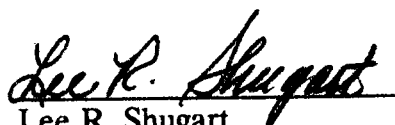
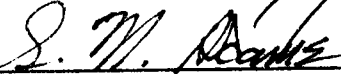


To the Graduate Council:

I am submitting herewith a dissertation written by Christine Gettys Hull entitled "Japanese Medaka (*Oryzias latipes*) Embryo Assay for Developmental Toxicity Incorporating Individual Embryo Incubations: Evaluation of Controls, a Pure Compound (BaP), Soil Extracts, and Stream Effluents." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Life Sciences.


Lee R. Shugart,
Major Professor

We have read this thesis
and recommend its acceptance:



Accepted for the Council:


Associate Vice Chancellor
and Dean of The Graduate School

**JAPANESE MEDAKA (*ORYZIAS LATIPES*) EMBRYO ASSAY FOR
DEVELOPMENTAL TOXICITY INCORPORATING INDIVIDUAL
EMBRYO INCUBATIONS: EVALUATION OF CONTROLS,
A PURE COMPOUND (BaP), SOIL EXTRACTS,
AND STREAM EFFLUENTS.**

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Christine Gettys Hull

May, 1993

Dedication

This dissertation is dedicated to my husband and best friend, CPT Barry S. Hull, USAR, for his continued support and understanding. It is also dedicated to my parents, Kenneth and Polly Gettys, for never telling me that there was anything in this world that I could not do.

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My dissertation committee members have been most patient and understanding with this project as it has taken numerous winding and twisting routes to emerge as this document. The committee members include Dr. Lee R. Shugart (ORNL), Dr. Walter Farkas (UT, Environmental Toxicology Program),

Dr. Richard Strange (UT, Fisheries Program) and Dr. S. Marshall Adams (ORNL). Also, Dr. Mark S. Greeley, Jr. (ORNL) served as a curtesy member. I appreciate the time and energy these individuals have dedicated to helping with this dissertation, however, I am solely responsible for any errors, either grammatical or technical, that may remain in this document.

I am indebted Ms. Glenda Tolliver for her help in editing this dissertation. Her encouragement and criticisms have made this document better than I alone could have ever achieved.

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ABSTRACT

While the embryos of many North American fish species are commonly used in toxicity testing, a non-indigenous, but equally useful fish species has been largely overlooked by organizations preparing protocols for such use in research and environmental regulation. The Japanese medaka (*Oryzias latipes*) has been used in basic and applied research in the United States and Japan for many years. All life-history stages have been used and studied, including numerous different types of embryo and embryo-larval assays. However, a medaka embryo assay has yet to be recommended by the American Society for Testing and Material, the United States Environmental Protection Agency or the American Public Health Association for use as a test species in water quality control.

We have developed and refined methods for evaluating toxicity and abnormalities in embryos exposed to contaminants in a variety of media. Individual embryos exposed to benzo[a]pyrene showed both increased mortalities and abnormalities. In the two field applications, individual embryos were exposed to: 1) *soil extracts* from two hydrocarbon-contaminated sites on U. S. Army Kwajalein Atoll, in the General Republic of the Marshall Islands, or 2) *water samples* from ten sites on East Fork Poplar Creek, in Oak Ridge, TN, a stream that originates inside the DOE Y-12 Plant.

The two soil extracts were from diesel fuel contaminated soil; treated embryos showed significant responses ranging from increased mortalities to abnormalities and developmental delays. The stream has a history of industrial

contamination, including polycyclic aromatic hydrocarbons, polychlorinated biphenyls, and heavy metals, (particularly mercury); other typical industrial discharges (i.e. chlorine, ammonia, and nitrates) are also present at some sites. Treatment groups showed high mortality and dose-dependent mortality in diluted samples compared to control and reference water samples. Medaka fry exposed to the water samples (96 h) did not have significant mortalities. This study demonstrates the flexibility of this medaka embryo assay for assessing the toxicity of complex environmental sites.

The incubation of individual embryos provided several advantages over group incubations. These advantages included (1) lower exposure potential for technicians using individual vials rather than Petri dishes, (2) increased ability to observe and track developmental abnormalities in developing fry during individual incubations, (3) greatly reduced problems with fungal, bacterial and parasitic contamination in individual vials, and (4) smaller samples sizes could be used with individually incubated embryos compared to either type of group incubation. Overall, the incubation of single embryos appears to be more applicable to toxicity testing, with both laboratory-generated solutions and in solutions obtained from soil extracts and stream effluents .

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ABBREVIATIONS

APHA	American Public Health Association
ASTM	American Society for Testing and Materials
AWWA	American Water Works Association
BaP	Benzo[a]pyrene
BNF	Betanaphthoflavone
d	Day(s)
DDT	Dichlorodiphenyl-trichloroethane
DEN	Diethylnitrosoamine
DENA	N-nitrosodiethylamine
DMSO	Dimethyl sulfone
DNOC	Dinitro- <i>o</i> -cresol
DOE	United States Department of Energy
2,4-D	2,4-Dinitrophenol
ED ₅₀	Effective dose for 50% malformed individuals = MC ₅₀
EFPC	East Fork Poplar Creek, in Oak Ridge, TN
EMS	Ethylmethanesulfonate
ENU	Ethylnitrosourea
EPA	United States Environmental Protection Agency
ERM	Embryo Rearing Medium (Kirchen and West, 1976)
EROD	Ethoxyresorufin- <i>o</i> -deethylase
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act
FWPCA	Federal Water Pollution Control Act
gal	Gallon
GSI	Gonado-somatic Index
Gy	Gray; 1 Gy 100 rad = 1J/kg
h	Hour
HPLC	High Performance Liquid Chromatography
LC ₅₀	Concentration causing 50% mortalities

50% LT	The first 24 h observation period during which at least 50% of the embryos were dead
MAM-Ac	Methylazoxymethanol acetate
MATC	Maximum Acceptable Toxicant Concentration
MC ₅₀	Concentration causing 50% malformed individuals = ED ₅₀
MEMC	Methoxy-ethyl-mercuric chloride
MEP	Fenitrothion = <i>O, O</i> -dimethy- <i>O</i> -(3-methyl-4-nitrophenyl phosphorothioate
MFO	Mixed Function Oxidases
MMC	Methyl mercuric chloride
MMS	Methylmethanesulfonate
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
MNU	Methylnitrosourea
μS/cm	microSiemens/cm; measure of conductivity
NOL(E)C	Concentration resulting in no observed lethalties (effect)
NOLC	Concentration resulting in no observed lethalties
NPDES	National Pollution Discharge Elimination System
PAH	Polycyclic aromatic hydrocarbon
PCB	Polychlorinated biphenyl
PCDD	Polychlorinated dibenzo- <i>p</i> -dioxin
PCDF	Polychlorinated dibenzofurans
PCP	Pentachlorophenol
PF	Postfertilization
PMA	Phenyl mercuric chloride
ppb	Parts per billion
ppm	Parts per million
ppt	Parts per trillion
R	Roentgen; unit of exposure; amount of ionizing radiation in air from γ- or X-rays
rad	Unit of radiation-absorbed dose

2,4,5-T	2,4,5-Trichlorophenoxyacetic acid
TCDD	2,3,7,8-Tetrachlorodibenzo- <i>p</i> -dioxin
TCLP	Toxicity characteristic leaching procedure
TL ₅₀	Median tolerance limit, 50% survival or abnormalities
TL _m	Median tolerance limit, 50% survival or abnormalities
TSCA	Toxic Substances Control Act
USAKA	United States Army Kwajalein Atoll, in General Republic of the Marshall Islands
UV	Ultraviolet light
WPCF	Water Pollution Control Federation
WSF	Water soluble fraction

GENERAL INTRODUCTION

The Japanese medaka (*Oryzias latipes*) is a killifish native to Southeast Asia. This fish is commonly used in studies of basic developmental biology, oncology, and toxicology. The primary goal of this study was to develop a medaka embryo assay protocol for evaluating developmental toxicity. This assay would differ from current and past medaka embryo assays in that it would:

- provide for the incubation of individual embryos, and
- investigate the use of stream effluents and soil extracts as test media.

The project had three phases.

- (1) Development of a protocol for the incubation of individual embryos, and comparison of data from control solutions to insure that it was feasible to utilize individual embryos.
- (2) Testing these protocols on single-compound, laboratory-generated solutions.
- (3) Using the protocols to evaluate the toxicity of both soil extracts and environmental water samples.

One rationale for using embryo assays to evaluate the toxicity of chemical pollutants has long been that early life-stages are the most sensitive life-stages (McKim, 1977). (The term embryo assays will be used in this document to include embryo-larval assays unless otherwise stated.) By testing a single, more sensitive life-stage, researchers could bypass the need for whole life-cycle tests, which are

both lengthy and more costly. Governmental regulatory agencies recommend early life-stage toxicity tests for chemical product testing and for bioremediation studies (EPA, 1989; ASTM, 1991).

The lists of embryo assays currently recommended for use by the United States Environmental Protection Agency (EPA), the American Society for Testing and Materials (ASTM), or the American Public Health Association (APHA) do not include a medaka embryo assay, even though the medaka has been used in embryo assays for at least 50 years (e.g. Waterman, 1939). These three lists include only species indigenous to North America. Not only do these three organizations publish recommended lists of species, but corresponding protocols for developmental toxicity tests are also available (APHA, 1985; EPA, 1989; ASTM, 1991). Therefore, the addition of the medaka to any list of recommended species would also include the determination of a standard set of protocols for a medaka embryo assay.

The protocols currently and historically in use for medaka embryo assays have varied widely between laboratories. The development of a standard set of protocols would provide more consistent data, which could lead to consideration of the medaka as a recommended test species. Consequently, increased use of a medaka embryo assay, resulting from standard protocols, would provide an increased volume of toxicity data for the species. The increased data would then provide more information on species sensitivity, and allow better direct

comparisons between the sensitivity of the medaka and currently recommended species.

The protocol developed and tested for this project differs from other literature-reported protocols in the following ways:

- (1) The protocol utilizes the incubation of individual embryos, eliminating any influence one embryo could exert on another, as well as eliminating the need for antifungal agents in the incubation media.
- (2) This protocol is then used to combine the traditional research applications (e.g. toxicity testing of laboratory-derived solutions) with less traditional, and more applied applications (e.g. stream and soil extract toxicity testing).

Studies designed to compare other protocols to this method will be presented and evaluations will be made on the advantages and disadvantages associated with the different types of incubation protocols.

Part I will contain a literature review focusing on (1) uses of fish embryo assays in toxicity testing, (2) EPA- and ASTM- recommended species for fish embryo assays, (3) uses of the medaka in basic research, and (4) uses of the medaka in toxicology. The first two sections will briefly cover the types and applications of fish embryo assays, and the third section will provide an overview

of the uses of the medaka in basic research. The fourth section will be the most detailed, describing historical toxicological applications from the literature.

Although this will deal with embryo and larval life-stages in depth, it will also include experiments involving whole life-cycle tests and adult life-stages. The information will rely almost exclusively upon literature that is available in English. However, a few articles that are available only in Japanese with English abstracts have been cited. This section of Part I will also include a survey of chemicals tested, an evaluation of the endpoints monitored, and comparisons of incubation methods used in different laboratories. The primary goal of Part I will be to discuss why and how embryo assays are used and why the medaka should be considered for recommendation as a test species. Part I will also include a discussion on the need to develop a set of protocols, particularly a protocol that utilizes the incubation of individual embryos.

Part II will present the data from preliminary studies. These studies were designed to develop the assay protocols and to determine if these protocols will provide adequate incubation conditions for control embryos. Although literature is available that provides information on ideal conditions for adult breeding and embryo incubation (temperature, photoperiod, embryo rearing medium etc.) for medaka embryos (Kirchen and West, 1976), few studies consider these conditions as they would relate to toxicity testing (Marty, 1990). The preliminary studies in Part II investigate the water quality needed for controls, comparing the commonly used embryo rearing medium (Kirchen and West, 1976) against deionized distilled

water. Part II includes experiments comparing data from aerated and non-aerated solutions as test media. These experiments establish the protocols to be used in the rest of the project. In addition, the experiments compare data on various endpoints to determine those that are both (1) most easily monitored, and (2) most sensitive to perturbation.

Part III will report the data from a study that used the proposed medaka embryo assay protocol in a toxicity testing situation. This study was designed to determine if the protocols could be used for the traditional types of toxicity testing, with mortality, the appearance of developmental abnormalities, and the time required from fertilization to hatching (time-to-hatch) being the endpoints monitored. The test solutions were varying dilutions of microparticulate suspensions containing benzo[a]pyrene (BaP), a common environmental pollutant. The first phase of the study was an evaluation of developmental toxicity of BaP. In this experiment, individual embryos were incubated in the various dilution strengths to determine if exposed embryos demonstrated any identifiable effects. The second phase of the study compared data obtained from the replication of literature-reported protocols to data obtained from the proposed protocol.

Part IV of this dissertation will report the results of toxicity studies on soil extracts from two potentially contaminated sites. These sites were located at the U.S. Army airbase on the U.S. Army Kwajalein Atoll, Republic of the Marshall Islands, in the South Pacific. One site was known to be heavily contaminated with diesel fuel; the second site appeared to have less diesel fuel contamination,

however, the site did show some heavy metal contamination and was by no means considered a pristine site (Siegrist, et al., 1991). The first phase of experiments duplicated those used for the BaP experiment in Part III. In these experiments, however, instead of exposure to a single compound, embryos were exposed to complex mixtures via soil extracts. Recently, researchers have tested soil extracts using embryo assays, and even using medaka as the test organism (Marty, 1991). Therefore, it is necessary that to show that the protocol proposed in this study will be feasible for testing soil extracts. The second phase of this study compared data obtained from group incubation protocols to data obtained from the proposed single embryo incubation, in an attempt to determine the advantages and disadvantages associated with each type of incubation.

Part V will report the results of a study investigating the applicability of the medaka embryo assay to testing the developmental toxicity of stream effluents. The source medium for these experiments was water collected from East Fork Poplar Creek in Oak Ridge, Tennessee. The upper reaches of the stream originate inside a Department of Energy Industrial Complex, receiving discharges from the plant. The stream is diverted into a lined lake (Lake Reality), and then passes through the City of Oak Ridge, TN. The first phase of the study was a survey test using the proposed protocol on 10 sites along the stream to determine if the effluent had any effects on the medaka embryos. A site in a nearby, unaffected river was used as a reference site. Once toxicity was established in the EFPC sites, further experiments were done to clarify the type of effects seen in

exposed medaka embryos. These included different sterilization procedures, exposure to serial dilutions, and comparison of embryo and fry sensitivity. Two different types of group embryo incubations were also compared to the single embryo incubation procedure.

The final section of this dissertation will include a summary of the overall project, tying the conclusions from experiments in Part II-V together. This summary will present an evaluation of the performance of the single embryo incubation as it relates to the uses of a medaka embryo assay for standard toxicity testing of pure compounds, soil extracts, and environmental effluents.

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PART I: LITERATURE REVIEW

1. FISH EMBRYO ASSAYS IN TOXICITY TESTING

The purpose of Part I is to critically review the use of a fish embryo assay incorporating the embryos of the Japanese medaka (*Oryzias latipes*). As an introduction to the critical review, the following sections (1 through 3) provide background information on fish embryo assays and toxicity testing.

1.1 Fish Bioassays in Toxicity Testing

Toxicity tests are commonly used to evaluate the effects of chemicals on living organisms. Researchers test for toxic effects using any of a variety of test organisms. Fish have been used as test organisms in traditional types of toxicity tests for many years. The use of fish offers several convenient modes of exposure. As with other test organisms, fish may be exposed by injection, or chemicals may be added to the food, so that exposure is via the digestive tract. Additionally, fish may be exposed to waterborne chemicals via their gills and skin. Many fish species offer advantages over rodents and some other test organisms (e.g. dogs) because they are cheaper and easier to maintain in the laboratory.

A traditional use for toxicity tests is the evaluation of new chemicals, either before or after commercial production. A less traditional, but increasingly useful application of fish toxicity tests, is in the evaluation of natural waters collected from the environment for potentially toxic effects. The goal of testing may be (1) to determine the impact of an industrial discharge on a stream ecosystem, or (2) to determine potential effects to humans exposed to the contaminated water or

fish. The testing methods may differ somewhat, depending on the endpoints monitored and the type of test conducted (i.e. laboratory prepared solution versus environmental effluent).

1.2 Standardized Toxicity Tests

Although toxicity tests have been in use for many years, in recent years testing has become much more prevalent and standardized due to the impact of federal legislation. These legislative acts include:

- the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA),
- the Federal Water Pollution Control Act (FWPCA) amendments of 1972, 1977 (Clean Water Act), and 1987 (Water Quality Act), and
- the Toxic Substances Control Act of 1976 (TSCA).

FIFRA requires testing of all pesticides prior to commercial production; TSCA requires the evaluation of all new chemicals in terms of their environmental impact (EPA, 1979). These tests are to be done before commercial production, by the EPA and by industries (McKim, 1977; McKim, 1985; Merrill, 1986). The 1972, 1977, and 1987 amendments to the Federal Water Pollution Control Act (FWPCA) were enacted to prevent the discharge of toxic chemicals into water systems. This legislation led to the use of acute and chronic toxicity testing as a means of regulating industrial discharges. In addition, beginning in 1984, the EPA

recommended using toxicity tests to enforce the National Pollution Discharge Elimination System (NPDES) permit program (EPA, 1989). The EPA recommendation caused changes in toxicity testing procedures and gave new importance to the use of standardized or recommended protocols.

