Craney Island Coast Guard Base | MILIT |
| 86 | Tidewater Yacht Club | M&D |
| 87 | Haven Creek | UR |
| 88 | Lilly Creek | UR |
| 89 | Sterns Creek | UR |
| 90 | Western Branch Storm Drain | UR |
| 91 | Waterside | M&D |
| 92 | Indian River Creek | UR |

Key to LUA Categories:

| | |
|---|---|
| CP | = Chemical processing industries |
| I&C | = General industrial and commercial sites |
| LF&DA | = Landfills and disposal areas |
| M&D | = Marina and dock areas |
| MILIT | = Military installations (other than shipyards) |
| OT | = Oil terminals |
| POTW | = Publically-owned treatment works (sewage treatment plants) |
| SY | = Shipyards |
| UR | = Urban runoff (and creek drainage basins) |

devices (termed "SCDs" for "sorbent containing devices") were deployed to concentrate organics from receiving waters in proximity to the various LUAs. Sample extracts were evaluated through a series of biological assays of toxicity, teratogenicity and mutagenicity including the Ames *Salmonella* mutagenicity test, a microbial respiration assay employing a common species of estuarine bacteria (*Vibrio alginolyticus*), and the medaka embryo-larval assay. These assays provided rapid turnaround of data and allowed the simultaneous evaluation of numerous sites to determine the relative patterns of effects. Multivariate statistical techniques and a quantitative ranking protocol were used to assess these patterns.

Phase II of this study involved the biological and chemical assessment of

several of the "worst" sites selected from the Phase I data as well as several "ambient" sites located at one nautical mile intervals throughout the study area and into the Chesapeake Bay. The Phase II assays included those used in Phase I in addition to an assay of cytotoxicity utilizing human cell cultures, a standard whole organism assay using the mysid shrimp *Mysidopsis bahia* in addition to numerous analyses of chemical content for organics, metals and nutrients. Statistical ranking protocols similar to those employed in Phase I were used to assess spatial patterns of biological effects and chemical content.

Here, I will report primarily on the results from the medaka assay and briefly on their relationship to the other biological assays and chemical analyses employed during the study. For a more detailed discussion of the assay methods other than the MELA test and the data comparisons, refer to the report by Alden *et al.* (1988).

## Materials and Methods

### Phase I

Organic pollutants are typically present in natural riverine, estuarine and coastal waters in trace amounts, at levels often undetectable by modern instrumentation. In order to concentrate these components to detectable levels, one typically had to resort to extracting large volumes of sample with large quantities of non-polar organic solvent. The methods employed for collection of samples in this study were designed to circumvent this problem. Solid phase extraction (SPE) resins have been employed in laboratory-based studies for concentrating or partitioning trace levels of organic compounds (Chladek and Marano, 1984; Ozretich and Schroeder, 1986; Junk and Richard, 1988; and others). Properly selected, these resins have the

capability of stripping numerous classes of non-polar organics from aqueous media. Following a thorough evaluation of six resins in the laboratory, this study employed three resins (cyclohexyl [CH], octadecyl [$C_{18}$] and XAD-2) in field collection samplers called sorbent containing devices (SCDs). A subset of the results of this laboratory evaluation is presented in Table 7.2. These results indicate that following exposure to several chemicals in spiked water samples for 24hr, the three resins selected for use in the SCDs concentrate a significant portion of these test components.

For the field studies, each resin was placed in one of three SCDs located on a sampling buoy (Figure 7.2) and the entire setup was deployed for a 72hr sampling period. One SCD buoy was deployed at each site. Each SCD was made up of a marine-grade stainless steel ring ($^3/_4$in wide x $1^7/_8$in O.D.) and two 125$\mu$m nitex mesh screens held to the steel ring with viton o-rings; each contained 5g of pre-cleaned resin, either CH, $C_{18}$ or XAD-2.

Following retrieval of the SCD buoys, the sorbents were removed and extracted with methylene chloride and the extracts from the three different sorbent types were combined for each site. Each extract was then divided into aliquots for chemical and biological analyses. A pilot study was carried out prior to the main studies to determine the effectiveness of these SCDs at concentrating non-polar organics in the field.

Rearing conditions for adult breeder *Oryzias latipes* were identical to those discussed in Chapter 2. Egg collection and rearing conditions were also analogous with the exception that the eggs were incubated at 27 $\pm$ 1°C. The topical treatment procedure was similar to that presented in the single toxicant investigations, except

**Table 7.2.** Results of laboratory sorbent uptake studies for six resins. The data are expressed as mean recoveries for three replicates ± the standard error of each mean.

| Compound | XAD-2 | $C_{18}$ | CH | $C_8$ | $C_2$ | Charcoal |
|---|---|---|---|---|---|---|
| Bis(2-chloroisopropyl)-ether | 61.8 ± 4.1 | 39.4 ± 2.8 | 37.1 ± 2.5 | 8.5 ± 2.1 | 7.5 ± 1.3 | 17.1 ± 8.8 |
| Nitrobenzene | 58.1 ± 1.8 | 30.1 ± 1.7 | 25.6 ± 3.7 | ND | 4.3 ± 0.8 | ND |
| Naphthalene | 44.5 ± 2.5 | 34.2 ± 1.7 | 22.2 ± 2.5 | 1.6 ± 0.1 | 11.3 ± 1.3 | ND |
| 4-Chlorophenylphenyl-ether | 42.8 ± 3.6 | 38.3 ± 1.7 | 16.3 ± 3.3 | 6.3 ± 2.4 | 24.7 ± 6.9 | ND |
| Fluoranthene | 32.1 ± 4.1 | 31.9 ± 7.0 | 43.2 ± 1.1 | 3.5 ± 1.3 | 14.5 ± 5.6 | ND |
| Benzo(b)fluoranthene | 31.3 ± 9.2 | 16.4 ± 7.0 | 11.3 ± 2.2 | ND | 8.3 ± 0.1 | ND |
| Benzo(a)pyrene | 27.1 ± 8.9 | 14.2 ± 6.3 | 9.4 ± 1.4 | ND | 6.3 ± 1.1 | ND |
| Dimethylphthalate | 84.8 ± 6.4 | 16.1 ± 6.2 | 20.1 ± 2.0 | 5.5 ± 2.1 | 15.6 ± 4.7 | 9.6 ± 3.4 |
| Acenaphthene | 65.6 ± 12.9 | 17.6 ± 6.2 | 19.8 ± 2.5 | 2.7 ± 0.5 | 19.1 ± 3.8 | 4.0 ± 1.4 |
| Fluorene | 61.8 ± 14.1 | 14.4 ± 7.1 | 19.5 ± 3.4 | 2.9 ± 0.5 | 20.0 ± 5.0 | 9.6 ± 7.3 |
| Anthracene | 59.9 ± 12.7 | 29.1 ± 12.6 | 15.3 ± 3.5 | 2.6 ± 0.6 | 15.8 ± 5.2 | ND |
| Butylbenzylphthalate | 83.7 ± 11.9 | 30.3 ± 12.5 | 21.4 ± 0.5 | 5.9 ± 1.1 | 24.0 ± 10.5 | ND |
| Benzo(ghi)perylene | 43.4 ± 21.6 | 47.6 ± 2.3 | 5.2 ± 1.1 | 3.6 ± 0.1 | 5.2 ± 0.2 | ND |

ND - Compound not detected

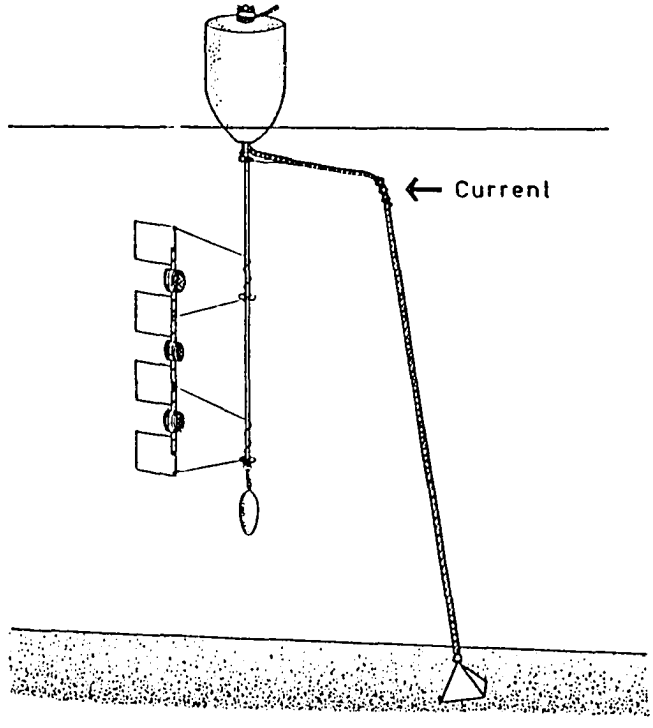



**Figure 7.2.** SCD sampling buoy deployed in Phase I studies for the collection of non-polar organics.

that a 10$\mu$l syringe was used in the Chaney repeater, resulting in a delivered dose of 0.2$\mu$l rather than the modification to provide 0.1$\mu$l as used in the toxicant assays presented previously in this dissertation. In addition, three MELA replicates of five eggs were treated for each site. The extensive nature of the program and limited funding precluded the inclusion of actual site replicates. Numerous untreated, DMSO, DMSO with methylene chloride solvent exchange, and $C_{18}$, CH and XAD-2 resin extract controls were evaluated in conjunction with the station treatments.

A 900$\mu$l aliquot of the 2ml final extract for each station sample was solvent exchanged to DMSO for the MELA evaluation. The extract aliquot was concentrated from 900$\mu$l to 25$\mu$l, 25$\mu$l of DMSO was added and the mixture was further concentrated to 25$\mu$l to remove all methylene chloride in the mixture. Eggs and hatch fry were evaluated daily up to 21 days post-treatment for acute and chronic mortality

as well as any developmental defects caused by the dose. The remainder of the extract was employed in the Ames *Salmonella* mutagenicity test (Ames *et al.*, 1975; Maron and Ames, 1983), a bacterial respiration assay and for organic chemical determinations. Pentachlorophenol was used as a reference toxicant to evaluate the viability of the eggs over a range of concentrations from 0.050 to 50.0$\mu$g/egg.

Phase II

Following statistical analysis of the Phase I data, the top quartile of sites with respect to total biological activity were selected for further evaluation as well as the top ranked sites for each biological assay. The funding agency (Virginia State Water Control Board) selected 12 of these sites for further, extensive evaluation in Phase II of the study.

Water samples for Phase II were collected at each of the 12 stations as well as at 15 ambient stations located at each nautical mile throughout the study region and a Chesapeake Bay sample to establish "background" toxicity levels (Figure 7.3). Fifteen liters of water were collected for organics analysis and the numerous biological assays; additional samples were collected separately for nutrient analysis, metals analysis and the toxicological assay with the mysid shrimp, *Mysidopsis bahia.* The fifteen liter sample for each site was extracted in the laboratory employing SPE techniques. A teflon column (37cm x 2.5cm) was packed with 20g of pre-purified XAD-2 and the sample was drawn through the column at a rate of 50ml/min. The column was then extracted with a non-polar solvent system and the extract was concentrated to a final volume of 5ml, which was stored as 5 separate 1ml aliquots.

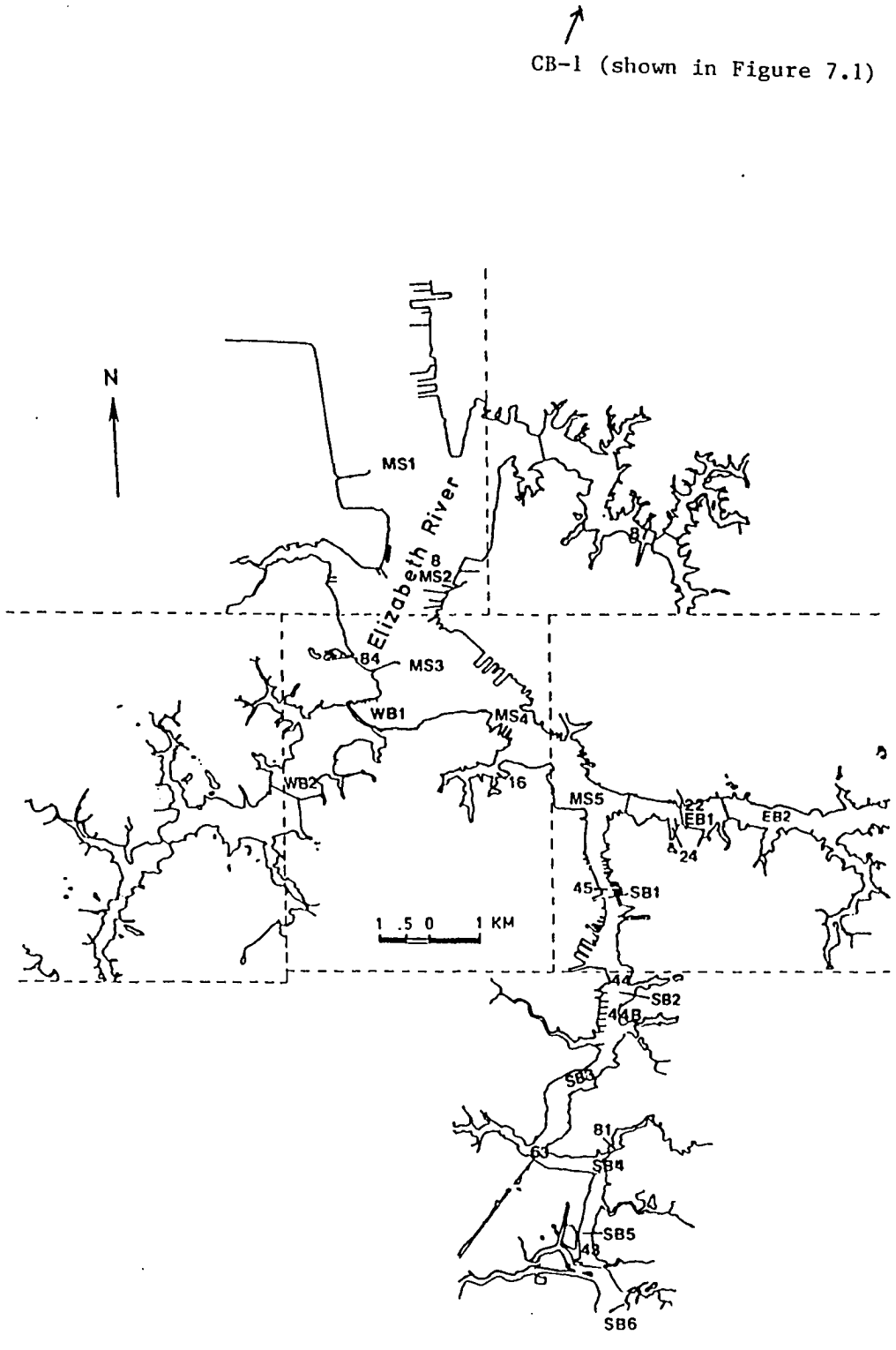



**Figure 7.3:** Map of the Elizabeth River indicating the Phase II study sites.

One aliquot was further concentrated and analyzed for organic pollutant content employing gas chromatography techniques with flame ionization and mass spectrometry detection systems. Another 1ml sample was used for the Ames *Salmonella* assay, 0.5ml was used for an animal cell culture cytotoxicity assay, and the remaining 2.5ml were used in the MELA evaluation. This aliquot was solvent exchanged to DMSO as described previously and the treatment procedures were identical to those in Phase I. As with Phase I, pentachlorophenol was employed as a reference toxicant in the Phase II studies.

A supplementary study was added to Phase II in response to the reduced occurrence of adverse effects relative to the Phase I data. This supplementary study entailed the deployment of SCDs at the Phase II LUA sites to attempt to reproduce the effects seen in the earlier samples collected employing the SCD technique. The methods for the SCD deployment and extraction were analogous to those described for Phase I. Extracts were evaluated for biological activity using the medaka and the Ames assays.

Statistical Analysis

The goals of the statistical analyses were threefold: 1) to determine relationships between general land use activity (LUA) types and potential ecological effects, 2) to characterize overall spatial patterns of potential ecological effects among sites and 3) to quantitatively rank the LUA and ambient sites with respect to each type of potential ecological effect as well as overall effects (i.e., to identify "hot spots"). Multivariate statistical techniques were employed for examining relationships between general LUA types and ecological effects. Multivariate analysis of variance

(MANOVA) and discriminant analysis were used to explore overall differences in response of the tests involving the various LUA categories. A regression model MANOVA with dummy class variables (SAS, 1985) was employed to determine whether there were statistical differences between LUA groups. Cluster analysis, followed by MANOVA and discriminant analysis, was used to define patterns of similar ecological effects among the sites. The continuity of the site groups were evaluated by discriminant classification analysis and any misclassified sites were relocated into the proper group(s). MANOVA/discriminant analysis was then employed to test for differences between groups with respect to all response parameters and to display confidence ellipses describing these differences. Finally, a quantitative ranking procedure was developed to represent relative effects on a standardized scale. Each biological variable to be included in any given ranking process was subjected to unit deviate standardization (i.e., standardized to have a mean of zero and a standard deviation of one). The standard scores for variables making up any given ranking category were then averaged. The scaled mean ranks for each ranking category were then summed to yield a "total" rank for each site (i.e., the higher the total rank, the higher the level of adverse biological effects). In addition to total rank, rank was also determined for each individual biological variable.

## Results

### Pilot Study

The results of the MELA evaluation of the pilot study extracts are shown in Figure 7.4. These results indicate that the SCD concentration technique appeared to

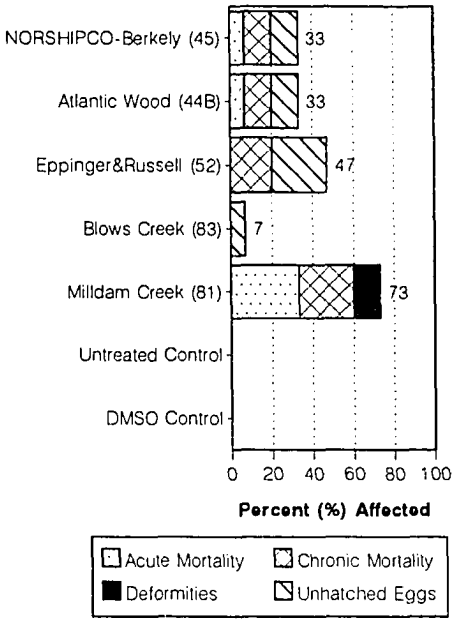



**Figure 7.4.** Results from the pilot study MELA exposures. Acute mortality is that occurring within 48hrs, chronic mortality is that occurring after 48hrs and sublethal effects include all deformities, unhatched eggs and dead fry.

perform well in the concentration of organic toxicants. Effects were observed in anywhere from 7 to 73% of the eggs dosed with extracts from each site, and included acute mortality (within the first 48hrs of exposure), "chronic" mortality (mortality occurring after 48hrs) and sublethal effects (deformities, unhatched eggs and dead fry). Perhaps the most surprising result was the level of effects resulting from exposure to the Milldam Creek extract. This site appeared to be urban runoff without a great deal of localized industry, however, the effects may have been the result of the burning down of a chemical industry (Chilean Nitrate) the year prior to sampling. In addition, a search of the USGS land surveys indicated that there are numerous potential hazardous waste sites located within the Creek's drainage basin. This sample also resulted in the highest level of mutation rate in the Ames test (fold

increase in mutation rate over background = 2.6 without S-9) and induced *Salmonella* colonies which displayed unusual morphological changes. Further, as with the medaka assay, the Ames assay results were also high in the Eppinger & Russell sample (fold increase in mutation rate over background = 2.5 without S-9).

Phase I

The results of the pentachlorophenol (PCP) reference toxicant exposure indicated a classical dose-dependent response. Though the concentrations employed were not identical to those used in the PCP studies presented in Chapter 5, the pattern and level of adverse effects appears to be very similar to the Chapter 5 data which were conducted with a different generation of breeders in excess of a year after these studies.

The MELA overall results of the Phase I samples are presented in Figure 7.5. The data for each site are broken down into acute and chronic mortality and sublethal effects. These are mutually exclusive effects which may produce an additive effect on the overall survival of a population. The extracts producing the greatest overall total effects were associated with Swan Oil (85%), Gilligan Creek (83%), Lambert's Point Sewage Treatment Plant (80%), Exxon (78%) and Lake Kingman (78%). It should be noted, however, that sample extracts associated with 19 other sites resulted in effects in over 50% of the test populations, indicating that adverse responses were fairly widespread for the study region. Common deformities included distended abdomens similar to those seen for polynuclear aromatic hydrocarbons (Chapter 2) and pentachlorophenol (Chapter 5), cardiac anomalies and numerous skeletal deformities similar to those seen for TBTCl (Chapter 6).

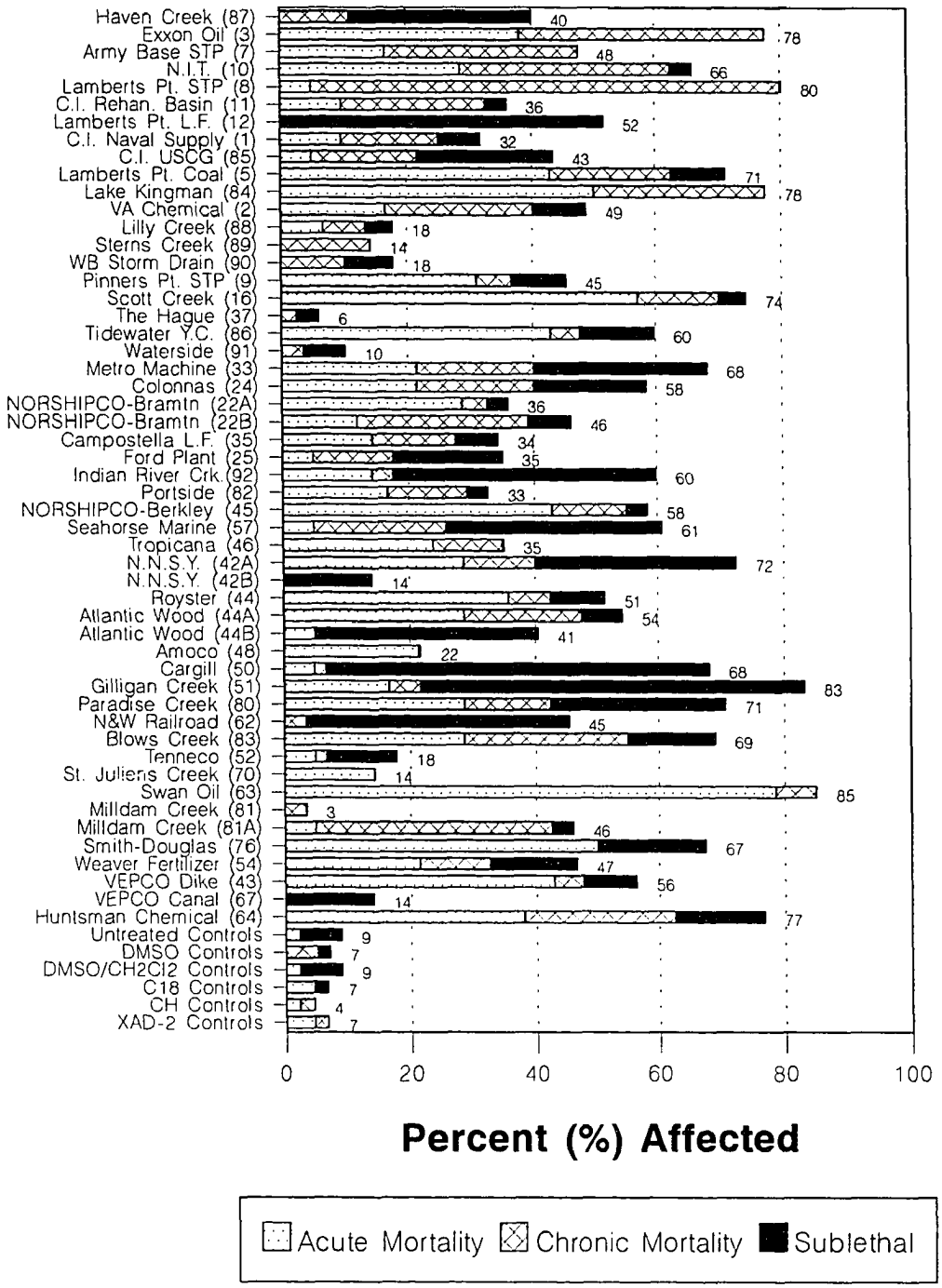



**Figure 7.5.** Results of Phase I extract exposures employing MELA. Acute mortality is that occurring within 48hrs, chronic mortality is that occurring after 48hrs and sublethal effects include all deformities, unhatched eggs and dead fry.

Three of the sites resulting in high sublethal effects also displayed mutagenic activity in the Ames assay (Indian River Creek - fold increase over background = 5.9 without S-9, Gilligan Creek - fold increase over background = 4.4 without S-9 and Atlantic Wood - fold increase over background = 2.5 for site A and 2.7 for site B without S-9), perhaps suggesting that some of the sublethal effects may be related to mutagenicity. The sites eliciting the greatest level of bacterial respiration depression (Portside = 57% depression relative to controls, Milldam Creek = 51%, NORSHIPCO-Brambleton = 44%, Haven Creek = 41% and Tropicana = 37%) were among the lowest in terms of overall effects on the medaka. However, the respiration assay did have a number of sites which correlated with the Ames mutagenicity data including Haven Creek, Lake Kingman, Colonnas, NORSHIPCO-Brambleton and Atlantic wood.

Cluster analysis of sites by biological effects (i.e., MELA, Ames and microbial respiration) identified six site groups (Figure 7.6). Although no clear patterns related to geographical location or LUA type were apparent, the discriminant analysis and MANOVA did indicate how the groups differed in terms of biological effects (Figure 7.7). The first discriminant function (DF1) was directly associated with depression in bacterial respiration and the second discriminant function (DF2) was directly associated with acute medaka mortality, with Groups 3 and 5 displaying significantly higher mortalities than the other Groups. The chronic egg mortality was negatively associated with DF1 and positively associated with DF2, indicating that Group 5 sites also tended to produce higher chronic mortalities. Groups 1 and 2 tended to be associated with depression in bacterial respiration, Group 4 was associated primarily with sublethal effects on medaka and the sites presented in Group

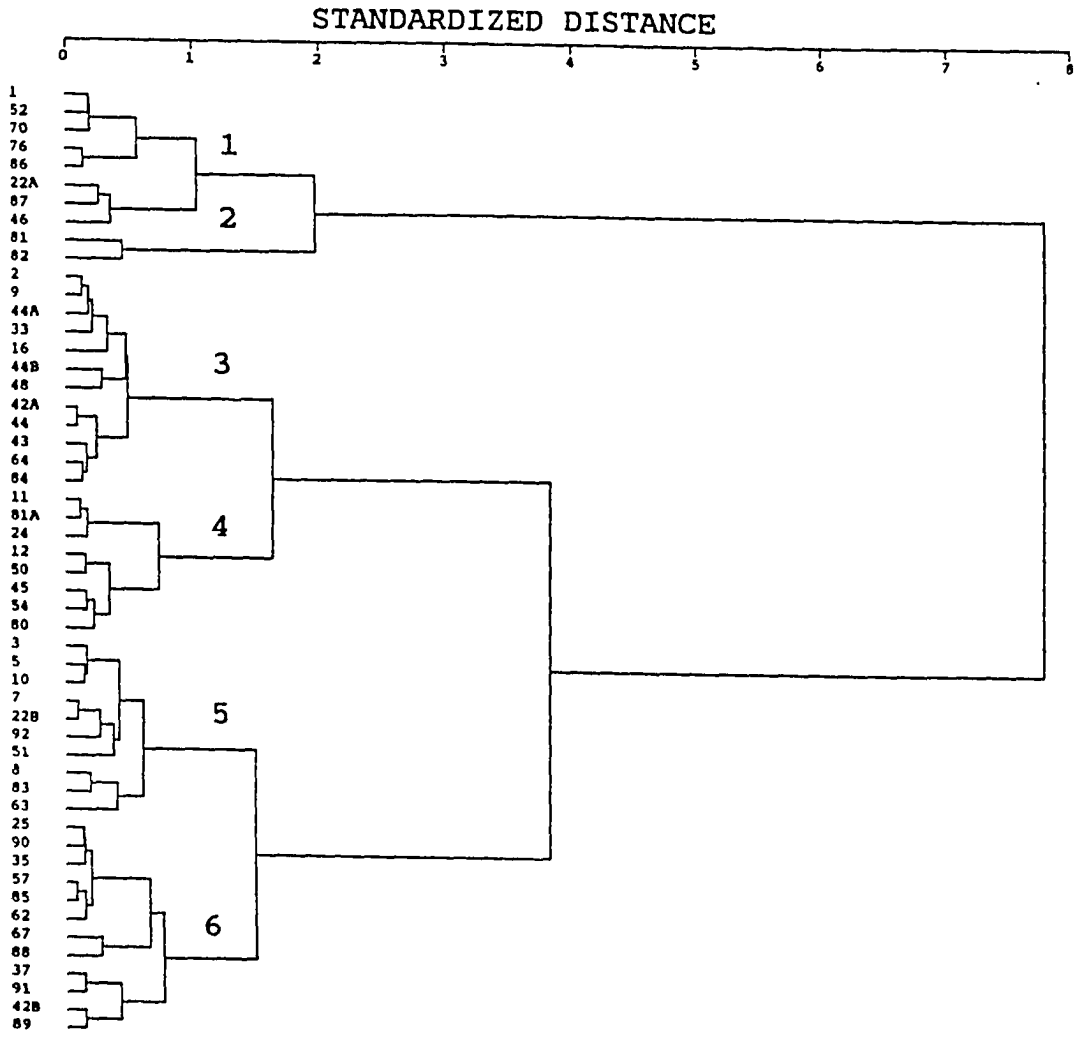



**Figure 7.6.** Standardized distance dendogram for classification of site groups with respect to biological data (i.e., MELA, Ames and microbial respiration) from Phase I studies. The numbers indicate site groups used in subsequent statistical analysis. LUA site names for numerical codes are shown in Table 7.1.