1.3 Fish Embryo Assays

Researchers have used fish embryo assays in various types of toxicity testing for many years. However, the use of these bioassays was very limited within the field of toxicology. Recently, the need for better toxicity testing methods and the use of bioassays in regulating and monitoring water quality have made the use of fish embryo assays both more prevalent and more important.

In fish bioassays, toxicity tests may be whole life-cycle tests (embryo-to-embryo) or partial life-cycle tests (Peakall, et al., 1983; Mckim, 1985; APHA, 1985; ASTM, 1991a). Although whole life-cycle tests are considered to be the "ultimate test in establishing long-term 'safe' environmental concentrations of toxic chemicals" (McKim, 1985), they are prohibitive in both cost and time. Whole life-cycle tests may range from 12 months for fathead minnow (*Pimephales promelas*) to 30 months for trout (McKim, 1985). The length of time needed for these tests increases the possibility that mechanical systems will fail or fish will become diseased, either of which could lead to early termination of the experiment and loss of data (Persoone, et al., 1990).

Early life-stage tests (embryo and embryo-larval assays) are being advocated and often used to replace the longer, more costly, whole life-cycle tests (EPA, 1989). McKim (1977) evaluated data from 56 life-cycle toxicity tests on four fish species, covering a range of 34 organic and inorganic compounds. From this, he determined that the maximum acceptable toxicant concentration (MATC) was equal, in whole life-cycle tests, to the MATC calculated for the embryo-larval stages, in 82% of the tests. McKim concluded that embryo assays could be used to predict MATC values within a factor of two, without the extensive time and cost involved with whole life-cycle tests. Later studies are in agreement with McKim (Danil'chenko, 1977; Benoit and Holcombe, 1978; Blaylock and Frank, 1979; Hall et al., 1984; Woltering, 1984). Today, many chemicals or effluents can be screened much more rapidly using embryo assays. Detailed information on testing procedures is readily available in Standard Methods for the Examination of Water and Waste Water (APHA, 1985), Fundamentals of Aquatic Toxicology (Rand and Petrocelli, 1985), 1991 Annual Books of ASTM Standards (ASTM, 1991b), and EPA's Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms (EPA, 1989). Standard Methods, 1991 Annual Books of ASTM Standards and Fundamentals of Aquatic Toxicology suggest testing methods and species. ASTM- and EPA- recommended species and protocols will be discussed in more detail in section 2 of this part.

Early life-stage tests generally use test organisms during the period of time beginning at or just after fertilization, continuing through embryonic development,

larval development, and the early juvenile period (Donaldson and Scherer, 1983; McKim, 1985). Embryo and embryo-larval tests encompass only a specific subset of the life-stages used in the early life-stage tests, and generally exclude the early juvenile period.

Endpoints of these tests may be either invasive or non-invasive. Non-invasive endpoints are collected without the destruction of the organism (e.g. observation of abnormalities). Invasive endpoints require the destruction of the organism (e.g. homogenization of embryo or fry for analysis of enzyme activity). Non-invasive endpoints for embryo assays may vary, depending upon the species and protocol, but will generally include observations of (1) embryo survival and mortality, (2) hatching success, (3) time-to-hatch (period of time between fertilization and hatching), (4) developmental abnormalities or terata, and (5) appropriately timed progression through embryonic development. When exposure is initiated prior to or during *in vitro* fertilization, additional endpoints include (6) successful fertilization and (7) rate of fertilization (Donaldson and Scherer, 1983; McKim, 1985; ASTM, 1991a). Invasive endpoints include specific biochemical and histological endpoints (Donaldson and Scherer, 1983; Peakall, et al., 1983; Schell, Jr., et al., 1987), requiring the preparation of light or electron micrographs of embryos at various stages of embryonic development or analysis of detoxication enzyme activity following histological preservation or homogenization.

1.4 Fish Embryo Assays in Use

Fish embryo assays have been used to evaluate both laboratory prepared solutions (Carlson, 1972; Benoit and Holcombe, 1978; Hall, et al., 1984), and environmental water sources receiving either mixed or unknown pollutants (Marty, et al., 1991). When the goal of testing is compliance with state or federal regulations, embryo assays are done according to recommended procedures. However, other species and test approaches are commonly used in independent research.

In some studies, researchers maintain cultures of test fish in a laboratory setting, and either naturally or artificially induce spawning (i.e. Carlson, 1972; Nebeker, et al., 1974; Rehwoldt and Karimain-Tehrani, 1976; Hemmer, et al., 1990; Middaugh, et al., 1990). Temperature and photoperiod may be used to naturally induce spawning; hormone injections may be used to artificially induce spawning. Depending on the species, the researchers then collect fertilized eggs immediately after spawning (as with medaka [e.g. Marty, et al., 1991] and fathead minnows [e.g. Nebeker, et al., 1974]), or they collect mature gametes from gravid adults and fertilization is *in vitro* (as with bluegill sunfish, *Lepomis macrochirus* [e.g. APHA, 1985], and striped bass, *Morone saxatilis* [e.g. APHA, 1985]).

Alternatively, researchers may use field-collected female and male fish from either contaminated or clean streams and lakes (i.e. Giesy et al., 1986). When fish are collected during the breeding season, assays can be performed on embryos produced by these fish (APHA, 1985; ASTM, 1991a); fertilization may be either *in*

vitro or *in vivo*. When fish are field-collected during non-reproducing seasons, hormone injections are often used to induce spawning (e.g. APHA, 1985). Studies can incorporate many permutations of the above methods, for example, field-collected fish may be used to test laboratory-derived, single-compound solutions. The multiplicity of variables provides the individual researcher with a great deal of flexibility in selecting an experimental approach.

1.5 Species Selection

Several important factors need to be considered when selecting a test species for fish embryo assays. These include

- (1) ecological significance of the species (Peakall, et al. 1983; APHA, 1985; McKim, 1985),
- (2) fish maintenance in the laboratory (Donaldson and Scherer, 1983; APHA, 1985; McKim, 1985), or
- (3) availability of fish in contaminated areas (Lennon, 1967; APHA, 1985),
- (4) sensitivity of the fish species (APHA, 1985; Goss and Sabourin, 1985; Mayer and Ellersieck, 1986; Cairns Jr. and Niederlehner, 1987), and
- (5) characteristics of the embryo, and embryonic development.

Each factor must be considered, both for laboratory testing and for field testing. It is likely that not every factor will be optimum for one species, thus researchers must prioritize the factors as they apply to each experimental setting. Some of these decisions may be dictated by the experimental approach, research priorities, or goals of the experiment. As these aspects of species selection are discussed, problems that could necessitate the use of alternative species will be examined within each category.

1.5.1 Ecological significance

A species may be considered to be ecologically significant when researchers can directly relate data from a particular species to a stream or ecosystem in question. For example, some would argue that testing an effluent on a non-indigenous fish species would lack ecological significance because this species would never be subjected to these conditions in its natural environment (Waldichuk, 1973). If the species can be proven to respond to toxicants similarly to indigenous species, non-ecologically significant species can still be used. Thus, side-by-side tests comparing ecologically significant and non-ecologically significant test species are important (APHA, 1985). Such comparative tests may be necessary where researchers incur difficulties using ecologically significant species. For example, the only species left in a stream may be (1) considered to be resistant to pollutants (Karr, 1981; Karr et al., 1986), (2) listed as threatened or endangered, or (3) present in the stream in only small numbers. In the case of

reproductive dysfunctions, there may be adults in the stream, but they may not be reproducing, and thus gravid females are unavailable. There may also be problems maintaining the ecologically significant species in the laboratory, or inducing spawning (Peakall, et al., 1983; Adams, 1990). Any of these situations could necessitate the use of alternative species.

1.5.2 Fish maintenance in the laboratory

The time, effort, and expense of maintaining a fish culture in the laboratory must be considered when a study requires a constant supply of embryos. In nature, most fish have a breeding season that does not extend throughout the year; thus laboratory conditions will need to mimic the environmental cues that stimulate breeding. Some fish, like *Fundulus grandis* (Gulf killifish) exhibit breeding behavior in response to tidal cues from lunar phases (Greeley, Jr, and MacGregor, III, 1983). The laboratory setup for their maintenance needs to have separate space available for holding fish prior to the induction of spawning. This space is not subject to the environmental cues used for spawning induction. Such a setup requires much more laboratory space than the maintenance of a species that spawns throughout the year. Many fish, like the fathead minnow and medaka, will produce eggs all year if maintained at the proper temperature, breeding ratio, and photoperiod. Some other species require hormone injections to achieve reproductive status in the laboratory. While hormone injections can allow for very precise timing for egg collection, there is some dispute about the

validity of the process for toxicity testing. Drawbacks associated with the use of hormone injections are that (1) it presents another variable within the test, and (2) information on the type of hormone and dosage needed for induction is not yet available for many indigenous species. Thus, much research would need to be done prior to successful induction of spawning and collection of embryos.

1.5.3 Fish availability in contaminated areas

When a study is designed to evaluate a particular environmental situation, for example a specific stream receiving effluent from a pulp paper mill, it is important to determine if an indigenous species is available for use in an embryo assay. In cases where the stream has been severely impacted by a pollutant, researchers may find no breeding adults in the stream, or may not find enough breeding adults to sample and from which to collect gametes. Although this later situation may imply a reproductive dysfunction beyond that of embryo survival, it does not necessarily exclude the need for such studies. Additionally, an indigenous species may be available for use, and yet may be unsuitable for a particular study. For example, the only fish in the stream may be considered a very hardy species. Presumably this species survived where more sensitive species could not. It can be argued that it would not be worthwhile to evaluate the toxicity of the effluent on a species known to be very hardy (Karr, 1981; Karr, et al., 1986). Other reasons for limited fish availability in impacted areas include some previously mentioned

situations where species may be present, but may be listed as threatened or endangered.

Where fish are unavailable in a contaminated area, there are two possible approaches:

(1) Alternative species that may or may not be closely related to the indigenous species may be used. These would most likely be from a laboratory-maintained culture.

(2) Fish that should be indigenous to the stream (but may no longer be there) can be collected from clean (reference) sites during the reproductive season.

The embryos produced from these adults can then be tested in water from the stream or lake in question.

Using an alternative species, the first approach, could allow researchers to study a field site year-round and monitor the changes seen in the toxicity of the effluent. The second approach, however, would allow such studies only during the reproductive season. Thus, if the study is designed to monitor a particular industrial effluent as it changes with time, either to insure regulatory compliance or to evaluate bioremediation efforts, the first alternative would be the more useful. Under other circumstances, fish collected from reference sites might be preferred. For example, if the researchers are interested in the effects of the

pollutants at different ecosystem levels, it would be more appropriate to initially use this second approach.

1.5.4 Species sensitivity

Selecting a species for use in toxicity tests requires that researchers consider the sensitivity of different fish species to various chemical pollutants (APHA, 1985; Mayer and Ellersieck, 1986; Cairns Jr., and Niederlehner, 1987). When Kenaga (1978) compared the results of toxicity tests using 42 chemicals on 3 fish species (bluegill, fathead minnow and rainbow trout), he concluded that no one species (of the three tested) was consistently the most sensitive to each compound tested. Mount (1982) went one step beyond Kenaga with this conclusion:

The point is that we have ample data to prove that a species can only represent itself consistently and not a group. . . . Thus, each species we test is not representing any other species but is one estimate of general species sensitivity. With several such estimates, the overall range of sensitivity for all species can be determined. Our problem is to know how many species and what type of species to test to adequately represent the whole range.

Birge and Black (1982) proposed a "biological response range" that is based upon the assumption that no one species is most sensitive. This "biological response range" would be determined from data of toxicity tests using 3 to 5 aquatic species (Birge and Black, 1982). The American Public Health Association (APHA, 1985)

suggests that researchers do preliminary tests to determine both the most sensitive species and the most sensitive life stage for specific geographical areas.

Many arguments remain unanswered as to whether some species are sensitive (intolerant) and others are insensitive (tolerant) to specific pollutants (Karr, 1981; Karr, et al., 1986). Various researchers have made attempts to compare the sensitivities of different species and life stages (Mayer and Ellersieck, 1986; McKim, 1977), but no definitive conclusions have yet been drawn.

Several complications could prevent the use of an indigenous species, necessitating the use of a sensitive alternative species. For example, researchers may be in a situation where the most sensitive indigenous species is (1) absent due to chemical pollutants (see section 1.5.3), (2) impractical to sample, due to low population numbers (see section 1.5.3), or (3) impossible to culture and to induce to spawn in the laboratory (see section 1.5.2). Another complication could be that the most sensitive species is *too* sensitive in regards to overall ecosystem protection (Cairns, Jr., and Niederlehner, 1987). In such cases, it would be necessary for researchers to use alternative fish species for the tests.

1.5.5 Embryo characteristics

The characteristics of different species' embryos may affect the researchers ability to monitor certain endpoints. For example, in studies concerned with the developmental effects of exposure to a particular compound, the clear chorion of the medaka would be an asset. This type of chorion allows for easy observation of

the development of the embryo. However, if a study is concerned only with the absolute survival of the embryos, the ability to observe embryonic growth and development is not important.

The time period during which development occurs is also a factor to be considered. Some embryos will progress rapidly through the early stages of development and hatch within 72 to 96 h (e.g. bluegill sunfish, striped bass, and *Brachydanio rerio* [zebra fish]). The resulting larvae may be free-swimming, but will continue to feed on their large yolk sacs for several more days. Conversely, embryos with longer incubation periods will hatch with smaller yolk sacs and spend a shorter amount of time as larvae. If a test is focused on the effects of exposure on early development and terminated with hatching, embryos with a longer incubation period may be better test organisms. These embryos would theoretically receive a larger dose of the test compound, and the slower developmental progression would allow better observation of transient effects. Where time and money are considered to be important, the shorter assays, using sensitive species, would be most desirable.

If an alternative test species is needed to replace an unavailable indigenous species, it is necessary to know the developmental time frame for the indigenous species. The best choice for an alternative species would need to have a developmental time-frame which matches that of the indigenous species as closely as possible.

2. ORGANIZATIONAL STANDARDS FOR TOXICITY TESTING

Several organizations, including government agencies, have produced guidelines or recommendations for toxicity tests. These include:

- the United States Environmental Protection Agency (EPA),
- the American Society for Testing and Materials (ASTM), and
- the American Public Health Association (APHA), in conjunction with the American Water Works Association (AWWA) and the Water Pollution Control Federation (WPCF).

Although this discussion will focus on early life-stage tests, these guidelines generally cover the full range of toxicity tests: chronic to acute, and whole life-cycle to single stage tests.

EPA guidelines for short-term chronic toxicity tests provide a means of monitoring industrial effluents and ensuring compliance with the National Pollution Discharge Elimination System (NPDES) permit program (EPA, 1989). But the use of ASTM standards for tests and procedures is voluntary, and does not have any legal or legislative implications. . .

An ASTM standard represents a common viewpoint of those parties concerned with its provisions, namely producers, users, consumers and general interest groups. It is intended to aid industry, government agencies, and the general public (ASTM, 1991b).

Standard procedures provided by the APHA (1985) in Standard Methods are to aid in the examination of. . .

waters of a wide range of quality, including water suitable for domestic or industrial supplies, surface water, ground water, cooling or circulating water, boiler water, boiler feed water, treated and untreated municipal or industrial wastewater, and saline water (APHA, 1985).

APHA notes that most of the methods included have been accepted by regulatory agencies. Individuals from the APHA, as well as AWWA, and WPCF, serve on the joint committees that accept methods for publication.

2.1 Recommended Species

The three early life-stage toxicity tests developed by EPA (EPA, 1989), ASTM (1991a) and APHA (1985) differ, not only in the intended use of the tests, but also in the species recommended for use in the tests. EPA recommends only the use of the fathead minnow and follows a modified protocol from Birge and Black (1981). ASTM, on the other hand, provides a longer list of recommended species and notes that. . .

whenever possible and appropriate, tests should be conducted with species listed . . . , mainly because these species have been used successfully in early life-stage tests. With appropriate modification of these procedures, other species can be used. Use of the species listed . . . is encouraged to increase comparability of results and availability of much information about a few species rather than a little information about many species (ASTM 1991a).

The ASTM recommended species are listed in Table 1.1 by both common and scientific names.

Table 1.1 ASTM recommended genera and species for early life-stage toxicity tests (ASTM, 1991a).

Common Name	(Scientific Name)
Bluegill Sunfish	(<i>Lepomis machrochirus</i>)
Channel Catfish	(<i>Ictalurus punctatus</i>)
Char	(<i>Salvelinus</i> sp.)
Fathead Minnow	(<i>Pimephales promelas</i>)
Gulf Toadfish	(<i>Opsanus beta</i>)
Northern Pike	(<i>Esox lucius</i>)
Salmon	(<i>Oncorhynchus</i> sp.)
Sheepshead Minnow	(<i>Cyprinodon variegatus</i>)
Silversides	(<i>Mendidia mendidia</i>)
Trout	(<i>Salmo</i> sp.)
White Sucker	(<i>Catostomus commersoni</i>)

The APHA (1985) presents a longer, although partial list of species that have been used successfully in other life-stage toxicity studies, but stops short of recommending specific species. APHA (1985) includes the following suggestion:

Few studies have been made to determine the important species most sensitive to a potential toxicant. To select a best species consider available information on sensitivity or determine sensitivity with short-term tests. . . .