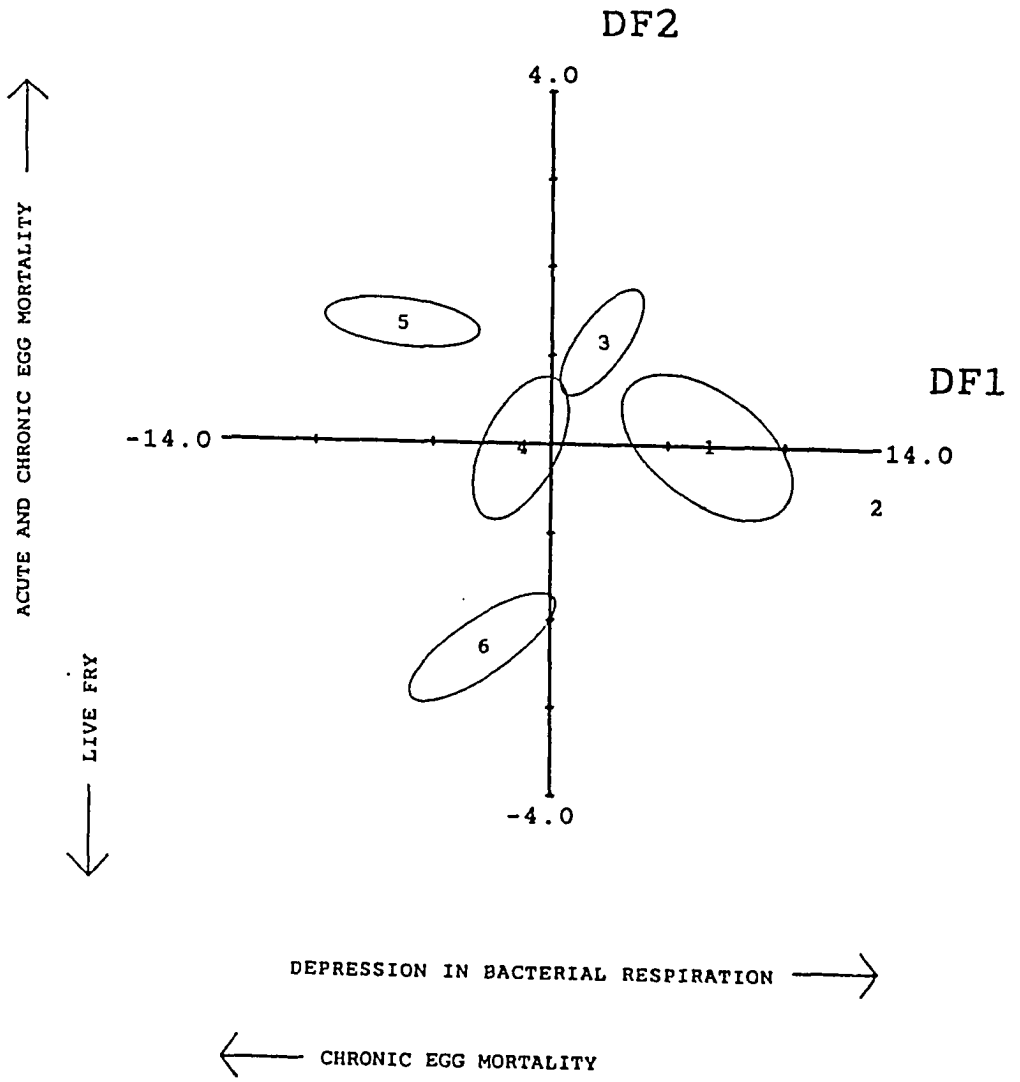



**Figure 7.7.** Confidence ellipses ($\alpha$=0.05) for canonical scores for discriminant functions (DF1 & DF2) describing differences in Phase I biological effects data between site groups defined by cluster analysis (see Figure 7.6). The number "2" represents the centroid location for this site group, since low group membership (2 sites) precluded ellipse calculations.

6 produced the lowest level of adverse biological effects.

Discriminant analysis and MANOVA models indicated that no overall biological effects pattern could be used to discriminate between LUA types. However, two minor patterns were observed with respect to the medaka data. Oil terminal LUA sites produced significantly higher ($p=0.03$) levels of acute egg mortalities than those from the other LUA categories, while the urban runoff sites produced significantly lower ($p=0.02$) acute mortalities than the overall site grand mean.

The estimated total mass of extractable organics collected by the SCDs at each site, calculated as a 1:1 response with the internal standard (2-fluorobiphenyl), are presented in Figure 7.8. The samples collected from the vicinity of the Craney Island Naval Supply Base (1) and NORSHIPCO-Berkley (45) displayed the greatest total quantity of organics. The samples from most of the remaining sites had considerably lower levels of organics.

The sites falling into the top quartile of total biological ranks plus any sites that were ranked in the top five values for any single effect were selected as candidates for the Phase II studies. These sites, representing seven LUA categories, are presented in Table 7.3 with their associated quantitative biological ranking, LUA category and selection criteria. The selection criteria provides an indication of which tests correlated best for a given site. The sites flagged with an '*' were selected for further evaluation in Phase II.

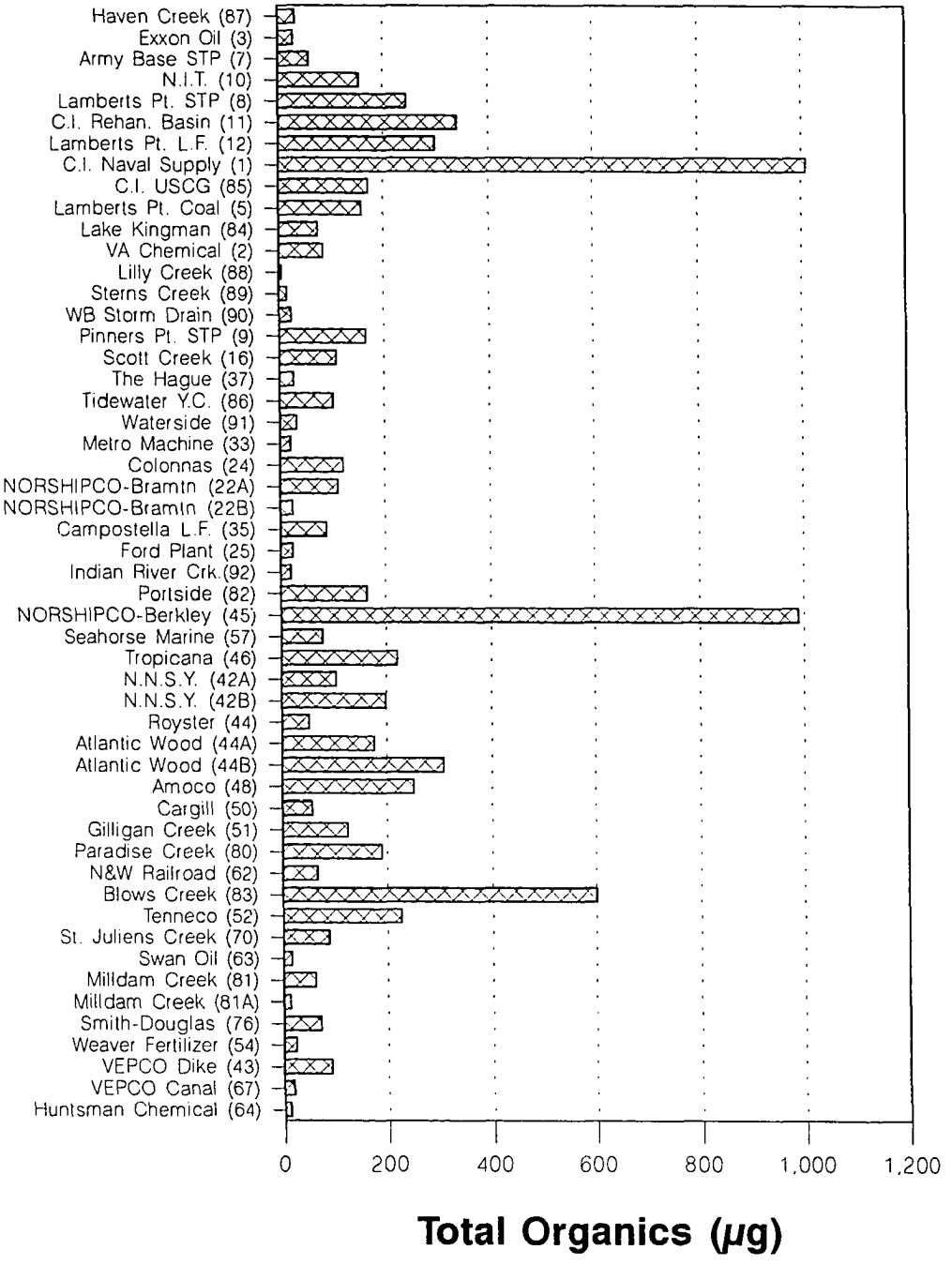



**Figure 7.8.** Total mass of organics determined in Phase I SCD extracts. Masses are total solvent extractable organics as a 1:1 response to the internal standard, 2-fluorobiphenyl.

**Table 7.3.** Phase I LUA sites proposed and selected for Phase II studies. Sites tagged with a '*' were selected for Phase II study.

| LUA Category | Site Name | Biological Index | Selection Criteria |
|---|---|---|---|
| Shipyards | NORSHIPCO-Bram.(22A)* | 7.35 | T,B,M |
| | NORSHIPCO-Berk.(45)* | 4.00 | A |
| | Colonna's Shipyard (24)* | 5.60 | T,C |
| Marinas & Docks | Portside (82) | 5.50 | T,B |
| | Scott Creek (16)* | 5.62 | T,A |
| | Waterside (91) | 2.65 | M |
| | Tidewater Yacht Club (86) | 4.67 | T |
| Oil Terminals | Swan Oil (63)* | 4.85 | T,A |
| | Tropicana (46) | 4.62 | T,B |
| Chemical Processing | Royster (44)* | 5.20 | T |
| | Huntsman Chemical (64) | 4.93 | T,C |
| | Smith-Douglass (76) | 5.87 | T,A |
| | Lake Kingman (84)* | 5.64 | T,A |
| | Atlantic Wood (44A)* | 6.47 | T,M |
| Publically-Owned Treatment Works | Lambert's Point STP (8)* | 2.31 | C |
| Industrial & Comm. | Ford Plant (25) | 4.21 | M |
| | VEPCO Dike (43)* | 4.60 | T,A |
| Urban Runoff | Gilligan Creek (51) | 3.52 | C |
| | Milldam Creek (81)* | 3.82 | B |
| | Haven Creek (87)* | 7.40 | T,B,M |
| | Indian River Creek (92) | 4.37 | C,M |

Key to Selection Criteria:

T = Total Biological Activity
A = Acute Medaka
C = Chronic Medaka
B = Bacterial Toxicity
M = Mutagenicity

Phase II

The pentachlorophenol reference toxicant data for Phase II were very similar to the Phase I reference toxicant data and those results presented in Chapter 5, indicating that the egg population remained in good and consistent overall health.

The 12 LUA and 15 "ambient" sites evaluated in Phase II are shown in Figure 7.3. In addition to the assays employed in Phase I, these sites were evaluated for biological activity with a cell culture cytotoxicity test and a whole organism test with the mysid shrimp (*Mysidopsis bahia*). Samples were also evaluated for basic water quality parameters including organics, metals and nutrients. The overall results of the medaka assay on the Phase II 15 liter extracts are presented in Figure 7.9. Extracts from 9 of the 27 sites resulted in adverse effects in greater than 50% of test organisms. Sublethal responses, primarily underdeveloped embryos and unhatched eggs, appeared to dominate the effects of the Phase II samples on the medaka. Moreover, it is apparent that the ambient samples in the highly polluted Southern Branch of the River appeared to induce lower effects than the nearby LUAs, indicating that there may be a dilution effect present in the ambient samples which were taken at mid-channel. The Chesapeake Bay sample (CB-1), added to establish "background" levels of lethal and sublethal effects, showed little adverse impact on the medaka eggs and chi-squared analysis indicated that it was not significantly ($p>0.050$) different from any control type.

Little toxicity was seen in the *Mysidopsis bahia* assay at any of the Phase II sites. This may have been due to the fact that these shrimp were exposed to whole water samples rather than the concentrated 15 liter extracts employed in the other assays. Similarly, little mutagenic activity was observed in the Ames test. Three

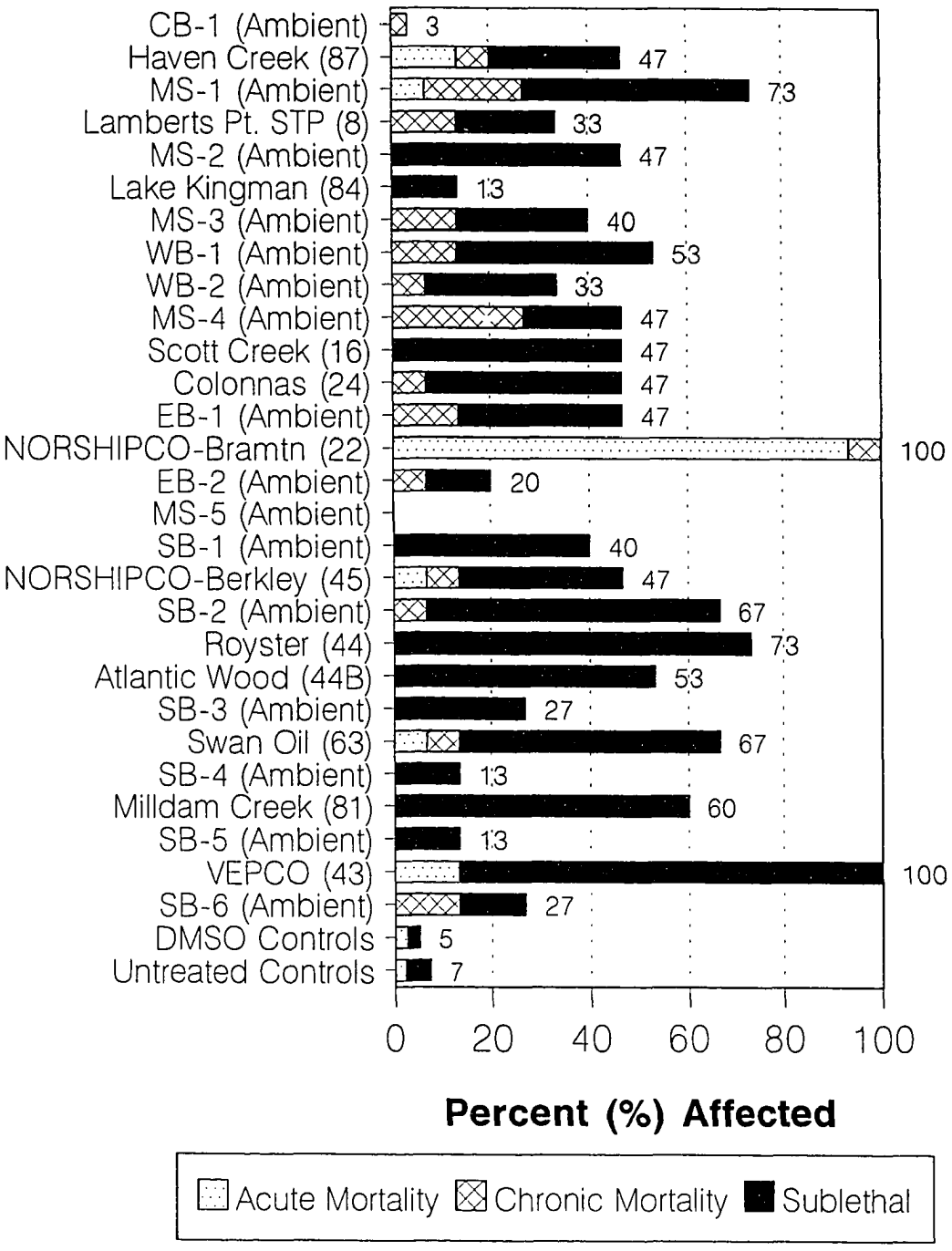



**Figure 7.9.** Results of Phase II extract exposures employing MELA. Acute mortality is that occurring within 48hrs, chronic mortality is that occurring after 48hrs and sublethal effects include all deformities, unhatched eggs and dead fry.