For studies to determine effluent requirements, use the most sensitive locally important species and most sensitive life stage. When using another species, make comparative tests to relate sensitivity of the selected test species to the most sensitive locally important species . . . (APHA, 1985).

The partial list of species in APHA (1985) includes 41 freshwater and 21 marine and estuarine fish species, and includes all 11 ASTM-recommended genera, except the gulf toadfish, (*Opsanus beta*). The only species common to all three lists is the fathead minnow.

2.2 Differing Philosophies

It appears that there is an inherent difference between the philosophies of ASTM and APHA as they address the issue of species for embryo assays. ASTM states that tests should acquire *large amounts of information on a few species*, while APHA promotes the use of the *most applicable species*. Thus APHA's philosophy includes consideration of the sensitivity, geographic distribution, and availability of species for use (APHA, 1985), and does not appear to be exclusively dependent upon the amount of previously accumulated data for the species.

As one can see, there is little consensus on ideal test species within the organizations that have produced standard protocols, except that most or all suggested species are indigenous to the U.S.. The final section of this part will examine the literature for uses of the Japanese medaka (*Oryzias latipes*) embryos as test organisms in early life-stage toxicity tests. While the medaka may not prove to be the ideal species for *all* test situations, it may prove to be a desirable alternative species for *many* situations.

3. GENERAL INFORMATION ON THE LIFE HISTORY AND CULTURING METHODS FOR JAPANESE MEDAKA (*ORYZIAS LATIPES*)

The Japanese medaka (*Oryzias latipes*, family Cyprinodontidae), a small killifish, is indigenous to the rice fields of Southeast Asia. Adults range from 2 to 5 cm long, and can provide an ample supply of embryos for embryo toxicity assays (Kirchen and West, 1976; Hirshfield, 1980). Medaka, which are hardy and easily

maintained in the laboratory, do not appear to establish territories within the aquaria (Kirchen and West, 1976; Hirshfield, 1980). Egami and Etoh (1969) reported a 4 year life-span for medaka, although adults maintained in the laboratory under breeding conditions experience shorter life-spans (1 to 2 years in our laboratory). Characteristics of older fish include large size, bloated appearance, and pale color (Egami, 1971). On a cellular level, aging is associated with enlarged hepatocytes (M. Yamamoto and Egami, 1974) and less polyploidization (Shima and Egami, 1978).

Adults may be purchased from supply stores (e.g. Carolina Biological Supply Company, Burlington, NC), or obtained from other laboratories. Kirchen and West (1976) have published a guide for the care and breeding of the medaka; this guide is available through Carolina Biological Supply Co. Other sources of such information are (1) Experimental Embryology Techniques and Procedures, 3rd edn. (Rugh, 1962), and (2) Medaka (Killifish) Biology and Strains (T. Yamamoto, 1975).

3.1 Secondary Sexual Characteristics

Adult medaka exhibit sexual dimorphism. There are three easily identifiable morphological differences between the two sexes: (1) the anal fin of the male is larger than that of the female, and has a convex, "lacy" margin, (2) the dorsal fin of the male has a deep notch in it that could be mistaken for a bite mark from another fish, and (3) the female will often still have several eggs

attached to the ventral surface, just anterior to the anal fin (Briggs and Egami, 1959; Kirchen and West, 1976). The external differences seen in the anal and dorsal fins are apparently a result of sexually dimorphic bone structures in those appendages (Oka, 1930a; Egami and Ishij, 1955). A more complete discussion of the differences in secondary sexual characteristics (both internal and external) between male and female medaka may be found in Okada and Yamashita (1943) and M. Yamamoto and Egami (1974a). Rare hermaphroditism has been reported, but these fish were not considered to be functional hermaphrodites (Oka, 1930b).

3.2 The Breeding Process

The ideal breeding ratio for each aquarium is generally three females for every two males (Kirchen and West, 1976). A clean, chlorine-free water source is necessary for culture maintenance. Medaka will live in water of various osmolalities, ranging from distilled water to sea water (Ikeda, 1934a). Medaka will breed in either flow-through or static aquaria; however, the flow-through systems reduce the potential for diseases. All aquaria need constant aeration to maintain an adequate oxygen level.

While the reproductive season for adult medaka in nature is usually mid-April through late September, adults in captivity will spawn for 8 to 10 months if given the correct environmental cues (Yoshioka, 1962, 1963; K. Yamamoto and Yoshioka, 1964). These environmental cues are an eight hour dark and sixteen

hour light photoperiod, with water temperatures of 24 to 26°C (Solberg, 1942; Kirchen and West, 1979; Awaji and Hanyu, 1987). Although the fish can live in water at ambient temperatures (Briggs and Egami, 1959), 24 to 26°C is optimum for breeding.

Under these conditions, the adult medaka will undergo daily physiological changes in preparation for spawning. Female medaka ovulate mature eggs in the lumen of the ovary approximately one hour *prior to* the onset of the light cycle (Iwamatsu, et al., 1988a). Maturation of the oocytes is controlled by the influence of the photoperiod and temperature on both the pineal and pituitary glands (Egami, 1959a; Urasaki, 1976; Iwamatsu, 1978). Oocyte development is asynchronous, and continues throughout the breeding season (K. Yamamoto and Yoshioka, 1964). Liver hepatocytes of female medaka undergo changes as a result of vitellogenesis. These changes include development of extensive endoplasmic reticula, increased amount of lipid droplets and a decrease in the amount of glycogen stored in the hepatocytes (M. Yamamoto and Egami, 1974a).

In the male medaka, environmental cues for spawning primarily cause physiological changes in the testis. Testicular tissue begins to produce primary spermatocytes and spermatogonia, and then produces sperm from the spermatogonia (Nishikawa, 1956, abstract only; Michibata, 1975; Awaji and Hanyu, 1987). The rate of spermatogenesis is temperature dependent, with increased rates at 25°C and decreased rates at 15°C (Egami and Hyodo-Taguchi, 1967). Spermatogenesis is reported to begin around 60 d posthatch (Hamaguchi, 1979).

Thyroid changes associated with the breeding cycle have also been noted in both male and female medaka (Nishikawa, 1975, abstract only).

Spawning generally occurs within 1 to 2 hours *after* the onset of the light cycle, and is controlled by the influence of environmental stimuli (e.g. presence of a male medaka and photoperiod) on the pituitary gland (Egami, 1959b; Egami and Nambu, 1961). The onset of breeding behavior is marked by a brief swimming courtship, after which the male medaka "approaches the female, and embraces the posterior part of the female body by means of the dorsal and anal fins" (Egami, 1959b). Thus, swimming side-by-side, the bodies form an S-shape. Next, there is rapid vibration of the fins of both fish, and release of the gametes (Ono and Uematasu, 1957; Egami, 1959b; Egami and Nambu, 1961). External fertilization occurs almost immediately at spawning.

Female medaka produce anywhere from 3 to 50 eggs each day (Egami, 1959c; Briggs and Egami, 1959; Kirchen and West, 1976; Hirshfield, 1980). This number appears to be dependent on the age and nutritional status of the fish. Once spawned, the eggs remain attached to the underside of the female by chorionic filaments.

3.3 Embryo Collection and Appearance

Egg collection is done in various ways. When adults are kept in glass aquaria, it is often possible to remove only egg-bearing females from the tanks. However, when adults are kept in large living streams, random netting is

necessary. Eggs are removed from the outer abdomen of the female by gently brushing either a gloved finger or a pair of blunt-ended forceps across the abdomen. Care must be taken to avoid putting pressure on the female fish, and the female should be kept out of the water for only a short period of time (i.e. 1 minute) (Kirchen and West, 1976).

Some laboratories also collect eggs by gently siphoning the bottom of the tanks and letting the siphon empty through a net (Gulf Coast Research Lab, personal communication). Still another means of egg collection is to place sponges (e.g. Tetra Twin Biological Filters, American Pet Sales, New Orleans, La) in the water with the adults. The females will scrape the eggs off onto the sponges following spawning, and the eggs are then easily removed from the sponges to incubation dishes (Gulf Coast Research Lab, personal communication).

Once the clutches of eggs have been removed from the female, the eggs are then placed under a dissection microscope. Here, fertilized eggs are easily identified by the perivitelline space surrounding the inner yolk. Oil droplets are visible within the yolks of both fertilized and unfertilized eggs. After fertilization, the oil droplets begin to coalesce, forming one to two large oil droplets (Briggs and Egami, 1959; Kirchen and West, 1976), as is commonly seen in teleostian eggs. Cryoprotection techniques have been developed for medaka embryos (Arii, et al., 1987), however no documented toxicological applications of cryoprotection were found in the literature.

3.4 Incubation: Media Considerations

The incubation or rearing media used for the medaka has varied between laboratories. The most commonly used hatching medium is a high salt medium recommended by Kirchen and West (1976), consisting of 17 mM NaCl, 0.400 mM KCl, 0.270 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and 0.660 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. Other incubation media include distilled water (e.g. Michibata, 1981a; Cameron and Hunter, 1984), dechlorinated water (e.g. Hiraoka and Okuda, 1983, 1984b), well water (e.g. Akiyama, 1970), and various high salt compounds other than that used by Kirchen and West (1976) (e.g. Heisinger and Green, 1975; Dial, 1978a, b; Cloud, 1981; Cameron and Hunter, 1984; Cameron, et al., 1985a, b; Chong and Vielkind, 1989; Belanger, et al., 1990). Such wide variation is possible since medaka embryos will develop normally and hatch in (1) concentrated sea water, (2) sea water, (3) fresh water or (4) distilled water. This is also true for the embryos of *Fundulus heteroclitus*, a close relative of the medaka (T. Yamamoto, 1934, 1941a).

Ikeda (1934b) and T. Yamamoto (1941a) investigated the permeability and osmotic properties of the medaka chorion and egg, and concluded that the chorion is readily permeable to water and electrolytes, and thus is not involved in maintaining the osmotic equilibrium of the eggs. Instead, the protoplasmic membrane on the surface of the embryo may be less permeable to ions than the outer chorion (T. Yamamoto, 1936a). The ability of the medaka embryo to develop normally in such widely varying solutions is an advantage for researchers who are interested in the effects of natural waters (e.g. streams, lakes, reservoirs).

However, there is some limited evidence that may suggest that the chemical properties of the incubation medium (pH, hardness, ionic content, salinity, etc.) may interact with toxicants and alter the effects seen in the embryos (Ikeda, 1936; Sakaizumi, 1980a; Michibata, 1981a; Cameron and Hunter, 1984; Michibata, et al., 1986; Chulakasem et al., 1989). Some of these factors (such as pH and hardness) may be results of pollutants, and the effects seen could be considered synergistic effects of toxicants. The observed interactions between toxicants and water chemistry may also be related to an electro-negative charge of the outer chorion, which makes the flow of cations into the egg higher than that of anions (Ikeda, 1934b; Kamuda, 1936); however, no definitive studies have examined the effect of different types of incubation media on the effects of toxicants. Historically, researchers have used various anti-fungal agents (e.g. methylene blue or malachite green) in the incubation media (Heisinger and Green, 1975; Kirchen and West, 1979), but more recently these have been deleted. Questions have been raised concerning possible deleterious effects of the anti-fungal agents, and possible interactions with the added toxicants or toxic effects of the anti-fungal agents themselves.

3.5 Embryonic Development

When fertilized eggs are removed from the females, and embryos are allowed to incubate in water or hatching media at 25°C, complete development usually requires 10 - 12 d. Although medaka embryos will tolerate a much wider

range of temperatures (7°- 38°C according to Kirchen and West, 1976), 25°C is most commonly used for incubations. According to Ogawa (1971), the temperature of incubation can have an influence on the number of vertebrae present in the resulting fry, with incubation temperatures less than 26°C resulting in an increase in the number of vertebrae. This was in agreement with an earlier study by Lindsey and Ali (1965), which included studies of the effects of "temperature breaks" during development. The "temperature breaks" occurred when embryos being reared at one temperature were abruptly moved to a new temperature and allowed to complete development at that second temperature. A temperature break differs from a "temperature pulse," in that for a temperature pulse, embryos are exposed to the second temperature for only a discrete amount of time, and are then returned to the original temperature to complete incubation. A decrease in the number of vertebrae present occurred if the temperature break occurred within 2 d postfertilization, but an increase in the vertebral count was seen if the temperature break occurred after day 6 postfertilization. Vertebral number is apparently unrelated to the size of the egg (Lindsey and Ali, 1971), although there is some evidence that the type and degree of variation seen in vertebral number may be heritable, to a degree (Ali and Lindsey, 1974). Lindsey and Arnason (1981) extended earlier data and information into a mathematical model in a attempt to determine the effects of various environmental influences on the vertebral count. The authors agree that the temperature effect may be a special case scenario that is not comparable to other environmental influences.

Complete discussions of the developmental stages observable in the medaka embryos are available in Kirchen and West (1976) and Rugh (1962). Kirchen and West (1976) note 36 stages, encompassing 264 h postfertilization at 25°C. A summary of these stages and times is included in Table 1.2. Other researchers have also attempted to define the development of the medaka in terms of discrete developmental stages, for example, Takeuchi (1987) describes 25 developmental stages centered around the development of cartilaginous and bony structures, while Ekanayake and Hall (1987; 1988) specifically studied the development of the vertebral bones. Observations and information on specific primordial germ cells and the origin of cell lines in medaka embryos can be found in Hogan (1978) and Gamo (1961).

Several studies have been done to determine the pattern of oxygen consumption, and the amount of oxygen required by medaka embryos during fertilization and throughout embryonic development (Nakano, 1953; Hishida and Nakano, 1954; Chulakasem, et al., 1989; Marty, et al., 1990a). The earliest of these studies (Nakano, 1953) established the overall pattern of oxygen consumption both of oocytes prior to and during fertilization, and of embryos throughout development at 25°C. Nakano (1953) determined that the oxygen consumption for oocytes before and during fertilization remains constant. However, the oxygen requirements of the developing embryo increase exponentially with time, from day 1 through day 9 of development (in this study, hatching occurred at day 9). Hishida and Nakano (1954) again demonstrated this

Table 1.2 Sequential stages of development observed in Japanese medaka embryos incubated at 25°C, from Kirchen and West (1976).

Stage Number	Time (h) post-fertilization	Developmental Events	Stage Number	Time (h) post-fertilization	Developmental Event(s)
1	0	oviposition	19	29	closed blastopore, optic vesicle
2	0.2	fertilization	20	33	anterior somites
3	0.8	germinal disc	21	36	pericardial cavity, optic cup
4	1.5	2 cell	22	40	optic lens, otocyst
5	2	4 cell	23	46	heartbeat
6	2.5	8 cell	24	52	clear circulation
7	3	16 cell	25	54	movement, otoliths
8	3.5	32 cell	26	56	retinal pigmentation
9	4	64 cell	27	62	pectoral fin bud
10	6	early high blastula	28	74	pink blood
11	9	late high blastula	29	84	vitellin veins sinus
12	12	flat blastula	30	102	urinary bladder
13	13	dorsal lip gastrula	31	121	pectoral fin movement
14	15	embryonic shield	32	128	liver rudiment
15	17	mid gastrula	33	144	swim bladder
16	20	late gastrula	34	168	jaw movement, yellow color
17	23	early neurula	35	200	spleen and mouth
18	26	late neurula	36	264	hatching

pattern of oxygen consumption, as well as the activity of various oxygen-requiring and oxygen-metabolizing enzymes. Marty, et al. (1990a) extended measurements through hatch and noted an increase in oxygen consumption in posthatch larvae, probably related to increased activity. The pattern of oxygen consumption does not appear to be affected by either incubation temperatures ranging from 23 to 29°C (Marty, et al., 1990a) or the ionic content (9 to 49 $\mu\text{S}/\text{cm}$) of low pH solutions (pH 4.2 to 5.6) (Chulakasem, et al., 1989). There are, however, variables which *have* been associated with alterations in the established patterns of oxygen consumption. These include (1) a decrease in the amount of oxygen used by embryos in low pH solutions (pH = 4.2 to 4.5, conductivity = 9 to 49 $\mu\text{S}/\text{cm}$) (Chulakasem, et al., 1989), and (2) an increase in oxygen consumption by embryos incubated at 20°C (Marty, et al., 1990a).

Hatching occurs at the completion of development, usually within 10 - 12 d postfertilization at 25°C, although the time of hatching is controlled by incubation temperature and light availability (Kirchen and West, 1976). Researchers have isolated the enzyme responsible for degradation of the chorion at the time of hatching (Ishida, 1944a, b; Yamagami, 1972, 1973, 1981; Yasumasu, et al., 1989 a, b). This enzyme is very heat stable, and will remain active following heat treatments of 50°C for up to 10 minutes. In comparison, the homologous enzyme isolated from rainbow trout becomes inactive after only 5 minutes at 50°C (Yamagami, 1973). Most recent studies report that the hatching enzyme is a zinc-protease dimer consisting of two isomers (HCE-1 and HCE-2), with a probable

combined molecular weight of 24 kDa (Yasumasu, 1989a, b). It is likely that several (yet unidentified) enzymes probably participate in the degradation of the chorion (Yasumasu, 1989b). Researchers can isolate the enzyme from solutions in which medaka have hatched, and it is thought that the enzyme(s) is (are) secreted from the embryo. The release of the enzyme from the embryo would likely be triggered by a signal for hatching, e.g. increased water temperature or salinity, or the onset of a light cycle after an extended period of darkness. When embryos were exposed to deoxycorticosterone, they exhibited a dose-dependent decrease in hatching time, leading the researcher to theorize that the release of the hatching enzyme may be stimulated by hormones (Cloud, 1981). Ishida (1944a) isolated discrete holocrine glands thought to be hatching glands along the outer surface of gill, eye, and mouth regions. These appear concurrent with the formation of melanin (day 3 or 4 in this experiment), and undergo growth and maturation until day 10, when the gland apparently disintegrates and releases the hatching enzyme.

3.6 Fry: Nutritional Recommendations

Fry will absorb their yolk sacs and begin needing exogenous food sources within 2 - 3 d posthatch. Either 24-h-old brine shrimp, mixed paramecium cultures, or mixed nematode cultures will provide adequate nutrition for the first 7 - 10 d posthatch. After this, 24- to 48-h-old brine shrimp, and fish flakes are adequate foods.

Fry will generally mature within 2 - 6 months (Kirchen and West, 1976; Hirshfield, 1980), depending upon the temperature of the water (22 - 25°C is optimum), the nutritional status, and the density of fish in the aquaria. There is not yet a standardized diet for the medaka; however, ongoing research is attempting to design such a diet (DeKoven, et al., 1991). Kirchen and West (1976) recommend the use of freshly hatched brine shrimp and flake food for the maintenance of adult medaka. Medaka are fed the brine shrimp every 48 hours, with flakes being fed daily. Commonly used flake foods include Tetramin (Tetra Werke) (Hamazaki, et al., 1985) and Stress Flakes (Cordon Oasis).

3.7 Basic Research Applications for the Japanese Medaka

The Japanese medaka has been used as a test organism in basic biological research for many years. A commentary and bibliography written by Briggs and Egami in 1959 included a list of potential advantages associated with the medaka and a bibliography of scientific articles relating to the medaka. This fish is indigenous to southeast Asia, and much of the early work done with it originated in that area of the world. Briggs and Egami's bibliography included both English and non-English citations dating back to 1913. The main objectives of the 1959 commentary and bibliography were (1) to advocate the use of the medaka in laboratories outside of Japan, and (2) to demonstrate the large amount of information already available on the species. Specific subject areas covered included studies on physiology, embryology, and genetics. A major shortcoming

noted by Briggs and Egami is that most of the articles were available only in Japanese.

In an effort to substantiate the claim that much information exists on the medaka and is readily available to researchers, the following paragraphs will briefly review several areas of research in which this species has been studied. It is by no means a comprehensive listing of available research, but it contains information on the primary areas of research that focus on the medaka. References that are in Japanese (but usually with English abstracts) will be noted as such in the reference section. This review primarily includes articles written in English, to provide the reader with easily accessible information on the biology of the Japanese medaka. Interestingly, many pertinent articles from various Japanese university journals, dating back to the late 1930's, are in English.

3.7.1 Endocrinology and reproductive biology

Adult medaka have been the subject of intense investigations on the reproductive cycle. Robinson and Rugh (1943) studied the anatomy of the female medaka reproductive tract and the photoperiodic nature of the ovulatory cycle. Studies that elucidated the effects of environmental cues on the medaka reproductive cycle include those by Yoshioka (1962, 1963), Urasaki (1976), and Egami (1954c, 1959 a, b). More in-depth studies of the physiology of the ovulatory cycle can be found in Hirose, 1971; Hirose and Hirose, 1972; Iwasaki, 1973; Iwamatsu, 1974; Iwamatsu and Fujieda, 1977; Iwamatsu, 1978; Iwamatsu,

1980; Iwamatsu and Ohta, 1981; Iwamatsu, 1983a; Iwamatsu and Onitake, 1983; and Hamazaki, et al., 1985. The overall morphology of developing oocytes has been investigated by M. Yamamoto (1964a), using electron microscopy, while Aketa (1954) specifically studied the polysaccharide-containing cortical alveoli within oocytes. Iwamatsu, et al. (1988a) provides an excellent review of the medaka ovulatory cycle and the stages of oocyte development.

Several researchers have focused their studies on the endocrine and exocrine glands of the medaka. The neurohypophysis, for example, can receive stimuli from the pineal gland and, in turn, send information to the gonads. Information is generally in the form of peptide or steroidal hormones. Both morphological and functional studies have been conducted on the glands and the different types of interactions between them. From a morphological viewpoint, Oota (1963) reported the results of an extensive investigation into the morphology of the hypothalamus and pituitary gland (both hypophysis and neurohypophysis) in the medaka. The hypothalamo-hypophysial system has been likewise investigated by Kasuga and Takahashi (1971). In a functional approach, Urasaki (1976) investigated the interactions between the eyes and pineal gland in relation to photoperiod, and Iwamatsu (1978) investigated the interactions between the pituitary gland, photoperiod and oocyte maturation. In a study designed to demonstrate the effects of stress on the thymus gland, researchers demonstrated the ability of deoxycorticosterone to damage the gland through the destruction of thymocytes (Ghoneum, et al., 1986).

The role of the steroid hormone, methyl dihydrotestosterone, in determining masculine secondary sexual characteristics in the medaka was investigated by Egami (1954 a, b). One study determined that the androgen can produce male-specific papillary processes on the anal fin-rays of female medaka in both transplanted and regenerating rays (1954a). The second study found that methyl dihydrotestosterone similarly influenced anal fin-rays on female medaka exposed to the steroid at temperatures above 18°C (1954b).

The lipoglycoprotein, vitellogenin, is known to be produced in the liver and transported to developing oocytes via the blood stream in amphibians, birds, and fishes. Vitellogenin has been isolated from the blood, liver, and ovaries of reproductively active females. In the medaka this protein has a molecular weight of about 420,000 mw (Hamazaki, et al., 1985). The production of this macromolecule probably accounts for the sexual dimorphism seen in the liver hepatocytes of breeding adult medaka (M. Yamamoto and Egami, 1974a).

3.7.2 Developmental biology and genetics

Researchers have focused investigations on the fertilized eggs of the medaka, from both the morphological and biochemical aspects of development. Iwamatsu (1983b) developed a method for removing the outer chorion of both fertilized and unfertilized medaka eggs. This technique was then available for both embryological and biochemical studies.

From a biochemistry standpoint, Hori (1958) found that medaka eggs (fertilized and unfertilized) were ideally suited for investigations into changes in the membrane potential associated with fertilization. T. Yamamoto (1939 and 1956) studied the physiology of fertilization in medaka embryos, describing the fertilization wave and the following breakdown of the cortical alveoli. Several researchers have used the medaka to study the fertilization wave, or calcium wave, which occurs within the egg concurrent with sperm entry or activation by physical agents (pricking or direct current) (T. Yamamoto, 1954; Maéno et al., 1956; Gilkey et al., 1978; Gilkey, 1983; Iwamatsu and Ito, 1986; Iwamatsu, et al., 1988b). An increase in the manganese content of medaka embryos during the first hour postfertilization has been reported (Hori and Iwaski, 1977). In an related area of study, Iwamatsu, et al. (1982) used mature medaka eggs and sperm to investigate the effects of forced polyspermy in the normally monospermic medaka. In other biochemistry-related studies, the medaka embryo has been used as a tool to study the presence of the neurotransmitter acetylcholine in early developmental stages (Fluck, 1978). These studies were later extended to develop techniques for the removal of embryonic cells and the study of intercellular communications in early embryonic cells (Fluck, et al., 1980; Fluck, 1982).

Medaka embryos and eggs have been used as tools to further the fields of developmental biology and embryology. For example, Ohta and Iwamatsu (1980, 1981) used medaka embryos to investigate the physiology and morphology of early embryonic cleavage, and the role of microtubules. Kageyama (1987) studied

embryonic cleavages in cells of older embryos (blastula and gastrula), as well as the projection of pseudopodia by these cells. Mizukami, (1987) concentrated primarily on the morula and blastula stages of the medaka embryos, demonstrating the effects of dissociation on the division patterns of the blastomere cells. The appearance of cells with either 1, 2, 3, or 4 nuclei varied with the time of development. Mizukami (1987) reports an increase in abnormally nucleated cells (2, 3, or 4 nuclei) as the embryo progresses from late morula to late blastula. Medaka embryos have also been used to study metamerism in mesodermal tissue (Martindale, et al., 1987). The induction, development and gradient of sexual differentiation of the testis in developing medaka embryos has been studied by Yoshikawa and Oguri, 1979. All of these studies have provided insight into complex questions in developmental biology.

Medaka embryos were used as the test species in investigations into the rhythmical movements of embryos (T. Yamamoto, 1930a, b, 1931, 1933a, b, 1934, 1936b, c, 1938, 1941b, c). According to T. Yamamoto (1931), the rhythmical contractions are apparent when the blastoderm covers about one-third of the egg. This stage is roughly equivalent to Kirchen and West's stages 14 and 15 (Kirchen and West, 1976). T. Yamamoto (1930a) describes the rhythmical contractions as thus;

Contraction waves arise at the edge of the blastoderm opposite the place where the embryonic shield is situated, and pass along the median surface of the blastoderm, finally disappearing on the opposite edge. . . .The time required for one contraction wave[s] at 25°C is about one minute and forty seconds. . . . The embryo as a whole rotates slowly in the direction of the contraction waves.

T. Yamamoto further concluded that these rhythmical waves were affected by temperature in at least two ways; (1) embryos exhibit an increased wave frequency with increased temperatures (T. Yamamoto, 1930b), and (2) embryos exhibit an increased wave amplitude with temperatures ranging from 7 to 15°C and a decreased wave amplitude when temperatures exceed 15°C (T. Yamamoto, 1933a). Although T. Yamamoto also concluded that these rhythmical waves could be affected by high concentrations of mono-, di- and trivalent cations, it should be noted that many of the cations used in the 1934 study are now known to be toxic to cells (e.g. Al^{3+} , Ba^{2+} , Sr^{2+} , and Li^{1+}) (T. Yamamoto, 1934). Thus the effects seen may not be directly related to the effect of cations, but instead may be related to metal toxicity. T. Yamamoto (1933c) also extended the studies of rhythmical movements of embryonic cells to pulsations in melanophores isolated scales from adult medaka. Matsuura (1934) and T. Yamamoto (1941c) both used medaka embryos to investigate the effects of KCl on the heart beat. They concluded that KCl can be toxic to the embryos by affecting the heart beat and rhythmical movements, and 72-h-old embryos are most resistant to KCl toxicity.

Medaka embryos have also been the objects of intensive microscopic investigations into the cellular events which occur during embryonic development. For example, M. Yamamoto (1964b) used medaka embryos and electron microscopy to examine the structure of the organelles and their progressive changes during development. Similar studies focused on the structure of the

periblast cells covering the embryo yolk (M. Yamamoto, 1965a) and the development of muscle cells and tissues (M. Yamamoto, 1965b).

Egami (1955) observed that estrogenized males could produce reproductive organs containing testis-ovum cells and tissue. This condition could be facilitated by a period of starvation followed by *ad libitum* feeding or X-ray exposure of the estrogenized males (Egami, 1955a, b). Estrogen-treatment or ovary-transplantation in male medaka will also cause the development of a female-type of urinogenital papillae (T. Yamamoto and Suzuki, 1955).

In the field of genetics, researchers have used the medaka as a test organism in various types of studies, including a dominant lethal mutation assay (Egami et al. 1983; Shimada and Egami, 1984a; Shima and Shimada, 1991), studies of specific genes and mutations (Oka, 1943; Lindsey and Ali, 1971; Yamamoto, 1975), and applications of molecular biology techniques (Ozato, et al., 1986; Inoue, et al., 1989). Hyodo-Taguchi and Egami (1985) also produced an inbred strain of medaka for studies concerned with the effects of chemicals on the DNA (i.e. cancer research and radiation biology). The development of melanophore cells under the influence of allelomorphic genes for body coloration has been studied in embryonic cells in tissue culture (Oka, 1943). Detailed information on the genetic strains and mutations documented and mapped for the medaka can be found in T. Yamamoto (1975). Lindsey and Ali (1971) cross-bred adult medaka to determine if the size of the egg produced was genetically controlled and if there was any correlation between the size of the egg and the

number of vertebrae in the resulting offspring. They concluded that wide variation was seen in both areas, and that the vertebral count did not appear to be dependent on or correlated with the size of the embryo. Molecular biology techniques now available to scientists have been applied to the medaka. For example, a group of researchers engineered transgenic medaka embryos which produced the chicken δ -crystallin gene (Ozato, et al., 1986; Inoue, et al., 1989).

3.7.3 Ecology

A few studies with more ecologically oriented approaches have also used the medaka as test species. Hirshfield (1980) used adult medaka as a model to study the energetics of the reproductive cycle. Awaji and Hanyu (1987) studied medaka that had been collected from the wild. This study focused on the life-history and annual reproductive cycle of the species in the wild. In a study that is more concerned with ethology, Ono (1936) conducted a series of experiments to determine various behavior patterns of medaka.

3.7.4 Carcinogenesis

Researchers frequently use the medaka as a test species for carcinogenic compounds. Such tests include the use of embryos, fry, and adults (Metcalf, 1989). The medaka has been proposed as an alternative and/or replacement species for the traditional rodent carcinogen models (Hatanaka, 1982; Hawkins, et al., 1988). This proposal is based on the apparent ability of the medaka to model

the responses of rodents when exposed to carcinogens (Hawkins, et al., 1986). Small aquarium fish are generally exposed to carcinogens through the water (Kyono and Egami, 1977; Klaunig, et al., 1984; Hawkins, et al., 1985; Walker, et al., 1985). Studies have also incorporated exposure via the diet (Hatanaka, et al., 1982), direct application of compounds to embryos (Maccubbin, et al., 1987), and microinjection of compounds into both embryos and free-swimming medaka (Metcalf, 1989). In at least one study, researchers were able to keep hepatocytes from DEN-generated (diethylnitrosamine) tumors viable in tissue culture (Mitani and Egami, 1980).

The use of aquarium fish (including medaka) as test species for carcinogens has several advantages over the traditional rodent models (Metcalf, 1989). Not only are the fish cheaper and easier to maintain, but researchers can also work with much larger numbers of fish than rodents in a test. Also, fish have a shorter latency period (period between exposure and the manifestation of carcinomas) than rodents. Finally, the small size of the fish allows for easy histopathological preparation. Several longitudinal slide sections of a single fish will allow for observation of all organs (Hawkins et al., 1988).

The carcinomas found in exposed medaka include cholangiolar neoplasms (Hawkins, et al., 1986), bile duct tumors (Maccubbin, et al., 1987), amelanotic melanomas (Hyodo-Taguchi and Matsudaira, 1984), and liver hepatocarcinomas (Klaunig, et al., 1984; Metcalf, 1989). A few of the compounds that have been shown to cause carcinomas in the medaka are shown in Table 1.3.

Table 1.3 Some compounds that have been shown to produce carcinomas and/or neoplasms in Japanese medaka.

Compounds	(Reference)
DEN ^a	(Kyona and Egami,1977)
MAM-Ac ^b	(Hawkins, et al.,1985)
MNNG ^c	(Hyodo-Taguchi and Matsudaira, 1984)
Benzo[a]pyrene	(Maccubbin, et al., 1987)
Aflatoxin B ₁	(Hatanaka, et al., 1982)
Aflatoxin G ₁	(Hatanaka, et al., 1982)
Sterigmatocysti	(Hatanaka, et al., 1982)

^aDEN = diethylnitrosoamine

^bMAM-Ac = methylazoxymethanol acetate

^cMNNG = N-methyl-N'-nitro-N-nitrosoguanidine

4. TOXICOLOGICAL APPLICATIONS OF THE JAPANESE MEDAKA

This section will focus on the toxicological studies that have used the Japanese medaka as a test species. The discussion will be broken down by the class of compound used in the test (e.g. metals and pesticides). Within each class, individual chemicals used in the tests will be discussed. Where applicable, there will be a discrimination of results obtained using different life stages (embryos, fry, adults).

4.1 Metals

Naturally occurring metals have long been known to cause toxic effects in living organisms; according to one source, "metals are probably the oldest toxins known to humans" (Goyer, 1986). Several heavy metals have been used in toxicity

tests with the Japanese medaka, including various formulations of mercury, cadmium and lead. Table 1.4 contains a summary of medaka studies using the metals.

4.1.1 Mercury

Inorganic mercury: When developing medaka embryos were exposed to the inorganic mercury salt, mercuric chloride (HgCl_2), Heisinger and Green (1975) reported teratogenic effects in the embryos and resulting fry that were exposed to concentrations ranging from 10 to 15 ppb HgCl_2 . The observed abnormalities included hemorrhaging, deterioration of blood vessels, and the loss of circulating blood vessels. Embryos exposed to HgCl_2 solutions with concentrations at or exceeding 20 ppb failed to hatch, with most dying prior to 16 d postfertilization. The researchers report a 16 d TL_m (median tolerance limit) value between 10 and 15 ppb for HgCl_2 .

Heisinger and Green (1975) also measured the ability of HgCl_2 to cross the outer chorion of the egg and to be concentrated in the embryo. Reported bioconcentration factors for HgCl_2 ranged from 1,590 to 2,677. This information indicates that the chorion is not impermeable to all compounds and does not necessarily confer protection to the developing embryo. Bowers III, et al. (1980) found that if the HgCl_2 -exposed embryos were treated with selenium *prior* to liver formation, the embryos exhibited high mortality as a result of HgCl_2 exposure.

Table 1.4 Summary of literature articles that have studied the toxic effects of metals on medaka.