Mainstem ambient samples (MS-2, MS-4 and MS-5) and one LUA sample in proximity to Virginia Power Dike resulted in mutagenic activity. Further, the minimum extract concentrations producing the mutagenic effects were higher and the levels of mutagenic activity (fold increase) were lower than those observed in Phase I. Similar to the Ames data, the cytotoxicity assay produced few results which corresponded to the MELA findings, however, the cytotoxicity data did correspond well with the Ames data, with the Mainstem sites producing the greatest level of effects.

The data from the Phase II assays were statistically analyzed in a manner analogous to those in the Phase I studies. Six site groups were identified from the cluster analysis of all biological effects data (Figure 7.10). The discriminant analysis and MANOVA of the biological effects indicated that Groups 4 and 6 were strongly separated from the other groups with 4 being associated with mutagenicity and deformed medaka fry and 6 associated with acute mortality in the medaka and cytotoxicity (Figure 7.11). Elimination of the variables related to mutagenicity (which overwhelmed the discriminant analysis) resulted in the ellipses shown in Figure 7.12. The separation of the first discriminant function (DF1) is clearly dominated by acute mortality in medaka associated with the Site Group 6 samples (triplicate analyses from the vicinity of NORSHIPCO-Brambleton). The second discriminant function (DF2) separated the site groups based on more chronic biological effects. This function was positively correlated to fry mortality and negatively correlated to unhatched eggs. These effects are somewhat mutually exclusive since fry mortalities are significant only in tests where the eggs hatched. Site Group 1 displayed significantly higher levels of fry mortality than the other

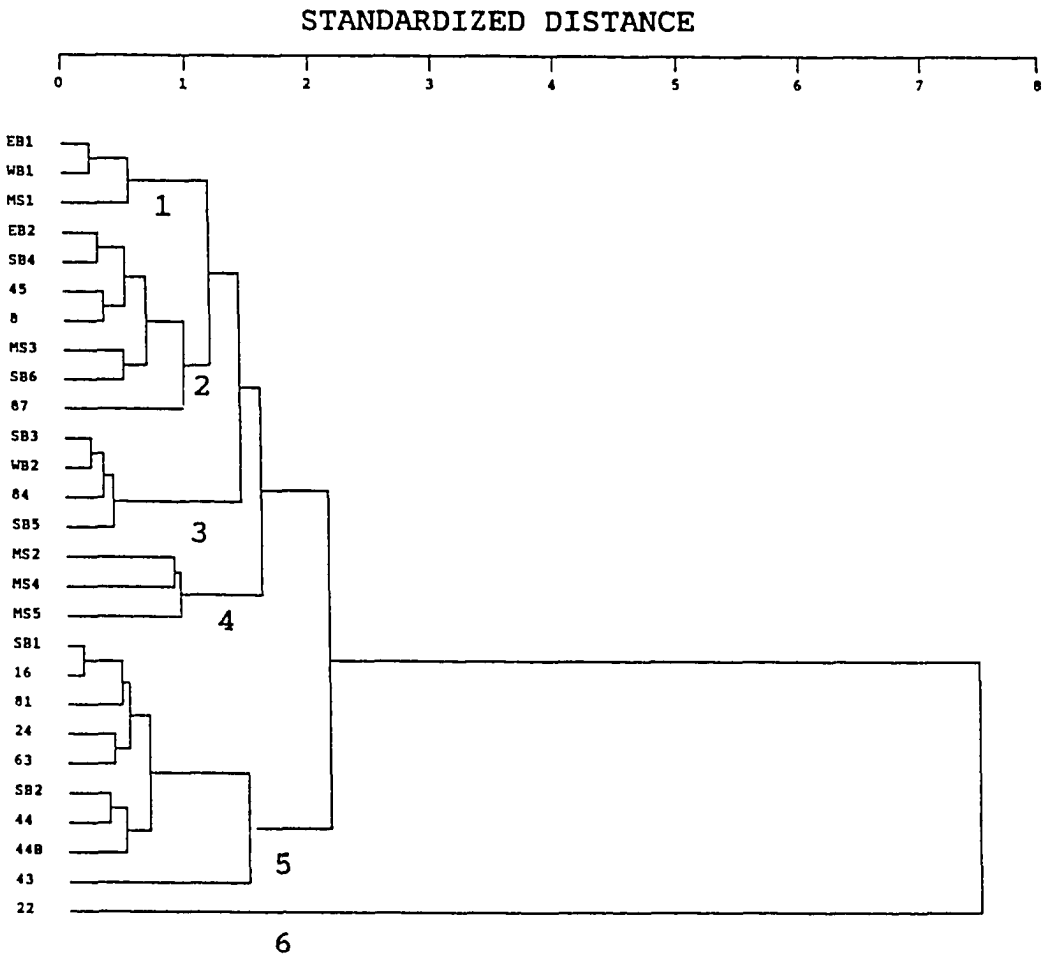



**Figure 7.10.** Standardized distance dendogram for classification of site groups with respect to biological data from Phase II studies. The numbers indicate site groups used in subsequent statistical analysis. LUA site names for numerical codes are shown in Table 7.2.

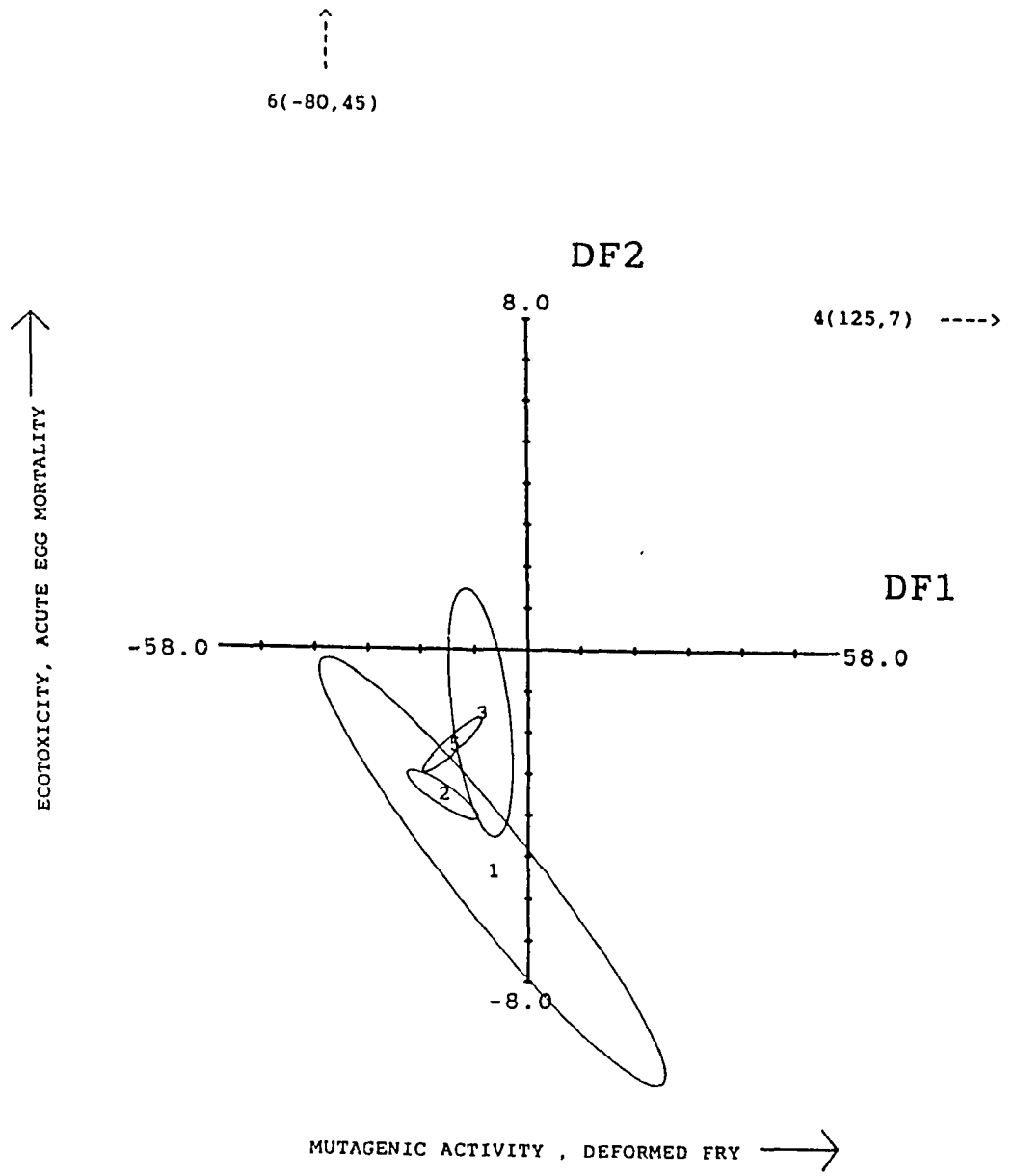



**Figure 7.11.** Confidence ellipses ($\alpha$=0.05) for canonical scores for discriminant functions (DF1 & DF2) describing differences in Phase II biological effects data between site groups defined by cluster analysis (see Figure 7.10. Site Groups 4 an 6 were so greatly separated from the other four groups that only coordinates and direction of centroid locations are indicated.

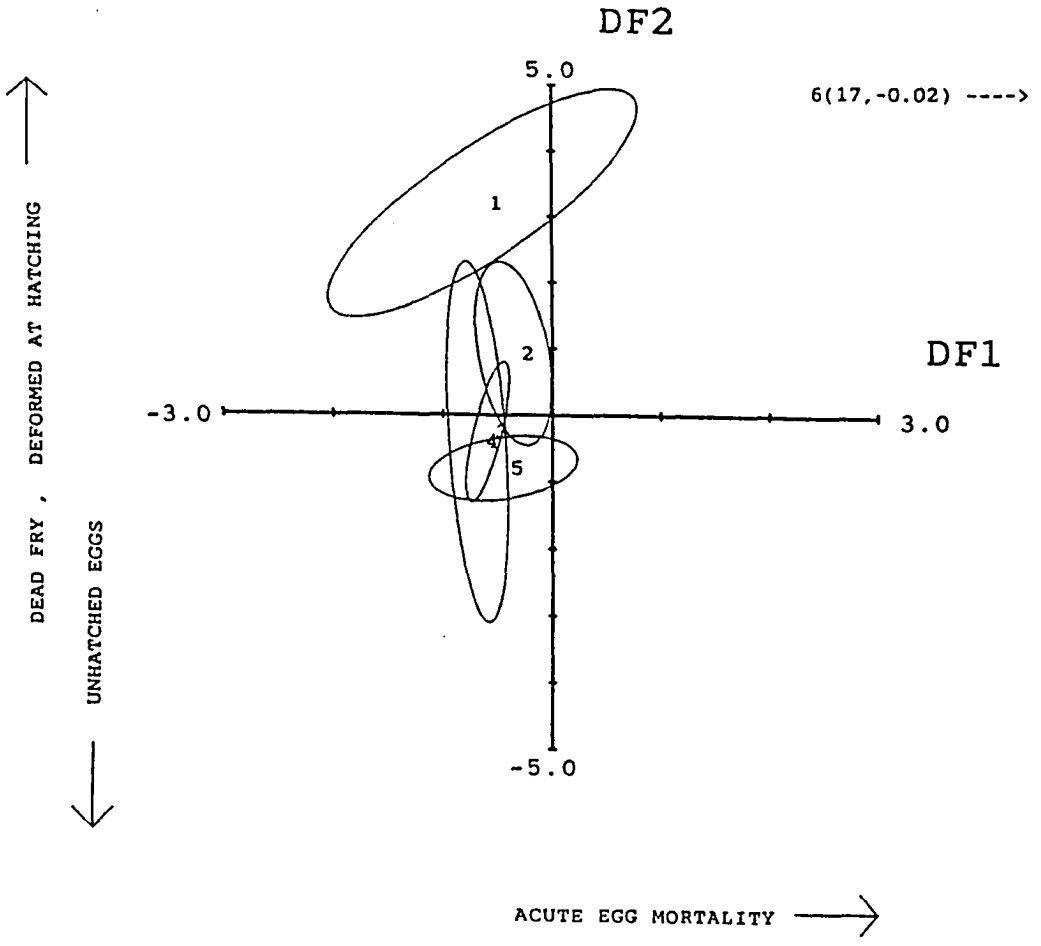



**Figure 7.12.** Confidence ellipses ($\alpha$=0.05) for canonical scores for discriminant functions (DF1 & DF2) describing differences in Phase II biological effects data other than mutagenicity between site groups defined by cluster analysis (see Figure 7.10). Site Group 6 was so greatly separated from the other five groups that only coordinates and direction of centroid locations are indicated.

Groups ($p < 0.0001$), while Group 5 had a significantly higher percentage of underdeveloped/unhatched eggs ($p < 0.0001$). Site Group 2 and, to a lesser extent, 3 displayed fewer of these chronic effects in the medaka assays. Seven of the eleven sites in these Groups were ambient sites.

The total extractable organics concentration data by site are presented in Figure 7.13. Complimentary discriminant analysis and MANOVA models were run to determine if there was any correspondence between the organic chemical data and biological effects data. The discriminant analysis and ANOVAs of biological effects data using site groups determined by organics cluster analysis failed to indicate any significant overall relationships. Likewise, the discriminant analysis of organics data with biological site groups failed to demonstrate any overall relationships, however, some significant relationships were observed between individual biological site groups and some chemical clusters.

Figure 7.14 shows the standardized, quantitative rank data for all parameters and all sites. This histogram indicates that multiple variables showed an adverse response for all sites (i.e., no site was dominated by one outstanding endpoint). In order to provide more insight into the overall patterns of biological and chemical effects, a cluster analysis/discriminant analysis/MANOVA sequence was run on the unit deviate standardized summary data sets used in the ranking evaluation. Five Site Groups were identified (Figure 7.15). The discriminant analysis/MANOVA indicated that Site Group 5 sites tended to display higher levels of cytotoxicity, mutagenicity and acute medaka mortalities (Figure 7.16). Site Group 4 showed higher levels of organics; Site Group 3 was intermediate for both of these functions; and Site Groups 1 and 2 tended to rank lower for most biological effects and chemical concentrations.

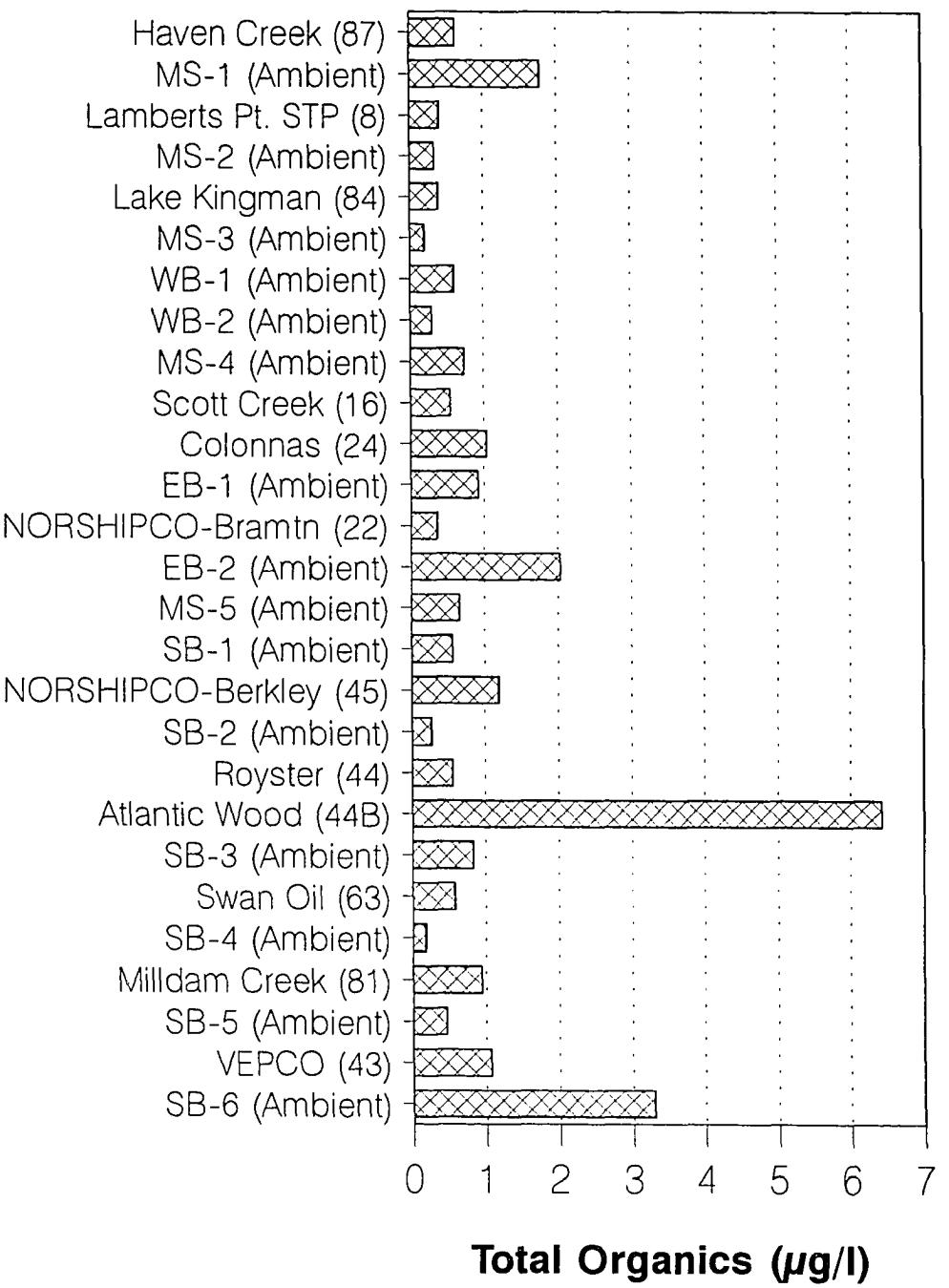



**Figure 7.13.** Total extractable organics concentrations in Phase II grab sample extracts. Concentrations are total solvent extractable organics as a 1:1 response to the internal standard, 2-fluorobiphenyl.

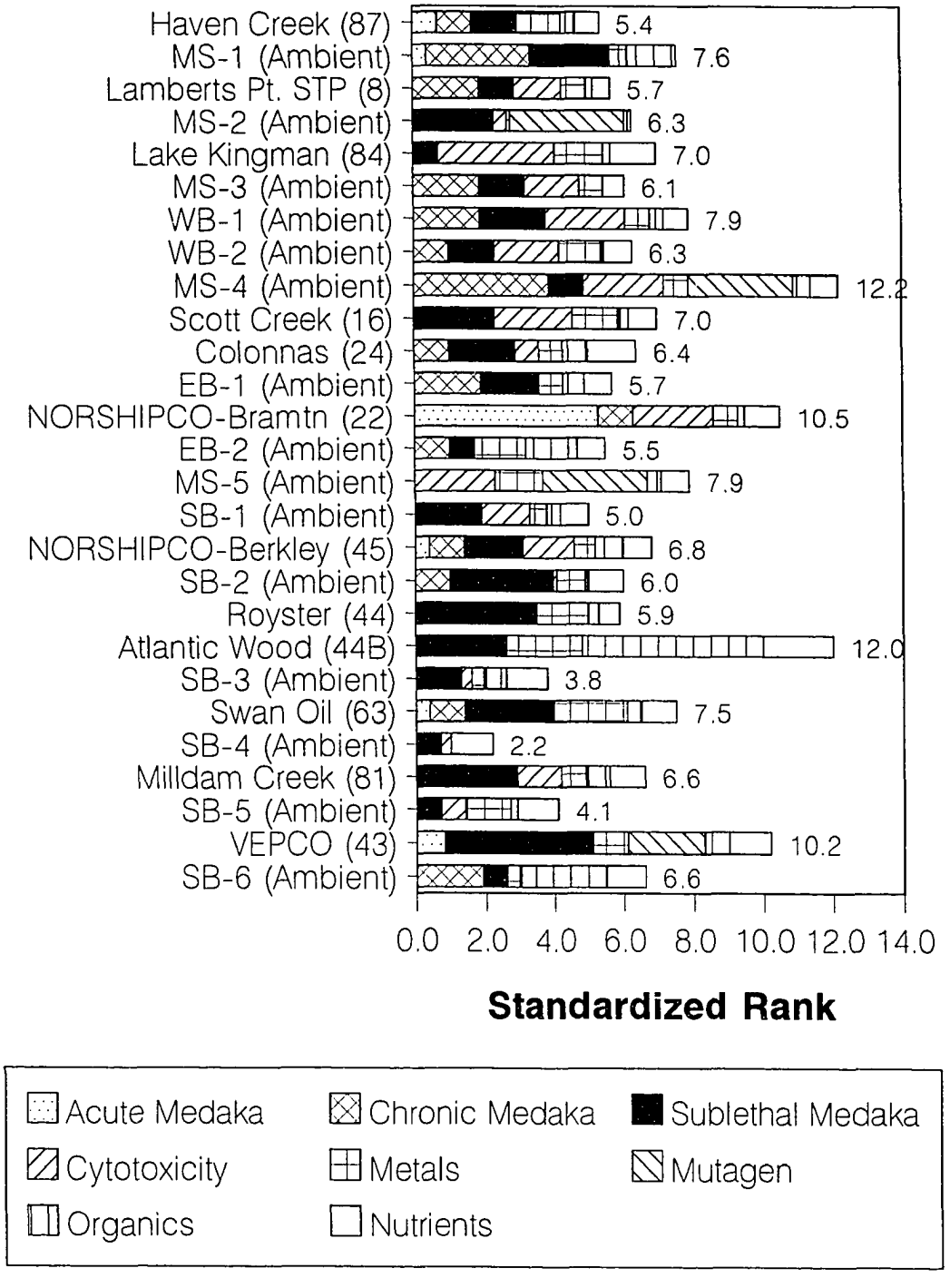



**Figure 7.14.** Quantitative ranks of all biological effects and water chemistry data for Phase II.

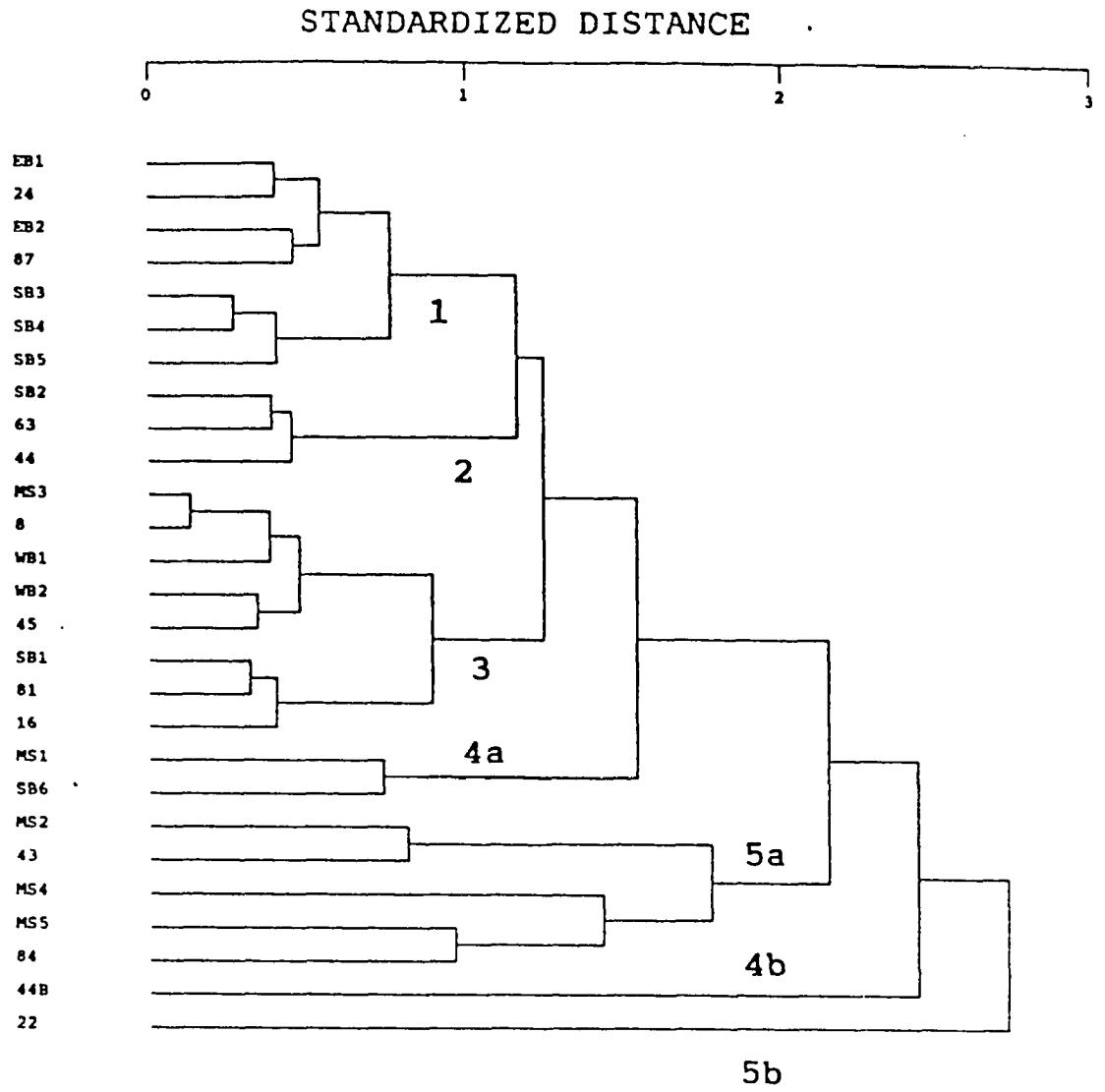



**Figure 7.15.** Standardized distance dendogram for classification of site groups with respect to standardized biological effects and water chemistry data from Phase II studies. LUA site names for numeric codes are shown in Table 7.2

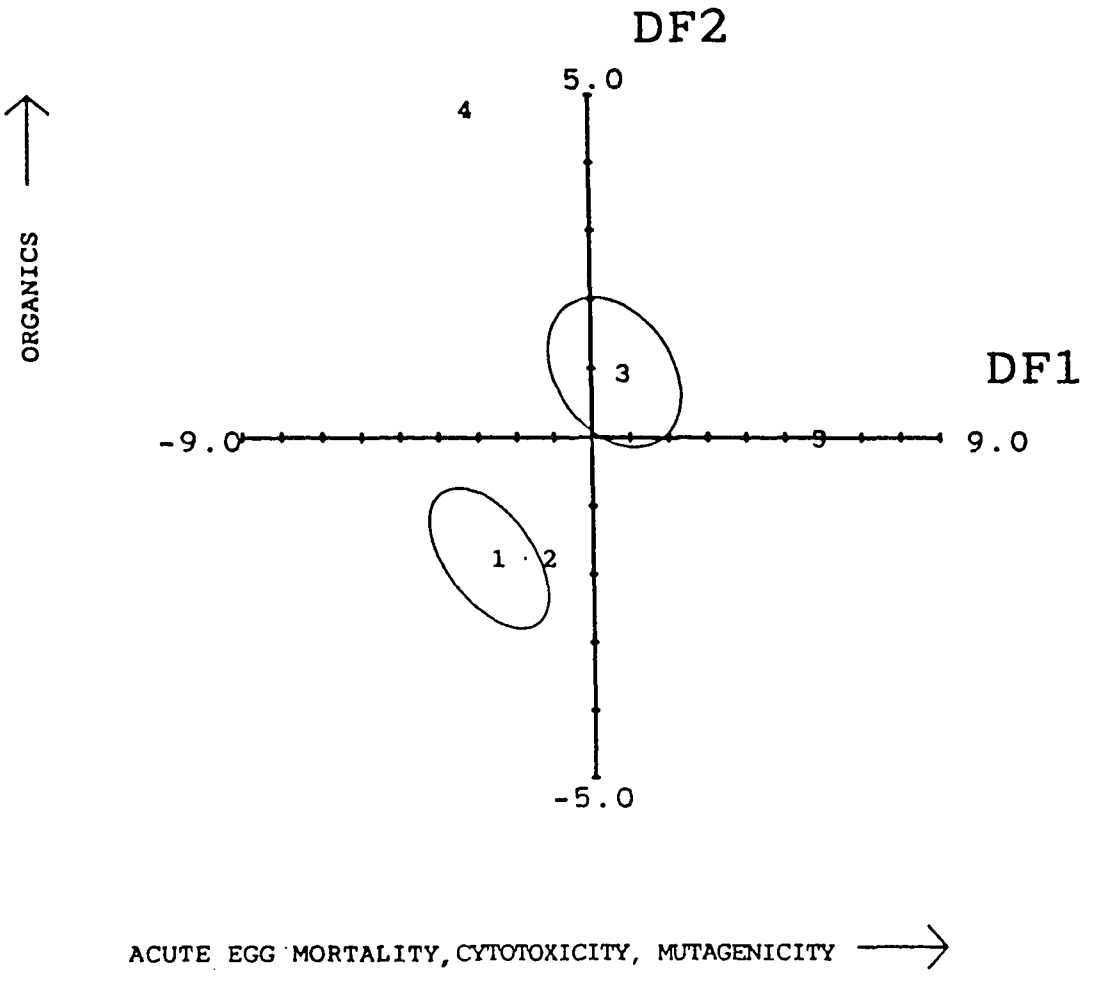



**Figure 7.16.** Confidence ellipses ($\alpha$=0.05) for canonical scores for discriminant functions (DF1 & DF2) describing differences in Phase II biological effects and water quality data between site groups defined by cluster analysis (see Figure 7.15). The number 4 represents the centroid location for this site group, since low group membership precluded ellipse calculation.