Metal^a	Life-stage	Reference
HgCl ₂	embryos	Heisinger and Green, 1975
HgCl ₂ & SeO ₂	embryos	Bowers III, et al., 1980
HgCl ₂	fry	Hiraoka, et al., 1985
²⁰³ HgCl ₂	NA ^c	Oh, et al., 1986a ^d
²⁰³ HgCl ₂	NA ^c	Oh, et al., 1986b ^d
HgCl ₂	fry and adults	Sakaizumi, 1980b
HgCl ₂	fry	Slooff, et al., 1983
HgCl ₂	adult	Nagashima, et al., 1984
MMC	embryos	Dial, 1978a
MMC	embryos	Dial, 1978b
MMC	embryos	Sakaizumi, 1980a
MMC	fry and adults	Sakaizumi, 1980b
MMC	adult	Nagashima, et al., 1984
PMA	embryos, fry and adults	Akiyama, 1970
MEMC	embryos, fry and adults	Akiyama, 1970
"Uspulum"	embryos, fry and adults	Akiyama, 1970
CdCl ₂	embryos	Michibata, 1981a
CdCl ₂	embryos	Michibata, et al., 1986
CdCl ₂	embryos	Michibata, 1981b
CdCl ₂	embryos	Michibata, et al., 1987
Cd(NO ₃) ₂	fry	Slooff, et al., 1983
Cd ^b	embryos, fry and adults	Hiraoka and Okuda, 1983
CdCl ₂	fry	Hiraoka et al., 1985
CdCl ₂	fry	Hiraoka, 1985
Cd	embryos	Hiraoka and Okuda, 1984b

Metal ^a	Life-stage	Reference
¹⁰⁹ Cd ^b	embryos	Aoki, 1978
Cd ^b	adult and embryos	Hiraoka, 1980f
Zn ^b	embryos, fry and adults	Hiraoka and Okuda, 1983
ZnCl ₂	fry	Hiraoka, et al., 1985
MnCl ₂	fry	Hiraoka, et al., 1985
(NH ₄) ₂ Fe(SO ₄) ₂	fry	Hiraoka, et al., 1985
KCr ₂	fry	Hiraoka, et al., 1985
KCr ₂	embryo-fry	Slooff and Canton, 1983
NaAsO ₂	fry	Hiraoka, et al., 1985
Na ₂ SeO ₃	fry	Hiraoka, et al., 1985
NiCl ₂	fry	Hiraoka, et al., 1985
CoCl ₂	fry	Hiraoka, et al., 1985
Pb(CH ₃ COO) ₂	fry	Hiraoka, et al., 1985
NH ₄ VO ₃	fry	Hiraoka, et al., 1985
CuSO ₄	fry	Hiraoka, et al., 1985
AgNO ₃	fry	Hiraoka, et al., 1985
Pb(NO ₃) ₂	embryos	Cameron, et al., 1985a

^aMMC = methyl-mercuric chloride; PMA = phenyl mercuric chloride; MEMC = methoxy-ethyl mercuric chloride; "Uspulum" = mercury containing fungicide

^bchemical formulation not given by reference.

^dCitation refers only to an abstract.

If, on the other hand, the HgCl_2 -exposed embryos were treated with selenium *after* liver formation, the mortality rate of HgCl_2 -exposed embryos decreased, and the LC_{50} values increased. Such data is consistent with the role of selenium in protection from HgCl_2 toxicity. This data also appears to indicate that the liver is an important component in this protective phenomenon (Bowers III, et al., 1980).

In newly hatched fry, the LC_{50} for HgCl_2 , determined by Sakaizumi (1980b), varied by concentration and length of exposure, i.e. 1 d LC_{50} was 1400 $\mu\text{g Hg/L}$, and 5 d LC_{50} was 300 $\mu\text{g Hg/L}$. (Exposure medium for the fry was a 10% Yamamoto's salt solution [Sakaizumi, 1980b].) When Hiraoka, et al. (1985) examined the effect of one 24 h HgCl_2 exposure (in tap water) on 8-day-old medaka fry, he found an LC_{50} value between 10 and 100 $\mu\text{g Hg/L}$. These results appear to indicate either an increase in sensitivity to the effects of HgCl_2 in 8-day-old fry compared to newly hatched fry, or an increased sensitivity in fry exposed to HgCl_2 in tap water compared to salt media. When Slooff, et al. (1983) exposed 4-5-week-old fry to HgCl_2 for 48 h in acute toxicity tests, the resulting NOLC (no observed lethality concentration) was 0.43 ppm, and the LC_{50} was 0.59 ppm.

Sakaizumi (1980b) also exposed adult medaka to HgCl_2 , and, like the fry, the LC_{50} varied by concentration and length of exposure, i.e. 3 d LC_{50} was 1000 $\mu\text{g Hg/L}$, and 5 d LC_{50} was 400 $\mu\text{g Hg/L}$. Most fish in this study apparently died of suffocation of the gill tissues by excessive mucus secretions. An added variable in these tests could have been overcrowding. The author reports that 10 adults were maintained in 2 L glass vessels that received water changes every 3 d. Exposure

of fish to such conditions for long periods of time (in this case 80 d) could lead to stress and potential disease susceptibility. When Nagashima, et al. (1984) exposed adult medaka to HgCl_2 for 24 and 48 h periods, they reported a 48 h LC_{50} value of 700 ppb HgCl_2 . The test conditions and fish density in Nagashima, et al. (1984) were identical to Sakaizumi (1980b). Adult medaka accumulated 41 - 58 ppm HgCl_2 from the aqueous environment during 48 h exposures (Nagashima, et al., 1984). This value did not differ for MMC exposures, however, HgCl_2 was excreted at a faster rate than was MMC (Nagashima, et al., 1984).

In both Heisinger and Green's (1975) and Sakaizumi's (1980b) studies, the median tolerance limit (TL_m) equates to the LC_{50} , although consistent days are not given for direct comparison. Thus the embryo appears to be much more sensitive (16 d $\text{TL}_m = 10 - 15 \mu\text{g/L}$) to HgCl_2 toxicity than either the fry (5 d $\text{LC}_{50} = 300 \mu\text{g Hg/L}$) or adults (5 d $\text{LC}_{50} = 400 \mu\text{g/L}$, and 26 d $\text{LC}_{50} = 400 \mu\text{g/L}$). Sakaizumi (1980b) concluded that the fry were more sensitive to HgCl_2 than the adult medaka. The comparison between fry and adults is much stronger than a comparison of either fry or adults to embryos, since, in this case, both fry and adult exposures were carried out within one laboratory and test.

When medaka (life stage not given) were exposed to $^{203}\text{HgCl}_2$ in situations which more closely resembled environmental conditions (HgCl_2 in association with humic acid), Oh, et al. (1986a, abstract only) reported that increasing amounts of humic acid caused decreasing amounts of mercury uptake in the fish. A similar trend with HgCl_2 in solutions of varying pH was also reported; the

bioconcentration of the mercury appears to decrease as the pH of the solution increases from pH 5 to 9 (Oh, et al., 1986b, abstract only).

Organic mercury: Several studies used medaka to examine the effects of organic mercury compounds. These compounds will cross cell membranes more easily than inorganic mercury compounds; thus it was expected that the effective concentrations (TL_m and LC_{50}) would be less for organic mercury compounds.

Dial (1978a, b) allowed newly fertilized embryos to incubate throughout development in solutions of 40, 60, and 80 ppb methyl mercuric chloride (MMC). In this study, incubation was at 21°C; thus embryonic development lasted for up to 25 d. Within 10 d postfertilization, there was 45% mortality, in embryos exposed to 80 ppb MMC. None of the remaining embryos hatched by day 25: effectively a 100% mortality. In the 60 ppb MMC group, there was only 15% mortality by day 10, and 94% mortality by day 20. For embryos in 40 ppb MMC, there was only 5% mortality by day 10, and 63% by day 20.

Abnormalities seen in the embryos included abnormal or reduced retinal pigmentation, abnormal incorporation of the heart, slowed development (observed by 3 d postfertilization), and gross morphological abnormalities in head and body development. Light and electron microscope observations included focal cytoplasmic lesions in both brain cells and cells of developing retina. The developing vascular system also appeared to be heavily affected by MMC. All exposed embryos that survived to hatch exhibited abnormal swimming behavior.

These results are in close agreement to those of Weis and Weis (1977), where embryos of a close relative of the medaka (the gulf killifish, *Fundulus heteroclitus*) were exposed to MMC.

Even the lowest concentration tested in this study (Dial, 1978a, b) resulted in a mortality rate greater than 50%. In a more detailed study, Sakaizumi (1980a) investigated the dose response and stage sensitivity of medaka embryos to MMC. When embryos were exposed to MMC in both a salt medium (10% Yamamoto's solution) and in deionized water, the LC_{50} differed depending upon the incubation media. In the salt media, the LC_{50} was 30 $\mu\text{g Hg/L}$, while the LC_{50} for embryos in deionized water was 55 $\mu\text{g Hg/L}$. Stage sensitivity also varied depending upon the incubation media. In the salt media, all embryonic stages appeared equally sensitive to the effects of MMC, while day 3 embryos were most sensitive when exposed to MMC in deionized water. Further experiments appeared to indicate that the NaCl , CaCl_2 , and MgCl_2 were responsible for the increased sensitivity of embryos to MMC in the salt media. While the movement of MMC across the chorion was greatest in embryos exposed in deionized water, most of the MMC was deposited in the yolk. Conversely, CaCl_2 reduced the overall MMC uptake. More MMC was deposited in the embryo and very little in the yolk sac when embryos were exposed in salt media (Yamamoto's solution or CaCl_2 solution).

Table 1.5 contains a comparison of the LC_{50} 's reported in Sakaizumi (1980a, b) and Nagashima, et al. (1984) by life-stages. Although the total incubation time for the embryos is not given, incubation at 23°C should result in a

Table 1.5 Reported LC_{50} data for medaka exposed to MMC at various life-stages, from Sakaizumi, 1980a, b and Nagashima, et al., 1984^a. (MMC = methyl mercuric chloride).

Life-stage	Conditions	Time and index	MMC $\mu\text{gHg/L}$
Embryos	salt media	total incubation LC_{50}	30
Embryos	deionized water	total incubation LC_{50}	55
Fry	salt media	1 d LC_{50}	120
Fry	salt media	4 d LC_{50}	50
Adults	dechlorinated tap water	1 d LC_{50}	120
Adults		48 h LC_{50}	155
Adults		18 d LC_{50}	80
Adults		27 d LC_{50}	40
Adults		41 d LC_{50}	20
Adults		51 d LC_{50}	10

^a LC_{50} for adult 48 h exposure is from Nagashima, et al., 1984. All other data from Sakaizumi, 1980a and 1980b. The LC_{50} value of 155 from Nagashima, et al. (1984) is reported as 155 ppb MMC. All other data is reported as ppb Hg.

16 - 25 d incubation period. Therefore, the LC_{50} 's reported for the embryos probably reflects this incubation period. The comparable LC_{50} for adult medaka would be between 40 and 80 $\mu\text{g Hg/L}$, indicating that the embryo is more sensitive than the adult. The author (Sakaizumi, 1980b) concludes that fry are more sensitive than adults. But because fry survive only up to 10 d without an exogenous food source, using this data for comparisons of embryo and fry may not be valid.

In addition to determining the 48 h LC_{50} for adults exposed to MMC, Nagashima, et al. (1984) also studied the accumulation and excretion of MMC and $HgCl_2$. Fish accumulated both mercury compounds equally, but excreted the MMC much more slowly. However, fish fed $HgCl_2$ - and MMC-spiked food did not accumulate it equally. The authors report accumulation with MMC-spiked food, but no accumulation with $HgCl_2$ -spiked food (Nagashima, et al., 1984).

The effects of MMC on the function of sperm and spermatogenic tissue in male adult medaka has been studied in conjunction with gamma irradiation (Sakaizumi and Egami, 1980). MMC treatment ($40 \mu g$ Hg/L) amplified one effect of gamma irradiation (reduced fertilization rate) and delayed recovery to the normal fertilization rate.

Three other organic mercury compounds, phenylmercuric acetate (PMA), methoxyethylmercuric chloride (MEMC), and the commercial fungicide "Uspulum" (which contains approximately 2.5% Hg as MEMC) were tested on medaka by Akiyama (1970). Results for adult exposures, expressed as 24 hour TL_m values, are shown in Table 1.6.

4.1.2 Cadmium

Numerous researchers have studied the effects of cadmium on various stages of developing medaka embryos (Michibata, 1981a, b; Slooff, et al., 1983;

Table 1.6 Summary of medaka TL_m values reported for 3 organic mercury compounds, from Akiyama, 1970. (PMA = phenylmercuric acetate; MEMC = methoxyethylmercuric chloride; Uspulum = commercial fungicide).

Compound	Life-stage	24 hr TL _m mg/L
PMA	Adult	0.21
	Fry	0.030
	24 hr-old Embryo	0.235
	5 day-old Embryo	0.263
	9 day-old Embryo	0.180
MEMC	Adult	1.71
	Fry	1.48
	24 hr-old Embryo	0.50
	5 day-old Embryo	1.30
	9 day-old Embryo	0.55
"Uspulum"	Adult	3.53
	Fry	1.26
	24 hr-old Embryo	0.72
	5 day-old Embryo	1.59
	9 day-old Embryo	1.31

Hiraoka and Okuda, 1983, 1984b; Hiraoka, et al., 1985, Hiraoka, 1985; Michibata, et al., 1986, 1987). When blastula-stage embryos were exposed for 1 h to varying concentrations of CdCl₂ (10, 50, 100, 150, 200 and 300 mg Cd/L), the highest concentration caused just less than 40% mortality within 5 d of exposure. However, when earlier-staged embryos (4 cell through morula stages) were exposed to 200 mg Cd/L for 1 h, these embryonic stages suffered 90 to 100%

mortality within 5 d. Thus early embryos showed an increased sensitivity to cadmium, with sensitivity decreasing with the age of the embryo. According to Kirchen and West (1976), the stages noted as early stages in this study should occur within 4 - 6 h postfertilization. Michibata, et al., 1987 also provided evidence that cadmium is bioconcentrated within the embryos, but that it is released from the embryo when the embryo is transferred to clean solutions. Conflicting data is reported by Aoki (1978, abstract only), in which the fry were reported to be more sensitive to cadmium than the embryos; although exposure regimes were not given.

In an experiment designed to determine the TL_{50} for chronically exposed embryos, Michibata (1981a) exposed blastula-stage embryos to varying concentrations of $CdCl_2$ throughout development. The 96 hr TL_{50} (= 3 d LC_{50}) for $CdCl_2$ was estimated to be 20.5 mg Cd/L. The highest concentration tested, 50 mg Cd/L, caused 100% mortalities. Further studies determined that most of the cadmium that entered the embryos was associated with the chorion, and also, that most movement across the chorion occurred within the first hour following exposure.

As with other fish species (Brown, 1968; McCarty, et al., 1977), medaka embryo sensitivity to cadmium appears to be related to the hardness of the water in which the exposure is conducted. Michibata (1981b) reported a decrease in toxicity in embryos exposed to $CdCl_2$ in hard water, and a higher toxicity in soft water. In fact, he reported a linear relationship between water hardness and the

96 h TL₅₀ for cadmium. Increased hardness resulted in an increased 96 h TL₅₀ value. Michibata (1981b) theorized that this was the result of a lower association of cadmium with the chorion in harder waters, leading to a decrease in the amount of cadmium being absorbed into the embryo. A later study of cadmium content of embryos supports this theory (Michibata, et al., 1986). The two ions which are responsible for the hardness of water are calcium and magnesium. By exposing medaka embryos to CdCl₂ in either high magnesium or high calcium solutions, these researchers were able to determine that the protective ion was calcium. Calcium prevents cadmium-chorion interactions, thereby protecting the embryos by decreasing the cadmium uptake and subsequent toxicity.

In 48 h acute toxicity tests, Slooff, et al. (1983) reported NOLC and LC₅₀ values for 4 - 5-week-old medaka fry that were exposed to varying concentrations of Cd(NO₃)₂. The reported NOLC was 45 ppm and the LC₅₀ value was 73 ppm (Slooff, et al., 1983).

Several studies have focused primarily on the developmental effects of exposing of embryos and adults to cadmium (Hiraoka, 1980f, abstract only; Hiraoka and Okuda, 1983, 1984b). Embryos appear to be more sensitive than adults to the occurrence of vertebral abnormalities. This not entirely unexpected, since embryonic exposure occurs during the period of rapid cell division and growth. Conversely, adults experience very little overall growth relative to embryos. Hiraoka and Okuda (1983) determined that embryonic exposure of 0.01 mg Cd/L, for the first 2 - 3 d postfertilization did not result in significantly higher

rates of deformity, damaged vertebrae or damaged centra. However the overall length of these fish at adulthood was significantly shorter than controls. These results conflict with a later study by the same authors (Hiraoka and Okuda, 1984b) where significantly higher rates of abnormal centra are reported for medaka embryos exposed to 0.01 mg/L Cd under the same conditions. In the later study (Hiraoka and Okuda, 1984b), observations for abnormal centra were made at 4 weeks postfertilization, however, in the earlier study (Hiraoka and Okuda, 1983) they were not made until 30+ d posthatch. The authors do not address any possible reasons for the lack of reproducibility between these studies.

When embryos were exposed to 0.001 and 0.01 mg Cd/L throughout embryonic development and into adulthood (Hiraoka and Okuda, 1983), the overall body length of the adult fish was again significantly shorter than controls. The incidence of fish with damaged vertebrae, and centra, was significantly higher than controls for fish at both concentrations. Adult medaka exposed to 0.01 and 0.05 mg Cd/L for 30 d showed no significant difference in body length, incidence of deformity or incidence of damaged vertebrae or centra.