Phase II Supplementary Study

The adverse biological effects observed in the Phase I medaka studies tended to be greater and more widespread than those observed in Phase II. This pattern existed despite the fact that the sites selected for, and evaluated in, the Phase II studies were among those eliciting the greatest effects in Phase I. One possible explanation for these differences resides in the difference in sample collection technique between the two phases. Phase I employed a long-term sampling design with adsorbing resins and Phase II involved the collection of single, 15 liter grab samples from each site. Therefore, it may be possible that substantially more organics were concentrated by the SCDs than were present in the grab samples. If the SCDs were exposed to volumes of water in excess of 15 liters at a similar level of contaminants, one would expect a higher level of organics to be collected as the resins were employed to adsorb chemicals in a manner similar to high organic content sediment partitioning or lipid partitioning in biota. To explore this possibility, a supplementary study was performed in Phase II whereby SCDs were deployed at the 12 LUA sites approximately 1 month following the Phase II grab collections.

Figure 7.17 presents the results of the medaka assay indicating that sublethal effects, primarily unhatched eggs, were the dominant type of response observed in these assays. The high effects sites in this supplementary study (Swan Oil, Atlantic Wood, Lambert's Point STP and NORSHIPCO-Brambleton) were among the highest in both the Phase I SCD extracts and the Phase II grab samples. Deformed hatch fry in both the grab sample extracts and the supplementary SCD extracts were very similar to those seen in Phase I with distended abdomens and skeletal deformities dominating the abnormalities. Ames assay was also performed on the Phase II

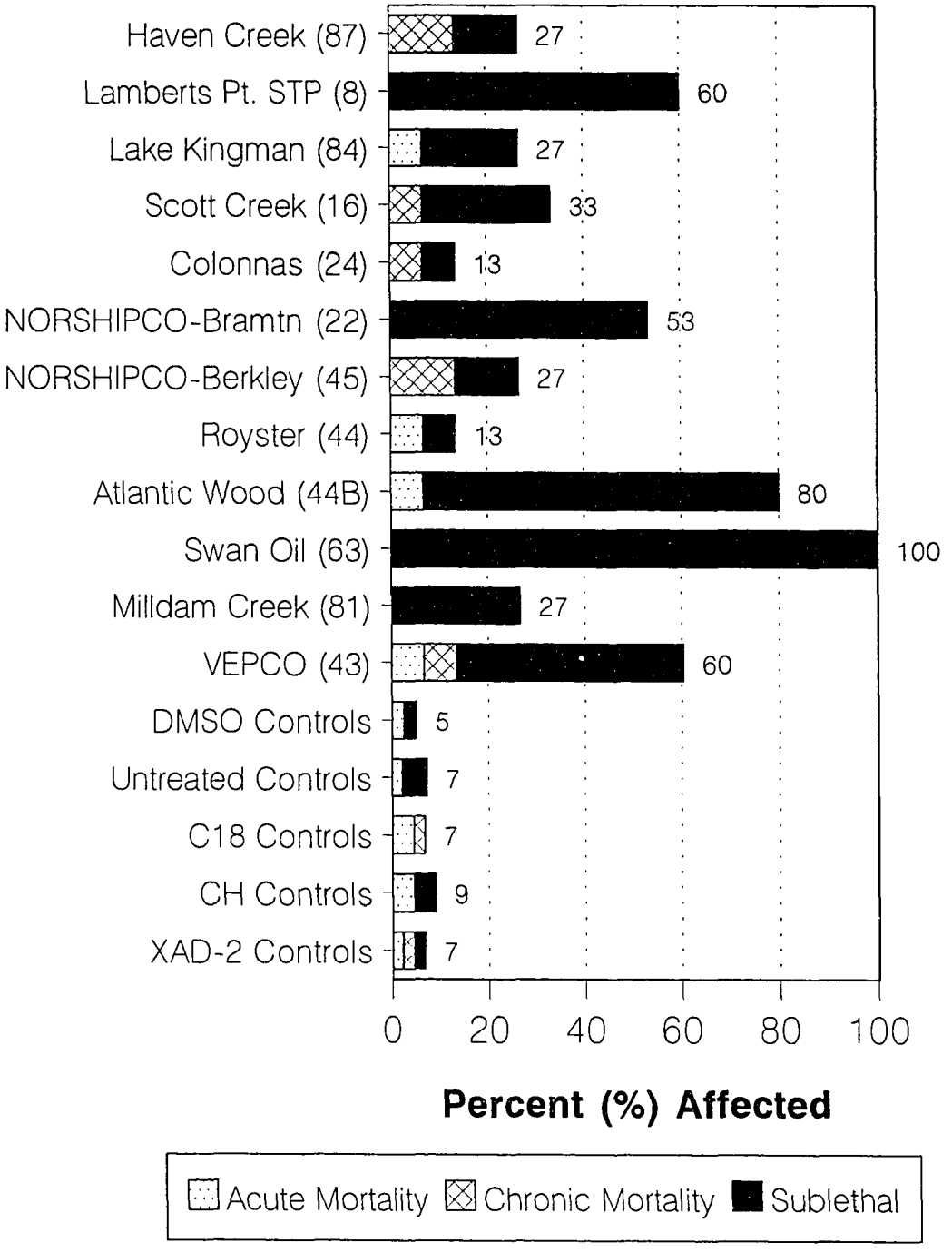



**Figure 7.17.** Results of Phase II supplemental study extract exposures employing MELA. Acute mortality is that occurring within 48hrs, chronic mortality is that occurring after 48hrs and sublethal effects include all deformities, unhatched eggs and dead fry.

supplementary extracts and no mutagenic activity was observed in any site.

A comparison of the level of organic contaminants observed in the three sampling events indicated that the Phase II grab samples contained substantially lower levels of organics than both of the SCD collection periods (Figure 7.18; the Phase II grab samples have been adjusted to reflect the concentration in the whole 15 liter sample). Moreover, the Phase II SCD extracts displayed higher concentrations than Phase I SCD extracts in approximately as many samples as they displayed lower concentrations. However, no mutagenic activity was observed in any of the Phase II SCD extracts and the level of effects was also reduced in the MELA exposures as compared to Phase I.

Discussion

This study was designed to assess the relative potential of a very extensive series of land use activities (LUAs) for producing adverse biological impacts in laboratory tests with sensitive indicators of toxicity, teratogenicity and mutagenicity. As a component of this comprehensive study of the Elizabeth River Basin, the medaka embryo-larval assay (MELA) proved to be a useful tool in identifying "hot spots" in the system relative to impact on fish early life stages. However, the patterns of effects were found to be both diverse and widespread. This is not surprising when one considers the large number of potential inputs into the system and the associated diverse number of chemicals introduced in close proximity to each other in this complex ecosystem.

This study also indicated that the different assay systems appear to respond with differing sensitivities to distinct types of pollutants. Previous investigations have

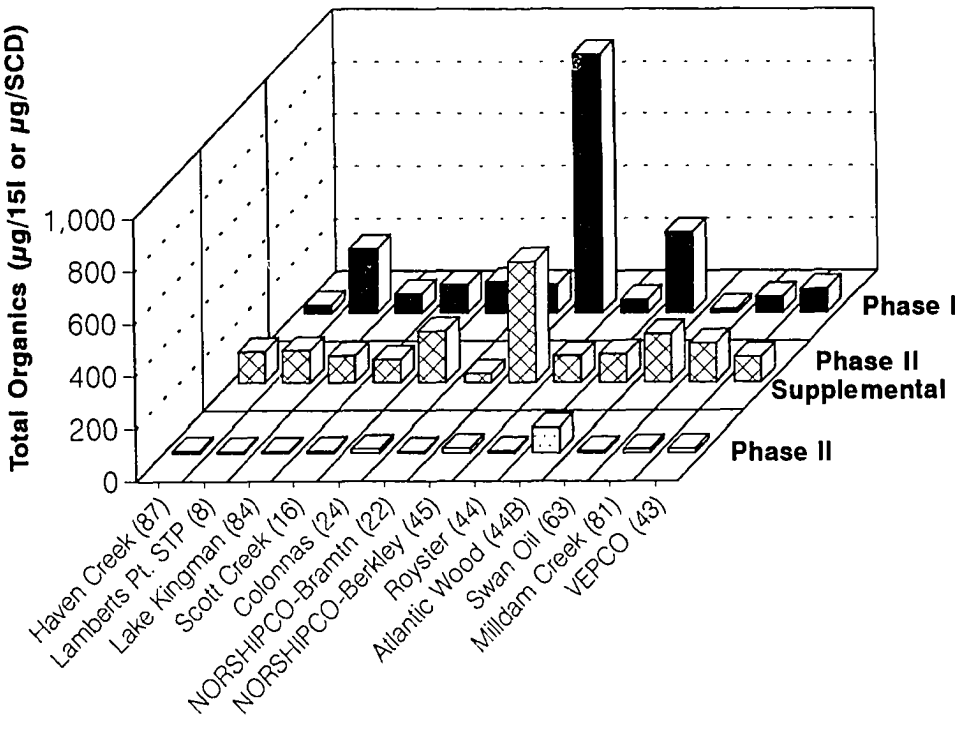



**Figure 7.18.** Temporal patterns in total organics data collected during Phase I, Phase II and the Phase II supplementary study. Phase I and II supplementary data are expressed in mass units ($\mu$g) per SCD and Phase II data are expressed as the concentration in the 15 liter grab sample.

also indicated that differing assay systems for carcinogenicity/mutagenicity display different sensitivities with respect to defining a substance as a "positive" carcinogen/mutagen (see, for example, Tennant *et al.*, 1987). Therefore, it is apparent that these assays complement each other in that each detects the adverse potential of different chemical types to differing degrees.

With the exception of a few subtle trends, the discriminant analysis and univariate comparisons failed to indicate a significant relationship between LUA type and adverse biological impact. Rather, LUA sites differed individually with respect to associated effects, suggesting that when evaluating a system for target "hot spots" one must focus on individual sites rather than LUA categories.

With respect to the temporal variability observed between the Phase I and II studies, there are several possible explanations. The most obvious possibility is the difference in collection method between the two Phases. The SCDs employed in Phase I apparently collected more biologically active organic contaminants than were present in the Phase II 15 liter grab samples. Although there was not necessarily a direct correlation between total organic compounds and biological effects, the fact that both displayed the same pattern suggests that the SCDs were more effective at sampling bioactive organics than were the extractions of grab water samples. This is not surprising, as the sorbent materials act as "surrogate" organisms by attracting the hydrophobic organics from the aqueous surroundings and thereby better represent actual biotic exposure conditions than a single "plug" of River water represented by the grab samples. This trend was confirmed, at least in part, when the SCDs were employed in the Phase II supplementary study. Total organic compound levels were more comparable to those concentrations determined in the Phase I collections. However, though the Phase II SCD effects on the medaka were somewhat higher than those seen in the grab sample extracts, the degree of adversity (particularly with respect to acute effects) was generally much less than that observed in the Phase I samples.

A second possible explanation for the differences in impact between the two study Phases was the temporal variability of the data observed over the course of the study. Figure 7.19 displays the general meteorological patterns during the sampling event periods. The Phase I SCDs were deployed during a period of fairly heavy rainfall (2.29in) which followed a relatively dry period (1.54in in the preceding 3 weeks). This scenario suggests that runoff from the rains could have contained high

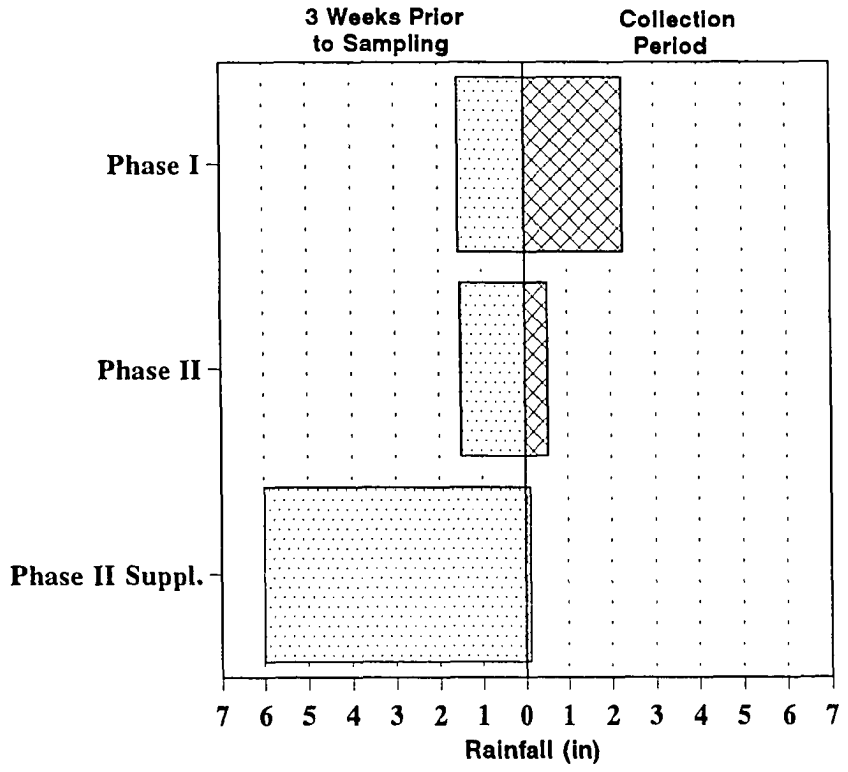



**Figure 7.19.** Rainfall data for the time period prior to and during the three sample collections.

levels of contaminants which were concentrated during the three week dry period prior to sampling. Conversely, the Phase II grab samples were taken during a relatively dry month, with little rainfall preceding the day of collection (0.54in the week of collection). Finally, the supplementary SCDs were deployed approximately two weeks following a very rainy period (6.00in in the 3 weeks prior to collection), with only a small amount of rain (0.12in) falling during the 72hr collection period. This pattern would suggest that the pollutants may have been flushed from the Basin during the rainy period prior to a virtually dry collection interval. Of course, other events may have contributed to the temporal differences, such as shipping and industrial activity, however, regardless of the sources, this type of temporal variability must be considered and evaluated in more intensive research studies.