Several sources agree that the period for maximum sensitivity of fry to cadmium toxicity is 7 - 10 d after hatching (Fuji and Sugiyama, 1983; Hiraoka and Okuda, 1984b; Hiraoka, 1985), but these studies do not compare cadmium sensitivity in fry and embryos. Hiraoka, et al. (1985) reported a 24 h LC_{50} value between 0.1 and 1.0 mg Cd/L for 8 day-old fry exposed to $CdCl_2$; thus it would

appear that fry may be more sensitive to cadmium than are the embryos (embryo 96 hr LC_{50} = 20.5 mg Cd/L according to Michibata, 1981a) .

4.1.3 Zinc

Hiraoka and Okuda (1983) examined the developmental effects of zinc exposure on medaka embryos and adults. Embryos exposed to solutions of 1 and 3 mg Zn/L had significantly shorter body lengths as adults, but not significantly higher incidences of deformities or damaged centra. There was a significantly higher incidence of damaged vertebrae in embryos exposed 1 mg Zn/L. If exposure was initiated in the embryo and continued into adulthood (0.3 and 0.5 mg Zn/L), both groups exhibited significantly shorter bodies and higher incidences of vertebrae damage. Only the embryos exposed to 0.5 mg Zn/L had a significantly higher incidence of damaged centra. Much like the cadmium experiments (Hiraoka and Okuda, 1983), embryos exposed to zinc for up to 2 - 3 d postfertilization did not show any significant changes in body length, deformity rate, or incidences of damaged vertebrae or centra.

Hiraoka, et al. (1985) exposed 8-day-old medaka fry to varying concentrations of $ZnCl_2$ and reported a 24 h LC_{50} value between 1.0 and 10 mg Zn/L. No studies examining the effects or sensitivity of medaka embryos were found.

4.1.4 Other metals

Hiraoka, et al. (1985) examined the sensitivity of 8-day-old medaka fry to a total of 14 different metals. The fry were exposed in tap water for a period of 24 h. The degrees of fry sensitivity were divided into four levels. Table 1.7 contains the three levels of sensitivity. These groupings appear to be based not only on the percent lethality at various concentrations, but also statistical comparisons (Fishers Exact Test) between test groups. Embryos were exposed to various concentration ranges of the metals. Tested concentration ranges are also given in Table 1.7.

Teratogenic effects of lead nitrate ($\text{Pb}(\text{NO}_3)_2$) to developing medaka embryos were reported by Cameron, et al. (1985a). Exposure of 100 ppm $\text{Pb}(\text{NO}_3)_2$ throughout development led to 100% mortality during early development; however, a lower concentration of 3.125 ppm $\text{Pb}(\text{NO}_3)_2$ led to no observable effects (NOEC). Abnormalities in embryos exposed to lead concentrations of 6.25 to 50 ppm included abnormally shaped heads, malformed hearts, abnormally shaped and pigmented eyes, spinal curvatures, and abnormal swimming behavior.

Table 1.7 Reported levels of sensitivity of medaka fry to 14 different metals, with the reported concentration ranges. Data is from Hiraoka, et al., 1985.

Level 1	Concentration Range (ppm)	Level 3	Concentration Range (ppm)
Ag	0.1, 1.0, 10.0	Se	1.0, 10.0, 100.0
Hg	0.1, 1.0	V	1.0, 10.0, 100.0
Cu	0.1, 1.0, 10.0	Fe	1.0, 10.0, 100.0, 1000.0
Cd	0.1, 1.0, 10.0	Co	1.0, 10.0, 100.0
Level 2	Concentration Range (ppm)	Level 4	Concentration Range (ppm)
Zn	0.1, 1.0, 10.0	Mn	10.0, 100.0, 1000.0
As	1.0, 10.0, 100.0		
Cr	1.0, 10.0		
Ni	1.0, 10.0		
Pb	1.0, 10.0		

In a study investigating the sensitivity of eleven different test species to eight chemical compounds, Slooff and Canton (1983) report a NOL(E)C ("the highest concentration at which no effect [NOEC] or lethality [NOLC] was observed") for KCr_2 in medaka embryos and fry. Embryos were exposed; exposure continued for 40 d. The reported NOL(E)C for mortality was 10 ppm KCr_2 , and, for hatching growth, 100 ppm KCr_2 . This differs slightly from Hiraoka, et al. (1985) in which the LC_{50} for KCr_2 was reported as 1 - 10 ppm.

4.2 Pesticides

Researchers have used the Japanese medaka as a test species in studies investigating the effects of various pesticides. Table 1.8 provides a summary and cross-reference for these articles. These studies will be classified by the type of pesticide: organophosphate, carbamate, organochlorine, fumigants and herbicides. As it is impossible to discuss experiments using these pesticides without also considering investigations concerning the toxicity of chemical intermediates, by-products and degradation products, these will be included in the discussion where applicable.

4.2.1 Organophosphate insecticides

Organophosphate pesticides share a common mode of action in biological tissues. The compounds act as inhibitors of the enzyme acetylcholinesterase and cause an accumulation of the neurotransmitter acetylcholine in the neural synapse. Acetylcholine normally acts to transmit the neural impulse across gap junctions, and this transmission is attenuated by the degradation of acetylcholine by acetylcholinesterase. The inhibition of acetylcholinesterase results in the continual stimulation of the receptors on the neuron or muscle. This leads to involuntary muscle twitching--the symptom most often associated with organophosphate poisoning (Murphy, 1986). Several studies have determined that teleosts

Table 1.8 Summary of literature articles using medaka to test the effect of pesticide exposure.

COMPOUND	CLASS ^a	LIFE-STAGE ^b	REFERENCE
Fenitrothion	OP	embryo, fry and adult	Hiraoka, 1980a ^c
Fenitrothion	OP	embryo through adult in F ₂ generation	Hiraoka, 1981 ^c
Fenitrothion	OP	embryo and fry	Hiraoka and Okuda, 1983
Fenitrothion	OP	embryo	Hiraoka and Okuda, 1984a
Fenitrothion	OP	embryo	Hiraoka and Okuda, 1984b
Fenitrothion	OP	embryo through adult	Takimoto, et al., 1984a
Fenitrothion	OP	embryo through adult	Takimoto, et al., 1984b
Fenitrothion and degradation products	OP	embryo and adult	Hiraoka, et al., 1989
Malathion	OP	embryo	Solomon and Weis, 1979
Malathion	OP	embryo	Solomon, 1977a, 1977b ^c and 1977c
Parathion	OP	embryo	Solomon and Weis, 1979
Parathion	OP	embryo	Solomon, 1977a, 1977b ^c , and 1977c
Dimethoate	OP	embryo-fry	Slooff and Canton, 1983
Carbaryl	CBMT	embryo	Solomon, 1977a, 1977b ^c , and 1977c
Carbaryl	CBMT	embryo	Solomon and Weis, 1979
β -Hexachlorocyclohexane (isomer of Lindane)	non-insecticidal isomer of OCl	embryo and fry	Wester and Canton, 1986
DDT	OCL	embryo, fry and adult	Takimoto, et al., 1984b

COMPOUND	CLASS ^a	LIFE-STAGE ^b	REFERENCE
DDT	OCL	embryo	Solomon, 1977c
2,4,5-Trichlorophenoxyacetic Acid (2,4,5-T)	HC	embryo	Schreiweis and Murray, 1976
2,3,7,8-Tetrachlorodibenzo- <i>p</i> -dioxin (TCDD)	contaminant in 2,4,5-T	embryo	Wisk and Cooper, 1990
2,4-Dinitrophenol (2,4-D)	HC	embryo	Waterman, 1939
2,4-Dinitrophenol (2,4-D)	HC	embryo	Ishihara, 1956
2,4-Dinitrophenol (2,4-D)	HC	embryo	Ishida, 1951
Dinitro- <i>o</i> -cresol	HC	embryo-fry	Slooff and Canton, 1983
Pentachlorophenol (PCP)	HC,INS, FNG	embryo-fry	Slooff and Canton, 1983
Pentachlorophenol (PCP)	HC, INS, FNG	fry	Slooff, et al., 1983
Paraquat	HG	embryo	Murray and Schreiweis, 1977 ^c
Methyl Bromide	FMGTS	embryo, fry and adult	Wester, et al., 1988

^aOP = organophosphate, OCL = organochlorine, CBMT = carbamate, FMGTS = fumigants, HC = herbicide, INS = insecticide, FNG = fungicide

^bLife-stage indicates the period during which exposure occurred.

^cCitation refers only to the abstract. In some cases articles were available, but only the abstracts were in English.

(specifically medaka) have acetylcholine and active acetylcholinesterase (Fluck, 1978; Fluck et al., 1980; Fluck, 1982; Takimoto, et al., 1984a), thus the medaka are susceptible to the effects of organophosphate insecticides.

Pesticides used in agricultural applications pose a threat to aquatic systems when they enter streams either through field run-off or aerial applications. The medaka has been used extensively in Japan to examine the effects of the organophosphate pesticide fenitrothion (= MEP = Sumithion® = O,O-dimethyl O-(3-methyl-4-nitrophenyl) phosphothioate [Takimoto, et al., 1984a]). Fenitrothion emulsions have been applied aerially on rice paddies in Japan to control pests. Since these same rice paddies are part of the natural habitat for the medaka, the species has been used to determine the effects of the pesticide to aquatic organisms. These studies have been extended and supplemented by those of U.S. researchers, who have used the medaka to study the teratogenic effects of several other organophosphates, including malathion and parathion (Solomon, 1977a, 1977b, abstract only; 1977c; Solomon and Weis, 1979) and dimethoate (Slooff and Canton, 1983).

Fenitrothion: The toxic effects of fenitrothion to medaka have been reported by Takimoto, et al. (1984b). Twelve different life-stages (ranging from embryos that were less than 24-h-old to sexually inactive adults) were exposed to varying concentrations of fenitrothion.

Embryo exposures were both pulse and continual: one group was exposed to the fenitrothion solution throughout embryonic development, while other groups were exposed for 3 d, beginning on days 0, 3, and 5 postfertilization. Embryos exposed to 5 ppm fenitrothion throughout development exhibited reduced hatching. Increased incubation time and mortality were evident in embryos exposed to 2 ppm fenitrothion. Embryos exposed to pulse doses of 10 ppm fenitrothion beginning on days 0 and 3 postfertilization experienced 100% mortality, whereas those groups in which exposure was initiated on day 5 experienced a lower mortality rate (75%).

Postlarval fry (at 10 d posthatch) were found to be the most sensitive to the toxic effects of fenitrothion. Postlarval medaka exposed for 96 h (beginning d 10 posthatch) were found to have an estimated LC_{50} value of 2.4 ppm fenitrothion (Takimoto, et al., 1984b). This stage follows yolk sac absorption, thus the fry are consuming external food. Takimoto et al. (1984b) measured cholinesterase activity for all exposure and control groups, and found that embryos have the lowest amount of acetylcholinesterase, accounting for their decreased sensitivity to organophosphates. The chorion does not appear to block the uptake of fenitrothion nor to prevent its bioaccumulation (Takimoto, et al., 1984a). Sensitivity increases as the yolk sac is absorbed and the nervous system completes development (Takimoto, et al., 1984b).

Various developmental abnormalities have been associated with fenitrothion exposure. For example, exposure of embryos to 0.4 ppm fenitrothion

through the retinal pigmentation stage resulted in a significant number of fry with damaged centra by the second week posthatch (Hiraoka and Okuda, 1984b).

These abnormal centra were observed through 16 weeks posthatch. The abnormal centra are considered developmental abnormalities since exposure was limited to early embryonic stages. Damaged vertebrae have also been reported in embryos and fry exposed to fenitrothion (embryo exposures = 0.1 - 4 ppm; fry exposures = 0.5 - 2 ppm) (Hiraoka, 1980a, abstract in English; Hiraoka and Okuda, 1983;). Exposure of adults to fenitrothion levels of 1 - 4 ppm did not result in a significantly greater number of adults with either damaged vertebrae or centra (Hiraoka, 1980a, abstract in English; Hiraoka and Okuda, 1983), although there did appear to be a trend toward increased abnormal vertebrae in the exposed adults (Hiraoka and Okuda, 1983).

There are some inconsistencies between two of Hiraoka and Okuda's studies (1983, 1984b). In the earlier paper (Hiraoka and Okuda, 1983), no significant centra-damage is reported, although it was a recorded endpoint. This conflicts with the later paper (Hiraoka and Okuda, 1984b) where the same type of embryonic exposure resulted in a significantly higher percentage of fry with damaged centra. Also, the authors do not report any data for damaged vertebrae in the later paper (Hiraoka and Okuda, 1984b). Another inconsistency that is not addressed is the effect of fenitrothion on the average length of the fry. In 1983, Hiraoka and Okuda report that most fenitrothion concentrations (0.1 - 4 ppm) resulted in fry with a significantly shorter mean body length. There was no

apparent difference in the mean body lengths reported in Hiraoka and Okuda, 1984b, with an equivalent dose of fenitrothion. The date of sampling could account for this inconsistency. Fry were sampled through 16 weeks posthatch in the 1984 study (Hiraoka and Okuda, 1984b), but the age at sampling is not given for fry in the 1983 study (Hiraoka and Okuda, 1983). Hiraoka has published extensively in this area, concentrating on vertebral abnormalities as an indicator of water pollution, however several of the papers have only been published in Japanese (Hiraoka, 1980c, d, e; Hiraoka et al., 1987). Still other articles are in Japanese, but with English abstracts (Hiraoka, 1980a, b; Hiraoka, 1981).

Hiraoka also monitored the offspring of medaka that had been exposed to fenitrothion for one week as newly hatched fry (Hiraoka, 1981, abstract in English). He reported no difference in vertebral abnormalities between controls and the F_2 generation, thus the abnormalities do not appear to be fixed in the gene line. This would also indicate that if an indigenous population of medaka was found to have an increased frequency of vertebral abnormalities, it would be a result of exposure during the embryonic or early fry life stages.

This concept was put to use in a field situation in Japan (Hiraoka, 1980b, abstract in English; Hiraoka and Okuda, 1984a). Newly fertilized embryos were placed in streams before and during aerial application of fenitrothion to surrounding crops. These eggs were then returned to the laboratory, incubated until hatching and then evaluated for the incidence of deformed or centrum-damaged fish. The fry resulting from embryos exposed at two different stream

sites showed significantly higher incidences of deformities and centrum-damage compared to laboratory controls and stream controls.

In an attempt to determine the distribution, fate and bioconcentration factor of the pesticide, various life-stages of medaka were exposed to C¹⁴-labeled fenitrothion, followed by a recovery period in clean water (Takimoto, et al., 1984a). Immediately following exposure, approximately 90% of the C¹⁴ label was in the form of fenitrothion, and 10% in the form of metabolites (demethy-fenitrothion and 3-methyl-4-nitrophenyl-glucuronide). Excretion of fenitrothion by the embryos was detected beginning after one day in clean water. No metabolites were detected in the water from the embryos. The ability of the embryo to metabolize the fenitrothion to glucuronide conjugates appeared to increase marginally following 3 d recovery. This may be due to the increased age of the embryo. The maximum bioaccumulation factor reported for the embryo at 0.1 ppm fenitrothion was 155. When the embryos were examined for the location of the C¹⁴, the majority of the radioactive label was located inside the embryonic chorion.

The percentage of radioactive label in the yolk sac fry consisting of fenitrothion was considerably lower than the embryo (approximately 30%), with the remaining 70 percent being glucuronide conjugates and demethy-fenitrothion. Thus, the yolk sac fry metabolized more of the fenitrothion than the embryo. Yolk sac fry also excreted fenitrothion and metabolites. The calculated maximum bioaccumulation factor for this life stage was 173, slightly higher than the embryos.

This corresponds to a slightly higher percentage fat content for the yolk sac fry compared to the embryo (5.2% compared to 4.3%). The majority of the radioactive label was associated with the yolk sac and internal organs (Takimoto, et al., 1984a), where most of the adipose tissue is located.

The relative percentage of glucuronide products and demethy-fenitrothion decreases throughout the postlarval, juvenile, and adult life stages. However, the excretion of glucuronides increases at all of these life stages during the period of recovery (2 - 3 d). Adults have the highest bioaccumulation factors (male = 520, female = 540), corresponding to the life stages with the highest percentage of fat content (adult male = 10.0%, adult female = 11.4%). In adults, most of the radioactive label was associated with the internal organs and the ovaries of the females (Takimoto et al., 1984a). Fertilized eggs from exposed adults were found to contain fenitrothion (bioaccumulation factor = 224), thus this may be a route of excretion for adult females.

Studies have also demonstrated the toxic effects of the degradation products of fenitrothion by sunlight in an alkaline solution (pH 10 and pH 14) (Hiraoka et al., 1989). Significant mortalities were seen at concentrations above 2.0 ppm in untreated fenitrothion and pH 10- and pH 14-treated fenitrothion compared to controls. The mean length of the fry from embryos treated with pH 10-treated fenitrothion was significantly shorter than untreated fenitrothion-exposed fish; a significantly lower mean body weight was also seen in the pH 10-treated group. While the degradation products appeared to affect some

groups, the effects are not clearly concentration-dependent and comparisons between groups are not always clear.

As can be seen in this series of papers, the medaka has been a useful tool in demonstrating some of the toxic effects of the insecticide fenitrothion. This research was extended in the U.S. to study the teratogenic effects of several other organophosphate insecticides: parathion, malathion, and dimethoate.