In addition to temporal variation, there was also a high level of spatial

variation observed over the course of study. Sites which were seen in close proximity to each other often displayed very different effects on the medaka and other biological assays and with respect to chemical content. Further, the Phase II studies included "ambient" samples collected from mid-channel sites along the study region. Except for those ambient sites found at the confluence of the Eastern and Southern Branches and the River's Main Stem (MS-4 & MS-5), the ambient samples appeared to induce fewer adverse effects than the nearby LUA samples, suggesting that there may be a dilution factor occurring and/or that the substances released from the LUAs are rapidly partitioning into the organic carbon-rich sediments.

No overall patterns were observed between biological and organic chemical data based on discriminant analysis between site groups. A number of factors may be responsible for confounding the relationship between organics and biological effects. The vast numbers of chemicals in any environmental sample make cause and effect relationships difficult to establish. Additive and/or synergistic relationships can further complicate this interpretation. Further, a complex matrix may be responsible for binding the component of interest or for acting as a carrier into biological systems. Another complicating factor is that analysis of sample components are limited by the ability to detect these substances with modern instrumentation (i.e., active binding may take place upon injection or with the chromatographic column phase). Ideally, one would want to identify the biologically active samples and then proceed through an analytical phase which includes fractionation followed by specific detection systems such as MS, NMR and LC with various visualization systems.

These results suggest that MELA is a practical and valuable tool in the assessment of the relative biological impact of numerous sites in a large aquatic

system. The ease in applying the assay to field collected samples allowed a very large number of stations to be evaluated simultaneously. This type of assay is ideal for screening numerous sites with a sensitive system, and at a low cost, from which several areas could be designated for more intensive, and costly biological and chemical evaluations. Further, MELA should prove very useful in evaluating temporal trends of individual sites to determine the relative impacts of these sites to the receiving system over the duration of seasonal and industrial activity fluctuations.

It should be noted that though the names of the nearby industries and facilities were used for many of these sites, this does not necessarily indicate that they are responsible, in whole or in part, for the observed contaminant levels and biological effects. Further, the degree of temporal variability observed between the study Phases suggests that it would be unwise to eliminate sites with reduced biological impacts from further evaluation based on this single study. Nevertheless, the results from the medaka assay indicate that several of the sites throughout the River system can be confidently designated as "hot spots" with respect to effects on teleostean early life stages.

## Study II: Sediment Samples

### Brief Overview of Methods and Results

The second bioassessment of natural samples employing MELA was conducted with sediments collected from eight sites in the Elizabeth, Nansemond and James Rivers as well as the mouth of the Chesapeake Bay (Figure 7.20). Again, this was a component of a much larger study which included numerous biological indicators of

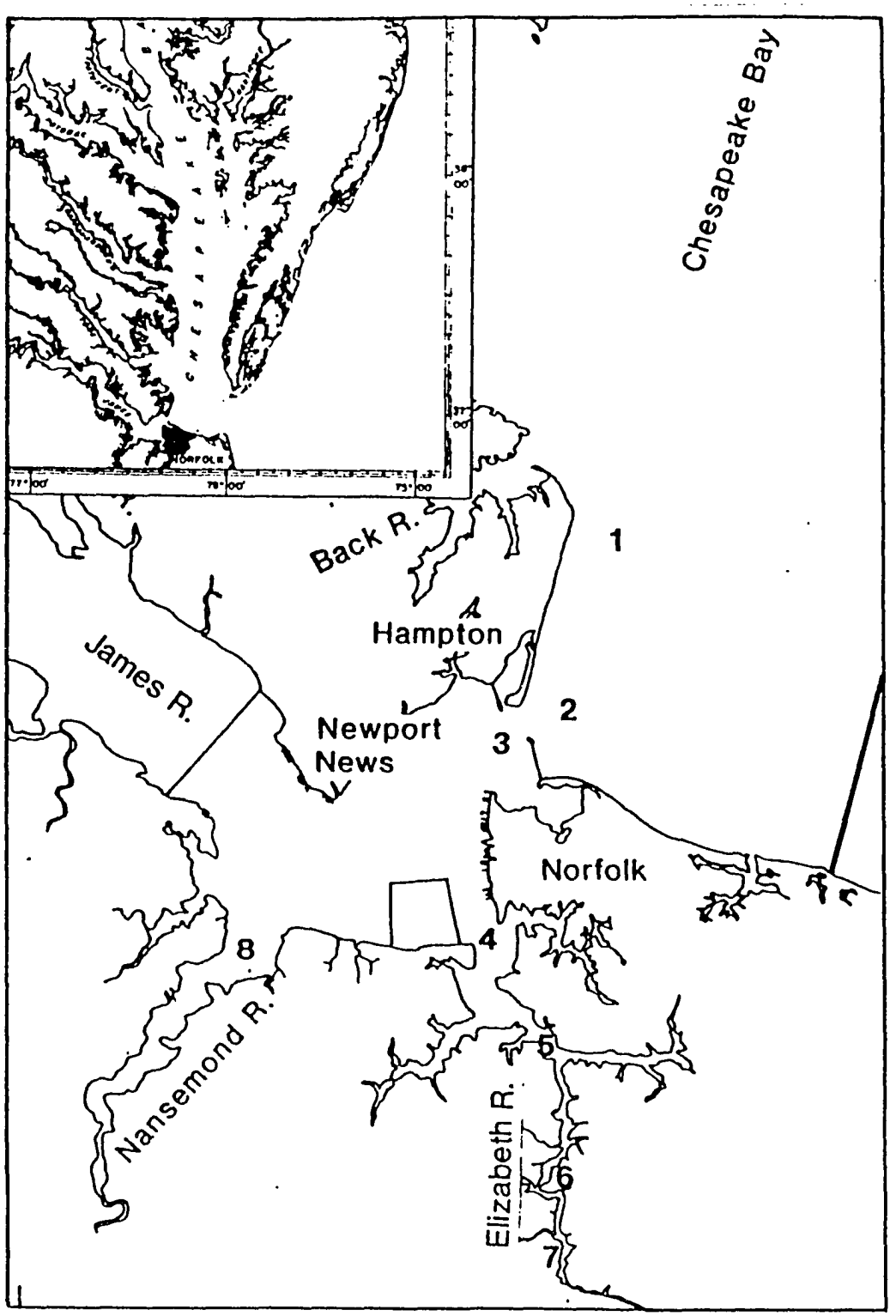



**Figure 7.20.** Map of lower Chesapeake Bay region, indicating the sediment evaluation study sites.

coastal pollution (i.e., "biomarkers") as well as chemical analyses. This research was a component of a project conducted by joint research teams from Old Dominion University, Virginia Institute of Marine Science, Oak Ridge National Laboratory, Chesapeake Biological Laboratory, Duke University, National Oceanic and Atmospheric Administration, U.S. Environmental Protection Agency (EPA), and University of Maryland.

Sediment samples were collected in triplicate at each station and were extracted employing U.S. EPA protocols (U.S. EPA, 1986) for semi-volatile (base/neutral and acid extractable) organic compounds in solid matrices. Briefly, approximately 30g wet weight of each sample was sonication extracted three times with a methylene chloride:acetone (1:1) solvent system. The extracts were filtered, pooled and dried over anhydrous sodium sulfate. The extracts were concentrated to approximately 100$\mu$l, 25$\mu$l of DMSO was added and each extract was further concentrated to 25$\mu$l with nitrogen to remove all methylene chloride and acetone. Three replicates of five medaka eggs were treated with 0.1$\mu$l/egg of each field replicate extract employing the methods described in Chapter 2 and were reared at 25 $\pm$ 1°C for 21 days post-treatment. Therefore, each dose represented the extract of approximately 0.12g of wet sediment.

The results of the sediment extract exposures are presented in Figure 7.21. The pattern of response was similar to what one would expect with the Elizabeth River stations (5, 6 & 7) eliciting the greatest impact, while the comparably pristine Nansemond site extracts resulted in a significantly reduced level of impact. Further, as one moves further out toward the mouth of the Bay, the level of adverse impact decreases relative to the Elizabeth River sites, however, the levels of effects are still

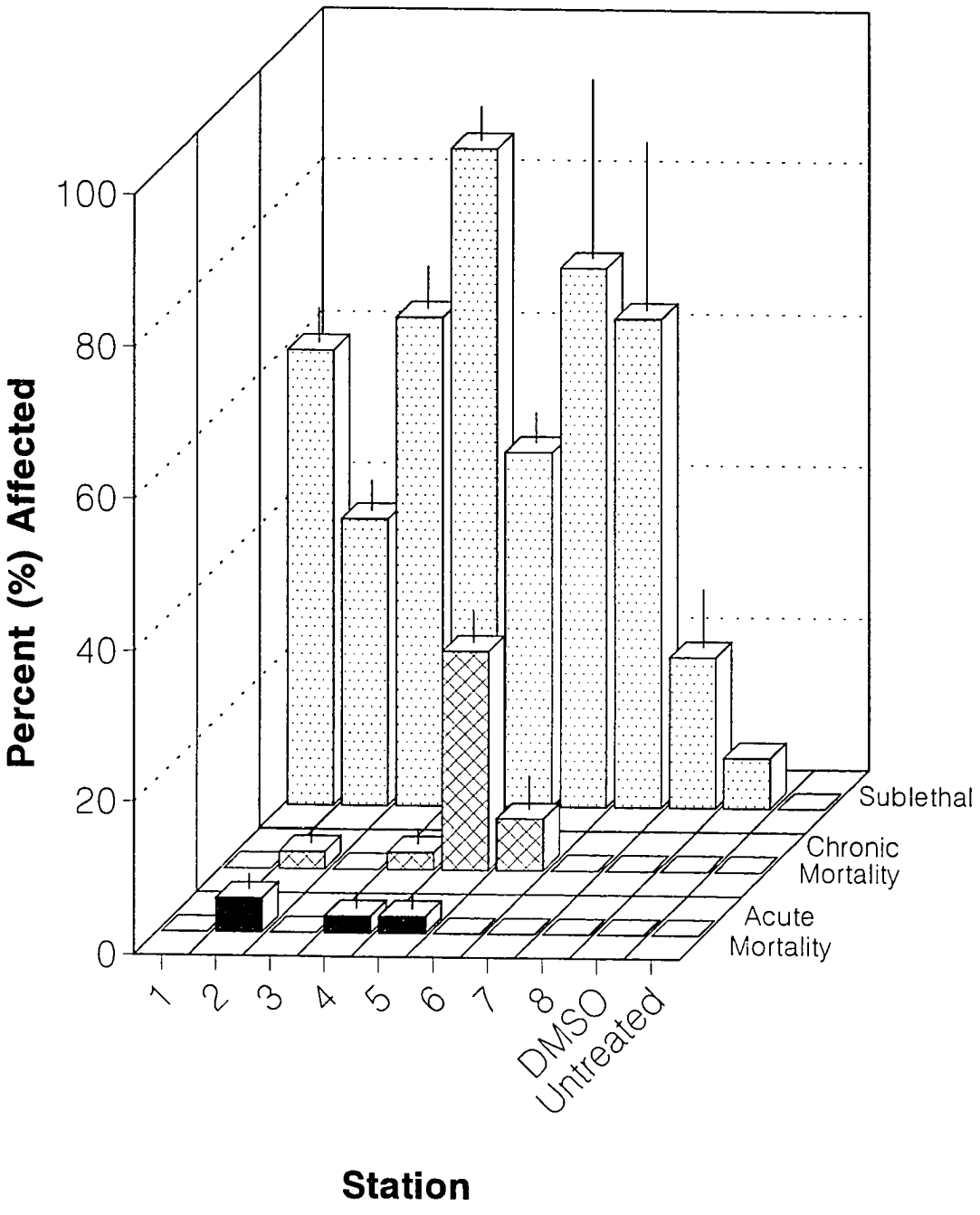



**Figure 7.21.** Results of sediment study extract exposures employing MELA. Acute mortality is that occurring within 48hrs, chronic mortality is that occurring after 48hrs and sublethal effects include all deformities, unhatched eggs and dead fry. Histogram bars represent means of field replicates. Error bars represent standard errors of each mean.

high relative to the Nansemond site (8) and the untreated and DMSO controls. This moderate level of toxicity in sites 1 through 3 may be the result of the flushing of pollutants introduced into the estuary by the Elizabeth River and or to those introduced via the James (e.g., residual kepone from historical introduction).

These results indicate that MELA is also very useful in the screening of sediment samples from systems suspect to be contaminated with biologically active pollutants. Extract from as little as 0.12g of sediment resulted in a significant decrease in viability in the majority of the sites and the spatial patterns of adverse impact appeared to correlate well with other studies of the biological hostility of the sediments and the level of hazardous contaminants contained within this matrix. The results also appear to correlate reasonably well with the water sample exposures discussed previously, in that the Southern Branch and Main Stem sites tended to elicit the greatest effects relative to Chesapeake Bay sites and numerous laboratory controls.

## CHAPTER SUMMARY

The *O. latipes* eggs were exposed to chemical extracts of water and sediment samples as a component of two studies of several Chesapeake Bay tributaries. The first was an extensive evaluation of the waters adjacent to a number of point and non-point sources of pollution to the Elizabeth River. This study incorporated the chemical characterization and examination of the chemical bioactivity of the three branches of the Elizabeth River (Eastern, Western and Southern) as well as the River's Main Stem, extending out into the mouth of the Chesapeake Bay. The second, was a more focused study of the sediments at numerous sites in the River, and lower Chesapeake Bay, in which a number of additional biological and chemical assays were employed for the evaluation of the system.