Parathion and malathion: The organophosphate insecticides parathion and malathion have been shown to cause abnormal circulatory development in medaka embryos exposed to 0.5 - 40.0 ppm of either compound throughout development (Solomon, 1977a; 1977b, abstract only, 1977c; Solomon and Weis, 1979). The reported ED₅₀ (effective dose for 50% abnormal individuals) reported for parathion was 2.0 ppm and 10.0 ppm for malathion. Circulatory abnormalities included poorly developed rudimentary hearts, blood clots and areas of edema. When malathion was combined with carbaryl (a carbamate insecticide), the percentage of abnormalities reported was greater than additive (Solomon, 1977a; 1977b, abstract only, 1977c; Solomon and Weis, 1979).

Dimethoate: Slooff and Canton (1983) exposed medaka embryos to the organophosphate insecticide dimethoate for 40 d (embryo through fry) and reported the NOL(E)C. The NOL(E)C for mortality was reported as 0.32 ppm dimethoate and 100 ppm for hatching growth.

4.2.2 Carbamate insecticides

Carbamate insecticides have the same mode-of-action as organophosphates (inhibition of acetylcholinesterase), however, the reaction of the carbamate with the acetylcholinesterase is short-lived in comparison to the organophosphates (Murphy, 1986).

Carbaryl: The teratogenic effects of the carbamate, carbaryl, to medaka embryos have been reported by Solomon and Weis (1979) and Solomon (1977a, 1977b, abstract only, 1977c). The resulting circulatory abnormalities were similar to those seen in embryos exposed to organophosphates, and the ED₅₀ was comparable to that of parathion (carbaryl = 2.5 ppm; parathion = 2.0 ppm). In embryonic stage studies, there appeared to be a decrease in the severity and number of circulatory abnormalities with the increased age of the embryo at the onset of exposure (Solomon and Weis, 1979).

4.2.3 Organochlorine insecticides

Several types of compounds are classified as organochlorine insecticides. These include cyclodienes, hexachlorocyclohexanes and chlorinated ethane derivatives (Murphy, 1986). While these compounds are not as acutely toxic as the organophosphates and carbamates, they tend to persist in the environment and bioaccumulate as they move up the food chain (Murphy, 1986).

Hexachlorocyclohexanes: Lindane is a hexachlorocyclohexane organochlorine insecticide. A noninsecticidal lindane isomer, (β hexachlorocyclohexane), often produced as a by-product of lindane (Wester and Canton, 1986), has been shown to have toxic effects on medaka exposed from early embryonic development through 1 - 3 months posthatch, and on 1-month-old fry exposed for an additional 1 - 3 months (Wester and Canton, 1986). The concentration range for the isomer was 0.032 - 1.0 ppm. While this study concentrated most heavily on the histopathological alterations seen as a result of exposure, it was noted that embryonic exposure of 0.056 ppm and fry exposure of 0.1 ppm resulted in decreased growth. Similar concentrations also affected the swimming behavior of the resulting fry. The most notable histopathological finding was the occurrence of intersexes or hermaphrodites following 3 months exposure at 0.18 ppm or greater. The authors hypothesize that this isomer may have an estrogenic effect in the medaka, causing a feminization of male medaka (Wester and Canton, 1986). This corresponded to an increased percentage of vitellogenic females in the study, as well as vitellogenesis in one intersex and one male medaka. Deposition of adipose tissue in the liver, kidney and testis of individuals was also noted in these exposure groups.

Chlorinated ethane derivatives: Possibly the best-known chlorinated ethane derivative is DDT (dichlorodiphenyl-trichloroethane or 1,1,1-trichloro-2,2-bis[p-chlorophenyl] ethane). This long-lived, bioaccumulated organochlorine insecticide

was reported to be responsible for the soft eggshells found in many raptor species in the 1970's (Murphy, 1986; Peakall, 1970). Takimoto, et al. (1984b) exposed twelve different life stages of the medaka to varying concentrations of *p,p'*-DDT. The postlarval stage was found to be the most sensitive life stage, with a 96 h LC₅₀ of 0.005 ppm. DDT was apparently stored in the adipose tissue of the embryos and utilized in the postlarval period. Although DDT did not result in decreased hatchability in exposed embryos, the survival of the postlarval fry was decreased with increasing concentrations of DDT (Takimoto, et al., 1984b).

In an experiment designed to determine the uptake of DDT by embryos, Solomon (1977c) exposed medaka embryos to ³H-DDT and documented uptake and storage of the lipophilic compound in both the yolk and embryo. Again, no developmental abnormalities were seen as a result of embryonic DDT-exposure (1 ppb for 1 - 8 d).

4.2.4 Herbicides

There are numerous classes of chemicals which are used as herbicides, but since only a few have been tested on medaka, they will be discussed as a group. When applicable, the chemical class of particular herbicides will be discussed.

2,4,5-Trichlorophenoxyacetic acid, PCDD's and PCDF's: The herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) is a chlorophenoxy compound and is used to control broad-leaf weeds and woody plants (Murphy, 1986). A common

contaminant of 2,4,5-T is the potent intermediate product 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Both of these compounds have some toxicity to vertebrates. However, it is thought that the original data for 2,4,5-T, which indicated that it was a potent teratogen, may have been attributable to contamination by TCDD (Murphy, 1986).

Both 2,4,5-T and TCDD have been studied using the medaka as test species (Schreiweis and Murray, 1976; Cooper and Wisk, 1990; Cooper et al., 1991). Schreiweis and Murray (1976) exposed medaka embryos in up to 50 ppm 2,4,5-T (with low TCDD contamination). Although it is noted that exposure was initiated in the early high blastula stage of development, no information is given for the duration of the exposures. Hatching was reduced from 96% at 14 ppm to 47% at 20 ppm. Developmental abnormalities were also present in embryos exposed to 2,4,5-T at concentrations of 20 ppm and greater. These abnormalities included cardiovascular problems in the heart and surrounding arteries and veins (Schreiweis and Murray, 1976).

2,4,5-T production and use are not the only sources of TCDD in the environment. Various polychlorinated dibenzo-*p*-dioxins (PCDDs) enter the environment from paper mills and other industries manufacturing or using polychlorinated phenols (Murphy, 1986; Emmett, 1986). PCDDs are polychlorinated-*p*-dioxins, of which TCDD is one. Wisk and Cooper (1990) exposed various stages of medaka embryos to TCDD to determine the toxic and teratogenic effects. This was the first reported use of the incubation of individual

medaka embryos. Embryos were exposed throughout embryonic development without renewal of the incubation solution. Wisk and Cooper (1990) reported an EC_{50} value for hatching of 14 ppt TCDD, while the EC_{50} for lesions was 3.5 ppt. The LC_{50} for survival to 3 d posthatch was reduced to 9 ppt. Much like 2,4,5-T (Schreiweis and Murray, 1976) and organophosphate exposure (Solomon and Weis, 1979), the primary abnormalities occurring in the developing medaka exposed to TCDD were found in the cardiovascular system (Wisk and Cooper, 1990). The most sensitive stage of embryonic development to the effects of TCDD appeared to be during liver formation (day 4 - 5). In another related study, Cooper et al. (1991) found contamination of PCDDs and PCDFs (polychlorinated dibenzofurans) in the oil of herrings from the Gulf of Bothnia, Sweden. PCDFs and PCDDs are both produced in the manufacturing and use of polychlorinated compounds [Murphy, 1986; Emmett, 1986]). Whereas TCDD is an intermediate product of 2,4,5-T, the herbicide pentachlorophenol (PCP) is often contaminated with PCDFs (Murphy, 1986). Individual medaka embryos exposed to solutions containing the contaminated oils exhibited a higher mortality than either those in rearing medium or those exposed to corn oil (Cooper et al., 1991).

2,4-Dinitrophenol: The effects of the herbicide 2,4-dinitrophenol (DNP) have also been demonstrated on the medaka (Waterman, 1939; Ishida, 1951; Ishihara, 1956). Exposed embryos were found to be the most sensitive to DNP during the early

cleavage stages, resulting in retardation of development. Embryos with pricked chorions responded the lower concentrations of DNP, indicating that the chorion may slow or retard the uptake of DNP into the embryo (Waterman, 1939). However, this outcome may be a result of injury to the embryo during handling. Abnormalities found in the exposed embryos include cardiovascular malformations and abnormal body shapes (Waterman, 1939). DNP increases oxygen consumption and ammonia production during the cleavage stages in a concentration-dependent manner and corresponds to the stage where retardation of growth by DNP generally occurs (Ishida, 1951; Ishihara, 1956). This phenomena is seen only if exposure is initiated prior to the formation of the optic vesicle (Ishida, 1951).

Dinitro-*o*-cresol: Another dinitrophenolic herbicide is dinitro-*o*-cresol (DNOC). Slooff and Canton (1983) exposed medaka embryos to DNOC for 40 d (embryo through fry) and reported NOL(E)C values for the exposure. The reported NOL(E)C value for mortality was 0.1 ppm and 1.0 ppm for hatching growth. Of the 11 test species, the medaka NOL(E)C for DNOC exposure was the lowest reported NOE(L)C for mortality (Slooff and Canton, 1983).

Pentachlorophenol: Pentachlorophenol (PCP) is a substituted aromatic that is used as an insecticide, fungicide and herbicide. This compound has also been found to be contaminated with PCDDs and PCDFs (Murphy, 1986). Slooff and

Canton (1983) determined the NOL(E)C for medaka embryos exposed to pentachlorophenol through day 40 (as fry). The NOL(E)C for mortality was 0.032 ppm, while the NOL(E)C for hatching growth was 0.32 ppm. In 48 h acute toxicity tests with 4 - 5-week-old medaka fry, the reported NOLC for PCP was 0.93 ppm, and the LC₅₀ was 1.1 ppm (Slooff, et al., 1983).

Paraquat: Paraquat is a commonly used herbicide (Murphy, 1986). This herbicide has been reported to cause malformations in developing medaka embryos at concentrations exceeding 23.3 ppm (Murray and Schreiweis, 1977, abstract only). Although detailed dose-response data is not given in the abstract, it is noted that embryos exposed to 50 ppm paraquat experienced 100% mortality (Murray and Schreiweis, 1977, abstract only).

4.2.5 Fumigants

Methyl bromide: Gaseous methyl bromide (CH₃BR) has been used as a fumigant for numerous years. Fumigants may be used as insecticides or rodenticides (Murphy, 1986). Wester, et al. (1988) investigated the histopathological effects of methyl bromide on medaka and guppies (*Poecilia reticulata*), and compared the toxic effects of methyl bromide to that of the bromide anion (in the form of sodium bromide, NaBr). Methyl bromide caused mortalities in the group of exposed medaka embryos and fry at 1.8 ppm, whereas

sodium bromide had a NOLC (no observed lethal concentration) value of 5,600 ppm for a 3 week exposure. When medaka embryos were exposed to concentrations of methyl bromide greater than 1.8 ppm, most died before hatching. The authors agreed that the bromide ion itself has a relatively low toxicity (Wester, et al., 1988), a point earlier demonstrated by Slooff and Canton (1983). When medaka embryos were exposed to sodium bromide for 40 d (embryo through fry), the NOL(E)C values for mortality were 3200 ppm and 10,000 ppm for hatch growth. Wester, et al. (1988) did report activation of the thyroid epithelium in medaka exposed to methyl bromide, but found no specific histopathological changes associated with chronic methyl bromide exposure.

4.2.6 Complex pesticide mixtures

The literature contains at least two studies focusing on the effects of complex pesticide mixtures on the survival and development of the medaka embryo. Marty et al. (1991) used a version of the medaka embryo assay to study and document bioremediation at a pesticide disposal site in the Sacramento Valley, near Davis, CA, U.S.A. Pesticides found at the site include organophosphates (carbophenothion, ethion, parathion, malathion, and methylparathion), organochlorines (*p,p'*-DDT, endosulfan sulfate, and endosulfan I and II), herbicides (atrazine and trifluralin) and a fungicide (chlorothalanil). Medaka were exposed to soil extracts from three different test pits at the site both before and after bioremediation measures (addition of lime, alfalfa meal, manure, blood

meal and wild rice hulls). There was a reduced mortality in the groups exposed to soil extracts taken after the bioremediation efforts (Marty, et al., 1991).

Another field study (Hiraoka, 1980b, abstract in English) examined the occurrence of vertebral abnormalities in indigenous populations of medaka living in streams receiving run-off from agricultural fields. Again, both herbicides and pesticides were known to be in use on the fields; zinc was also present in higher than normal concentrations at one site. In all three cases, the incidence of vertebral abnormalities was significantly higher the rate for medaka reared in the laboratory. This study does not provide any data from reference streams. The inclusion of such data would address the argument that the vertebral abnormalities are a result of xenobiotic exposure. Since some fish species have been reported to have a high incidence of spontaneous vertebral abnormalities (i.e. spinal curvatures) as a result of normal stressors (Heath, 1987), it is important to know the rate of such abnormalities for medaka exposed to normal environmental conditions, without the xenobiotics.

4.3 Aromatic Chemical Compounds

This section will focus on investigations into the effects of several different types of aromatics on medaka. The discussion will include experiments using polychlorinated biphenyls (PCB's), chlorophenols, toluene and *p*-nitrotoluene, 2,4-dichloroaniline, tetrapropylene benzene sulphonate, and crude oil and its aromatic components (see Table 1.9).

Table 1.9 Summary of literature articles using medaka to test effects of various aromatic chemical compounds.

Compound	Life-stage	Reference
Polychlorinated biphenyl (PCB)	adult	Ando and Yand, 1982 ^a
14 different chlorophenols	embryo	Shigeoka, et al., 1988a ^a
2,4-dichlorophenol	embryo, larval and juvenile	Shigeoka, et al., 1988b ^a
Tetrapropylene benzene sulfonate	embryo-fry	Slooff and Canton, 1983
2,4-dichloroaniline	embryo-fry	Slooff and Canton, 1983
<i>p</i> -nitrotoluene	embryo-fry	Slooff and Canton, 1983
Toluene	embryo and fry	Stoss and Haines, 1979
Crude oil and aromatic components	embryo	Leung and Bulkley, 1979
Aniline	fry	Slooff, et al., 1983
Benzene	fry	Slooff, et al., 1983
Pyridine	fry	Slooff, et al., 1983
<i>o</i> -Cresol	fry	Slooff, et al., 1983
Salicylaldehyde	fry	Slooff, et al., 1983
Trypan blue (azo dye)	embryo	Briggs and Wilson, 1959

^a Only the abstract is available in English.

4.3.1 Polychlorinated biphenyls

Polychlorinated biphenyls, a group of aromatic halogenated compounds containing 12 - 68% chlorine (Menzer and Nelson, 1986), are often referred to as PCBs, . The commercially produced "Aroclors" are mixtures of various PCBs, and are identified by four digit numbers, the last two digits identifying the percent of chlorine in the mixture (Windholz, et al., 1983; Nimmo, 1985). Discussions on the toxicology of PCBs may also be included with that of pesticides since they are closely related chemically and toxicologically (Nimmo, 1985). Ando and Yand (1982 abstract only) studied the effects of PCBs (no specific formulation or Aroclor given in the abstract) on the sexual maturity of the female medaka. By observing the time required to begin spawning, the gonado-somatic index (GSI) and the estrogen levels in the water, the authors determined that the PCB-treated females experienced a delay in sexual maturation in comparison to the controls.

4.3.2 Chlorophenols

Another group of aromatic halogenated hydrocarbons contains the chlorophenols. These include the isomers of the mono-, di-, tri-, tetra- and penta-chlorophenols. Shigeoka, et al. (1988a, b, abstracts only) investigated the toxicity of several of these compounds to medaka embryo, larval and early-juvenile medaka, and reported exposed embryos exhibited an increase in toxicity of the chlorophenol with increasing chlorination. When early life-stages (embryo, larval and early-juvenile) were exposed to 2,4-dichlorophenol, (Shigeoka et al., 1988b,

abstract only) the most sensitive endpoints and stages studied were embryonic survival and hatching. They reported a MATC value (maximum acceptable toxicant concentrations) of 0.32 - 0.63 ppm for the embryo life-stage.

Three other aromatic compounds were included in a study done by Slooff and Canton (1983): tetrapropylene benzene sulphonate, 2,4-dinitroanaline, and *p*-nitrotoluene. In this study, embryos were exposed for 40 d (through fry stage) and the NOE(L)C values were reported for each compound. The NOE(L)C values for these compounds are as follows:

	NOL(E)C value for mortality (ppm)	NOE(L)C value for hatching growth (ppm)
<u>Tetrapropylene benzene sulphonate</u>	3.2	10
<u>2,4-Dichloroanaline</u>	0.32	3.2
<u><i>p</i>-Nitrotoluene</u>	1	32

4.3.3 Toluene

Toluene, a small aromatic compound, is present in some fuel oils. Stoss and Haines (1979) exposed medaka embryo and fry to the water-soluble fraction (WSF) of toluene to determine the TL₅₀. Exposures were pulse doses of 24, 48 and 96 h durations. Early embryos (up to 3.5 h postfertilization) were the most sensitive to the toxic effects of toluene, with the stages just preceding hatching and 24-h-old fry also being more sensitive than other embryonic stages. The reported average TL₅₀ for embryos exposed to toluene was 54 ppm. Toluene apparently

interfered with the normal hatching of the embryos, resulting in erratic and extended hatch times. Abnormalities seen in toluene-exposed embryos included disruption in the cleavage pattern, malformed eyes, hearts and visceral organs, and cardiovascular abnormalities (Stoss and Haines, 1979).