The results of both studies indicate that the MELA approach is very useful as a "surrogate" organism test to establish the relative toxicity/teratogenicity of several sites, simultaneously. In addition, the results of the water sampling indicate that the data for the medaka assay often correlate well with other biological assays, however, a poor spatial correlation was seen between the MELA results and results of certain of the bulk chemical analyses. Site specific relationships with chemistry were observed. The data were used to rank numerous sites in this region to determine potential "hot spots" for further, more intensive biological and chemical evaluations.

The developmental anomalies seen during these studies were very similar to those reported previously for the individual toxicant studies (see Chapters 2, 5 and 6) including skeletal and cardiac anomalies as well as swollen abdomens and developmental arrest.

# CHAPTER EIGHT

## Summary Comments

## SUMMARY COMMENTS

The eggs of the Japanese medaka (*Oryzias latipes*) were used to develop a standardized assay, the medaka embryo-larval assay (MELA), designed to assess acute and sublethal effects of chemicals in variable salinity systems. Numerous previous investigations with medaka eggs have indicated that short-term embryo-larval assays can be used to rapidly evaluate the toxicokinetics of single chemicals or of complex mixtures of substances, such as those seen in field-collected samples from highly industrialized areas (Solomon, 1979; Hatanaka *et al.*, 1982; Klaunig *et al.*, 1984; Takimoto *et al.*, 1984a&b; Maccubbin *et al.*, 1987; Hinton *et al.*, 1988; Shigeoka *et al.*, 1988a&b; Hawkins *et al.*, 1990; Marty *et al.*, 1990a&b, 1991; Wisk and Cooper, 1990; Cooper *et al.*, 1991; and others).

The medaka early developmental stages were subject to exposure to a number of chemicals and water and sediment sample extracts, and evaluated for a broad spectrum of toxicant-induced responses, including effects on biochemical and physiological mechanisms associated with cellular differentiation, proliferation and growth, basic metabolism and systematic functions, as well as the hatching process and initial period of liberated existence. Exposure was through topical treatment of 48hr old eggs with a membrane-permeable solvent, dimethylsulfoxide (DMSO) containing the chemical or extract of interest. The eggs were exposed to nine chemicals in a pilot study to identify two representatives for further, intensive evaluation in studies of salinity-related response, immersion equivalence of the topical exposures as well as identification of significant sublethal responses.

Following the pilot study evaluations, and prior to conducting the extensive

toxicological and teratogenic evaluations of pentachlorophenol (PCP) and tributyltin chloride (TBTCl), a series of experiments was conducted to optimize the rearing condition of the developing eggs. This research was driven by the unexpected delays in the embryonic hatching rate relative to previous studies with the medaka under similar rearing conditions. Following elimination of variability in rearing solution chemistry, temperature and egg population size, three variables remained for evaluation. These were the presence or absence of aeration and solution changes as well as the use of two different rearing vessel types. The results indicated that, in general, the use of a reduced diameter vessel (i.e., a scintillation vial versus a Petri dish) with aeration was the optimum condition for rearing these eggs and that solution changes were not necessary to obtain optimum hatching rates (i.e., being the shortest possible rates while producing normal, healthy fry). Though these results indicated that a static test was preferable, immersion studies of PCP and TBTCl indicated that renewal was required due to potential toxicant loss and possible oxygen stress in higher salinity exposures. Although employed in this study to provide data comparable to the immersion exposures, it is apparent that renewals are not necessary for successful embryo development when the MELA topical exposure is employed (as long as sufficient aeration is present). It may be that the solutions do not need to be changed daily in immersion studies. However, future research in this area is needed to determine if the predicted uptake of test substances, based on topical dose concentrations, is an accurate estimate of the solution depletion.

In addition to the rearing condition research, a study was carried out in the area of the trans-chorionic permeability or "permeability factor" (PF) of several test substances. Numerous replicates of eggs were treated in a manner analogous to that

used in the toxicological assays and were subsequently extracted and the extracts analyzed employing gas chromatography with multiple specific detectors. Four of the chemicals evaluated in the pilot toxicity study were evaluated in addition to three surrogate substances of similar chemical structure and extractability. The findings indicated that the PF for these chemicals varied significantly with values ranging from 10.5 to 70.0%. Further comparison of these data with a physico-chemical coefficient frequently used in studies of bioconcentratable ability, the octanol-water partition coefficient ($K_{ow}$), indicated that these chemicals are apparently passively diffusing into the eggs at a rate well correlated to their $K_{ow}$. These observations suggest that the amount of toxicant penetrating each egg is actually based on the lipid affinity of the substance rather than active transport induced by the carrier solvent. Future study should be performed in this area with a more thorough range of toxicants to verify the regression developed in this study (i.e., $PF = 10.8\ logK_{ow} + 2.06$). However, these preliminary results indicate that it may be possible to approximate the amount of toxicant to be applied to a given test egg to represent an ambient environmental concentration employing a theoretical approach such as that indicated in equation 3.1 of Chapter 3.

The toxicological evaluations produced a wide range of responses to the nine substances evaluated in the pilot, freshwater range-finding study. As suspected, the carcinogens (e.g., benzo[a]pyrene, diethylnitrosamine) produced little acute response as seen in previous studies with medaka eggs and similar doses (Klaunig *et al.*, 1984; Maccubbin and Black, 1986; Maccubbin *et al.*, 1987; Marty *et al.*, 1990b; and others). These and other studies indicate that the response to these chemicals is typically detected at a later stage of development (typically 2 to 6 months) expressed

as neoplasia in numerous organ systems. The most common sublethal response detected in the present study was delayed development and/or reduced embryo activity reflected in the inability of the embryo to hatch from the restrictive chorion. Embryonic movement is presumed to stir and circulate the perivitelline fluid, improving the distribution of oxygen to the embryo (Rosenthal and Alderdice, 1976). The reduced embryo size and/or activity may have been insufficient to circulate the oxygen and/or hatching enzymes, resulting in the high number of unhatched eggs.

Of those toxicants tested, PCP and TBTCl, produced the most distinct responses in the developing embryos. PCP, a moderately lipophilic, relatively non-persistent environmental contaminant, displayed primarily an acute impact, while TBTCl induced widespread sublethal afflictions in addition to acute impact at higher concentrations. The results provided by these two toxicants promoted their use in the more extensive studies of salinity-related effects and immersion equivalent concentration evaluations.

In addition to the freshwater pilot study, PCP was evaluated at a salinity of 20ppt, representing a typical salinity of industrialized estuarine seaports, where this chemical is often found (Cirelli, 1978; Crosby, 1981). Eggs were treated both topically and through immersion, with medium concentrations developed from the topical doses and a bioconcentration factor (BCF) of 770 (Veith *et al.*, 1979). The results for the 20ppt topical treatment were compared to those generated in the pilot study and indicated that salinities up to 20ppt apparently had little overall effect on PCP toxicity, with 21 day $ED_{50}$ values of 0.056 and 0.062$\mu$g PCP/egg for freshwater and 20ppt water, respectively. This is interesting to note, in that previous investigations have suggested that the bioconcentration of PCP varies significantly

with salinity, with higher levels being found in the tissues of freshwater exposed organisms (Trujillo *et al.*, 1982; Tachikawa *et al.*, 1991). The similar levels of adverse effects seen in this study may reflect the fact that pH was controlled throughout the treatments, whereas the previous studies were carried out in media of wide pH range, which may have produced an interaction with varied salinity (an interaction is suggested by the fact that Tachikawa *et al.* [1991] found that pH had a smaller effect on bioconcentration than salinity). In addition, conversion of the immersion concentrations to their topical equivalents, followed by regression analysis and slope comparisons, indicated that the BCF of 770 is very similar to the apparent BCF for the medaka eggs.

One final conclusion drawn from the PCP study is that the response of the medaka is a classic response, presented as acute lethality at high concentrations which drops off with reduced concentration; and sublethal effects which are highest at the intermediate concentrations and decrease with decreasing concentration. This, in addition to the similarity in response between the pilot study exposure (Figure 2.14) and the two reference toxicant exposures in the field validation studies (Chapter 7), indicates that PCP may be a prime candidate as a reference toxicant for MELA. The reference toxicant would be employed on a routine basis to serve as an indicator of the general health of the test population of eggs to eliminate problems associated with "weak" eggs produced from breeding adults which are unhealthy or simply suffering from "reproductive burnout".

The second toxicant of focus was TBTCl, an organotin most commonly employed as a stabilizer in PVC and as an additive in antifoulant paints. MELA was used in a similar manner as that discussed for PCP. However, due to the potential

for elevated open ocean concentrations of TBTCl, due to increasing shipping activity, 35ppt salinity medium was added in addition to the 20ppt medium representing estuarine conditions. Further, as with the PCP study, immersion concentrations were developed (based on a TBT BCF of 4000) to provide for side-by-side comparisons of the topical treatment and immersion exposure.

The results from the TBTCl exposures are sobering in that concentrations as low as 0.0000187$\mu$g TBTCl/egg produced a response significantly different from the laboratory controls and mean 21 day $ED_{50}$ values of 0.001, 0.0001 and 0.0002$\mu$g TBTCl/egg for the 35ppt, 20ppt and 0ppt exposures, respectively. Immersion exposures indicated that the NOEC and MATC for both the 20ppt and 35ppt exposures lie below 0.025$\mu$g TBTCl/l and, in 20ppt medium, concentrations as low as 1ng TBTCl/l produced a response significantly different from control groups. These values are some of the lowest values reported to date; the lowest previous concentration resulting in an adverse biological response was 5ng TBT/l, which induced imposex in the dogwhelk (*Nucella lapillus*; Gibbs *et al.*, 1988).

A comparison of the topical and immersion data indicated that there was a statistically significant difference between the two exposure modes, but that the slopes of these lines for all adverse effects were not significantly different, suggesting that the mode of action was approximately the same for both treatments, but that there was a displacement due to an underestimated BCF. The results of this study suggest that the BCF for medaka eggs may be orders of magnitude higher than the estimated value of 4000 and may possibly be one of the highest established for TBT. This is not surprising, as the eggs contain a high concentration of lipid material, with very little mechanism for elimination of accumulated materials. These results suggest that

further study is warranted to establish the magnitude of concentration of TBT in fish eggs, particularly since most eggs spend their developmental duration either in the surface microlayer or on the sediments, both of which have been shown to concentrate organotins (Salazar, 1986; Cleary and Stebbing, 1987; Harris and Cleary, 1987; Unger *et al.*, 1987; Hall *et al.*, 1988; Krone *et al.*, 1991; and others).

The practical implications of these results for PCP and TBTCl are enormous, as concentrations in excess of the experimental concentrations are commonly found, even to the present day, following significant legislation attempting to reduce environmental levels of these and other substances. For example, in addition to the water concentrations of TBTCl discussed in Chapter 6, sediment samples taken in August, 1992 from a tributary of the Chesapeake Bay contained PCP concentrations as high as 35,200$\mu$g PCP/kg and TBT in excess of 400$\mu$g TBT/kg. Environments such as this study site may very well be undergoing severe perturbations in ecological structure as a result of exposure of sensitive early life stages to these and other potentially toxic and teratogenic substances.

In addition to the laboratory-conducted, individual toxicant exposures, two field validation studies of the MELA technique were conducted in the Chesapeake Bay (and associated tributaries), one with water organic extracts, the other with sediment organic extracts. Though the methods employed in these studies were precursory to those used throughout the laboratory portion of this research, the initial indication provided by the data is that the MELA technique can be successfully applied to concentrated field extracts. The evaluations also indicated that the MELA results are often correlated to other studies of bioactivity. Further research needs to be conducted whereby the extracts and corresponding topical treatments are directly

compared to immersion and sediment exposures for representation, similar to the approach employed for the individual toxicant laboratory studies.

It must be kept in mind that toxic and teratogenic effects observed in laboratory evaluations do not necessarily relate directly to natural conditions. In the field, the bioavailability of the chemical of concern may be altered by such factors as temperature, salinity, total suspended solids and biotic activity to produce increased or reduced effects. Increased stress due to synergism with natural biotic and abiotic factors can render some organisms susceptible to stresses to which they otherwise would not succumb (Oyewo, 1989). It is well known that the marine environment is a very complex system in which nature maintains a delicate and dynamic balance. Where continuous exposure to a toxicant stress occurs in nature, populations of organisms may become either acclimated or weakened, and may therefore behave differently from laboratory populations. However, although acclimation or debilitation may occur in adult life stages, resistance to a toxicant is unlikely to be transferred to the embryonic and larvae stages (Beaumont and Budd, 1984). Nevertheless, where comparisons have been made for pollutants between laboratory and field toxicity data, a reasonable agreement between the two has been observed (see, for example, Adams *et al.*, 1983; Chapman, 1983).

With respect to the application of data produced with the MELA technique to other species and potentially to man, the medaka can only define sensitivity and response to a chemical for the medaka. The toxic responses of any two species would not be expected to reflect each other exactly over a wide range of chemicals and field samples. However, despite these variations, the induction of adverse acute and sublethal responses in any species probably involves mechanisms at the molecular

level which should be similar, if not identical (Black, 1988) and entails similar organ systems and pathways of action. Further, ecotoxicity testing is particularly relevant to man because the ecosystem is, in many cases, more sensitive than the human system and thus serves to set thresholds of exposure which will, in turn, protect humans (Solomon, 1981).

Previous investigations have indicated that salinity stress patterns appear to be species specific; some may display no visible effect of salinity changes while others may show an adverse response to either increasing or decreasing sea salt concentration (Rosenthal and Alderdice, 1976). The medaka appear to exhibit limited stress related to variable salinity. High breeder egg production, in addition to the hatching success of control eggs and low incidence of osmotic swelling and dehydration from the various exposures, indicate that the medaka has a high tolerance for salinities up to 35ppt and is an ideal "surrogate" test species for studies of the relative toxicity/teratogenicity of various estuarine and coastal systems and their source and receiving waters. In addition, it appears that the medaka is extremely sensitive to two toxicants commonly found in these environments. This finding is particularly important in that concentrations in the ocean are often too low to induce effects in standard short-term acute assays, and often require a sensitive, longer-term assay which includes the observation of sublethal effects.

Finally, future study with the MELA assay and immersion tests with the medaka should continue to refine the approach developed here through more intensive evaluations of salinity response and refinement of the permeability and immersion equivalent models, followed by validation in field studies. Field studies conducted in this project used medaka eggs reared in freshwater, even though the field samples

were collected from elevated salinity environments. Based on the results obtained in the single toxicant studies, indicating no apparent salinity-related stress on the eggs, future field studies should incorporate the additional variable of salinity. This protocol would allow the determination of embryotoxicity in a system similar to that where the samples were taken and, therefore, would account for any elevations or reductions in toxicity due to increased seasalt concentration. Lastly, it would be interesting, and valuable, to extend the duration of the assay to incorporate the endpoint of carcinogenesis in these studies, thereby increasing the power of this assay to detect single toxicants or complex mixtures which are potentially harmful to similar indigenous species and possibly to man.

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**Select Publications:**

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