4.3.4 Petroleum crude oil WSF

The effects of the water-soluble fractions (WSF) of petroleum crude oil on developing medaka were reported by Leung and Bulkley (1979). Exposures were pulse-doses of 24, 28, and 96 h duration. Embryos treated with WSF in 24 and 48 h pulse exposures exhibited a significantly shorter hatching time than did controls or embryos exposed to WSF for 96 h. The authors noted an increase in opercular movement in exposed embryos, as well as other signs of stress. Embryos that survived to hatching were deformed or had curved tails.

In a 48 h acute toxicity test, Slooff et al., (1983) determined the LC_{50} and NOLC values for several aromatic compounds. All of these except salicylaldehyde are used as solvents; salicylaldehyde is used in the perfume industry (Windholz, et al., 1983). Medaka fry (4 - 5 weeks old) were used in the study. The reported NOLC and LC_{50} values were as follows:

	NOLC (ppm)	LC ₅₀ (ppm)
<u>Aniline</u>	100	165
<u>Benzene</u>	126	250
<u>Pyridine</u>	1420	1560
<u><i>o</i>-Cresol</u>	32	41
<u>Salicylaldehyde</u>	3.6	4.2

Data obtained by Briggs and Wilson (1959) established the teratogenic and toxic effects of the azo dye, trypan blue, in the medaka. Teratogenic effects in mammals were previously determined. Both 24 h and 48 h exposures of 4-cell-stage embryos (2.5% trypan blue solution) resulted in mortality rates approaching 100%. Abnormalities were evident at lower exposure times (1 - 2 h). Results for the exposure of 2-cell-stage embryos were similar. The mortality and abnormality rates increased when trypan blue exposure was coupled with low temperatures (6 - 7°C). Abnormalities included optical malformations, curvatures in tail and body portion of the spine, and developmental retardation.

4.4 Alkylating Agents

Several studies have been conducted in which the teratogenic effects of alkylating agents were evaluated on medaka embryos (Faustman-Watts and Solomon, 1985, abstract only; Solomon and Faustman, 1987; Shi and Faustman, 1989; Marty, et al., 1990b). Alkylating agents are compounds which can donate alkyl groups, and most alkylating agents are considered to be carcinogenic and

mutagenic (Williams and Weisburger, 1986; Solomon and Faustman, 1987). Investigations into the carcinogenicity of these compounds using medaka are referenced in Section 3.7.4 of Part I. Solomon and Faustman (1987) exposed medaka embryos to one of four different alkylating agents for a 2 h pulse-dose, during organogenesis. Of the four alkylating agents tested, two were N-nitroso compounds (methylnitrosourea [MNU] and ethylnitrosourea [EMU]), and two were sulfate ester compounds (methyl methanesulfonate [MMS] and ethyl methanesulfonate [EMS]). The most toxic was MNU, followed by ENU, MMS and EMS in decreasing order of toxicity. These agents caused abnormalities in the cardiovascular system, abnormal eyes and head development, and abnormal tail development (Solomon and Faustman, 1987; Marty et al., 1990b). In tests designed to determine the LC_{50} and MC_{50} (concentration resulting in 50% malformed embryos) for MNU in both rat and medaka embryo cultures, Faustman-Watts and Solomon (1985, abstract only) report a tenfold greater sensitivity of medaka embryos to MNU (rat LC_{50} = .12 mM, rat EC_{50} = .04 mM). In a very detailed study, Shi and Faustman (1989) attempted to design a specific scoring system for the morphological development of the medaka, thus providing a more quantitative method for determining the extent of developmental effects, such as retardation. Embryos were exposed to MNU for 2 h, during organogenesis. The authors assigned specific numerical values to developmental events within each developmental stage. Reported abnormalities were similar to other MNU-exposed embryos (Solomon and Faustman, 1987). Mortalities in the

experiment done by Shi and Faustman (1989) reached 50% at MNU concentrations between 1.0 and 2.5 mM. The study also demonstrated the dose-dependent occurrence of abnormalities in the head, heart, and tail (Shi and Faustman, 1989).

In a related study (Marty, et al., 1990b), medaka embryos were exposed to one of 3 alkylating agents (MNU, MNNG = N-methyl-N'-nitro-N-nitrosoguanidine, and DENA = N-nitrosodiethylamine). Abnormalities described for these compounds are similar to those observed in Solomon and Faustman's (1987) MNU experiment, but also include failure to inflate swimbladder, and partial hatching followed by death (Marty, et al., 1990b). By exposing only discrete developmental stages to the alkylating agents, the researchers determined that MNU and MNNG could cause lethality (at early stages, i.e. stage 10), teratogenesis (i.e. stage 20), or have relatively little effect (later, prehatching stages, i.e. stage 30). Marty et al. (1990b) also reported the results of studies on chorion permeability and uptake of several radiolabeled organic compounds. The ability of the compounds to cross the chorionic and embryonic membranes corresponded to the octanol/water coefficients of the compounds, with the following relationships: dimethylbenzanthracene > benzo[a]pyrene > naphthalene > benzene > phenol > DENA > $^3\text{H}_2\text{O}$ (Marty, et al., 1990b).

4.5 Miscellaneous Chemicals, Pharmaceuticals, and Physical Agents

Over the years, the medaka has been used to test a wide variety of agents, including some pharmaceutical and physical agents. A summary of these references may be found in Table 1.10.

4.5.1 Tolbutamide

Tolbutamide, a pharmaceutical used as an antidiabetic (Windholz, et al., 1983), has been shown to have teratogenic effects on developing medaka embryos (Smithberg, 1962). Abnormalities included abnormal cardiovascular development, degeneration of the eye cup and curved tail and trunk regions. Smithberg (1962) determined that the earlier stages of embryonic development were most sensitive to tolbutamide, but that both the duration and concentration of the tolbutamide exposure were important factors. In a later study, Smithberg and Dixit (1968) determined that glucose uptake was inhibited during the exposure of embryos to tolbutamide, and exposed embryos had significantly lower levels of glycogen after 48 h exposures.

4.5.2 Ethyl urethane derivatives

T. Yamamoto (1938) extended his study of the rhythmical movements of the early embryo (see Section 3.7.2 of Part I) to include a series of carbamates: ethyl carbamate, ethyl methyl carbamate, ethyl propyl carbamate, ethyl butyl carbamate and ethyl phenyl carbamate. Although these are carbamates, they do

Table 1.10 A summary of literature articles using medaka to test the effects of miscellaneous chemicals, pharmaceuticals, and physical agents.

Chemical	Use/Occurrence or chemical class	Life-stage	Reference
Tolbutamide	antidiabetic	embryo	Smithberg, 1962
Tolbutamide	antidiabetic	embryo	Smithberg and Dixit, 1968
Ethyl Urethane and six derivatives	antineoplastic; anesthetic	embryo	T. Yamamoto, 1938
Beta-naphthoflavone		adult	Schell, et al., 1987
<i>n</i> -Propanol	solvent/alcohol	fry	Slooff, et al., 1983
<i>n</i> -Heptanol	solvent/alcohol	fry	Slooff, et al., 1983
Ethylacetate	solvent	fry	Slooff, et al., 1983
Ethylpropionate	solvent	fry	Slooff, et al., 1983
Acetone	solvent	fry	Slooff, et al., 1983
Trichloroethylene	solvent	fry	Slooff, et al., 1983
Allylamine	used in mfg. of mercurial diuretics	fry	Slooff, et al., 1983
Aflatoxin B ₁			Llewellyn, et al., 1977
Asbestos	insulator/fire-retardant	embryo, fry and adult	Belanger, et al., 1990
Electromagnetic fields	exposure to levels found beneath high voltage power lines	embryo	Cameron, et al., 1985b

not necessarily have insecticidal applications. The parent compound (ethyl carbamate = ethyl urethane) is used as an antineoplastic (Windholz, et al., 1983). Embryos exposed to these compounds exhibited a transient increase in the rhythmical movements, followed by narcosis (arrest of rhythmical movements). These effects were apparently dose- and time-dependent. The chemicals varied in ability to produce narcosis, and in order of decreasing effectiveness the chemicals ranked as follows: ethyl phenyl carbamate > ethyl butyl carbamate > ethyl propyl carbamate > ethyl dethyl carbamate > ethyl methyl carbamate > ethyl carbamate (T. Yamamoto, 1938).

4.5.3 Betanaphthoflavone

Beta naphthoflavone (BNF) is a chemical known to induce the mixed-function oxidase (MFO) system of detoxification enzymes (Schell, et al., 1987). Other inducing agents include phenobarbital, 3-methylcholanthrene, halogenated pesticides, and TCDD (Schell, et al., 1987; Sipes and Gandolfi, 1986). Schell et al. (1987) investigated the ability of BNF to induce the MFO and cytochrome P₄₅₀ systems of adult medaka exposed via food spiked with BNF. Exposed adults exhibited higher concentrations of cytochrome P₄₅₀ and NADPH-cytochrome c reductase activity within 48 h of exposure, and higher levels of EROD (ethoxyresorufin-*o*-deethylase) within 96 h. Sex differences were seen in all three endpoints, with males typically exhibiting higher levels of activity and concentrations than females.

4.5.4 Nonaromatic organics

Slooff, et al. (1983) determined the NOLC and LC₅₀ values for several nonaromatic organic compounds in 48 h acute toxicity tests. The tests were conducted on 4- to 5-week-old medaka fry. All of these compounds are used as solvents except for allylamine, which is used in the manufacturing of mercurial diuretics (Windholz, et al., 1983). The NOLC and LC₅₀ values were as follows:

	NOLC (ppm)	LC ₅₀ (ppm)
<u><i>n</i>-Propanol</u>	4400	5900
<u><i>n</i>-Heptanol</u>	41	48
<u>Ethylacetate</u>	100	125
<u>Ethylpropionate</u>	46	58
<u>Acetone</u>	9500	14300
<u>Trichloroethylene</u>	220	270
<u>Allylamine</u>	13	16

4.5.5 Aflatoxin B₁

Medaka embryos exposed to varying concentrations of the mycotoxin, aflatoxin B₁ (0.05 - 15.10 ppm) exhibited 100% mortality (Llewellyn, et al., 1977). While mortality was not dose-dependent, the time of death appeared to be dose-dependent. Embryos exposed to lower concentrations of aflatoxin B₁ (0.05 - 0.50 ppm) experienced 100% mortality ($n = 8/\text{exposure concentration}$), but most lived for 504 - 720 h postfertilization. On the other hand, embryos exposed to higher concentrations (1.00 - 15.10 ppm) experienced 100% mortality within 72 h postfertilization. Abnormalities noted included abnormal or incomplete

development of the circulatory system, failure of hemoglobin to develop, and optical malformations.

4.5.6 Asbestos

Asbestos, a constituent of many industrial and commercial products, is actually a group of chemically inert, fibrous, silicate minerals (American Geological Institute, 1975). The properties of heat resistance and electrical insulation led to its active use; however, it has been determined that the inhalation of asbestos fibers can lead to pulmonary fibrosis and mesotheliomas (Williams and Weisburger, 1986; Menzer and Nelson, 1986). Although generally considered to be a human health risk via respiratory exposure, asbestos is found in drinking water and ambient waters (Menzer and Nelson, 1986). Belanger et al. (1990) investigated the effects of asbestos to aquatic populations, exposing embryo, fry, and adult medaka to solutions containing chrysotile asbestos in concentrations normally found in drinking water (10^2 - 10^{10} fibers/L). Asbestos-exposed embryos did not show significant mortality. Although there was an alteration in the time required to hatch, it does not appear to be dose-dependent. Medaka fry appeared to be much more sensitive to asbestos exposure, with almost 100% mortality seen at 10^{10} fibers/L concentration. In the groups of exposed fry, mortality did appear to be dose-dependent. In exposed adults, although mortality was not significantly different from controls, the reproductive success of the adult females was reduced in asbestos-exposed groups (reduced number of eggs

spawned and reduced number of viable embryos). Asbestos fibers were also found in the tissue of epidermal lesions. The authors concluded that asbestos may present a hazard to aquatic organisms, especially the early life-stages (particularly fry).

4.5.7 Electromagnetic fields

Cameron, et al. (1985b) investigated the effects of electromagnetic fields on the development of medaka embryos to determine if this agent could produce biological effects. Developing embryos were exposed for 48 h to fields similar to those found beneath high voltage power lines (60 Hz magnetic field at 1.0 gauss RMS, a 60 Hz electrical field of 300 mM/m², or a combination of the magnetic and electrical fields). Neither developmental abnormalities nor increased mortalities were seen as a result of the exposures; however, developmental delays were observed in groups of embryos exposed to the magnetic field and the combination magnetic and electrical field.

4.6 Radiation

The toxicology of the effects of various types of radiation has developed into a very specialized field. This discussion will not be an attempt to delineate all of the parameters of that field, but instead will cite those studies which have focused on the effects of radiation to the medaka. Table 1.11 contains a list of references to these studies, including the exposed life-stage and the exposure dose

(or range of doses). Table 1.11 is subdivided into two types of radiation, electromagnetic radiation and particulate radiation. Electromagnetic radiation includes X-rays, γ -rays, and UV irradiation, whereas particulate radiation includes α and β particles, electrons, protons, neutrons, and positrons (Hobbs and McClellan, 1986). This discussion on testing the toxicological effects of radiation on the medaka will be dealt with by the life-stage of exposure. The main subdivisions will be embryonic, fry, and adult exposures.

Developing Japanese medaka embryos are sensitive to irradiation by X-rays, exhibiting reduced hatching success when exposed to a dose of 2 kR (kilo Roentgen) (Egami and Hama, 1975). The embryos showed some resistance to that dose of X-rays if exposed during hours 8 - 9 or at 1.2 - 1.5 d postfertilization (Egami and Hama, 1975). When one-cell-stage embryos were exposed to varying doses of X-rays (25 - 500 R), researchers observed a concurrent increase in the number of chromosomal bridges (Suyama, et al., 1981). An estimated LC_{50} value of 360 R (X-rays) for one-cell-stage embryos is reported by Suyama, et al. (1981), although no reference is made to the effect of the dose-rate of the exposure. The effects of γ -rays on developing medaka embryos have also been investigated (Egami and Hama, 1975; Ijiri, 1977; Suyama, et al., 1981; Shimada and Egami, 1982; Shimada and Egami, 1984b; Shimada, et al., 1985a; Shimada, et al., 1985b). Egami and Hama (1975) investigated the effect of the dose-rate on the hatching success of exposed embryos (dose-rate = time period over which a specific cumulative dose is administered). They found that varying the dose-rate

Table 1.11 Survey of articles using medaka to investigate the effects of various types of irradiation.

Type of irradiation	Medaka life-stage/dose	Reference
Electromagnetic radiation		
X-rays	adult female/2kR	M. Yamamoto and Egami, 1974b
X-rays	early embryo/0-500 R	Suyama, et al., 1981
X-rays	fry/1000 R	Hamaguchi and Egami, 1975
X-rays	adult female/2000 R	Hama, et al., 1976
X-rays	adult female/2-16kR	M. Yamamoto, 1972
X-rays	embryo and adult/2 kR	Egami and Hama, 1975
X-rays	adult male/260 r/min	Egami, 1955b
γ -rays (heat)	embryo/2.5 Gy/min	Shimada, et al., 1985a
γ -rays	embryo/4-kCi ^{137}Cs	Shimada and Egami, 1982
γ -rays	embryo/4-kCi ^{137}Cs	Shimada and Egami, 1984b
γ -rays (heat, hypoxia, Cd, and Ar)	embryo/2.5 Gy/min	Shimada, et al., 1985b
γ -rays	embryo/250 Rad/min	Ijiri, 1977
γ -rays	sperm/0.25-74 kR/min	Ijiri, 1983
γ -rays	adult/0.8-250 R/min and 540-27.7 R/d	Hama and Egami, 1977
γ -rays	early embryo/0-74 rad/h	Suyama, et al., 1981
γ -rays	adult/50 R/min	Ghoneum, et al., 1979
γ -rays	embryo and adult/1.7-250 R/min	Egami and Hama, 1975

Type of irradiation	Medaka life-stage/dose	Reference
UV irradiation	sperm/7.5-200J/m ²	Ijiri, 1983
UV irradiation	embryo, egg and sperm330-500 J/m ²	Ijiri, 1980
UV irradiation	embryo/25-300 J/m ²	Hyodo-Taguchi, 1983
Particulate Radiation		
β -particles	early embryo/tritium, ⁹⁰ Sr- ⁹⁰ Y	Suyama, et al., 1981
β -particles	adult male/10 ⁻¹ -10 ⁻³ tritium/L	Hyodo-Taguchi and Egami, 1977

does vary the LC_{50} value for γ -rays, with the higher dose-rate (same dose administered over a shorter period of time, i.e. 250 R/min compared to 1.7 R/min) resulting in higher mortality rate. When embryos were exposed to continuous doses of γ -irradiation (78 - 591 rad), a significant increase in the occurrence of chromosomal bridges was observed, although fewer in number than those seen with X-ray exposures of 25 - 500 R (Suyama, et al., 1981). Changes in the development of the primordial germ cells have been reported in association with γ -ray exposure (Ijiri, 1977; Shimada and Egami, 1982; Shimada and Egami, 1984b). When exposed to γ -rays at 4 d postfertilization (2 kR), Shimada and Egami (1982) report an initial inhibition in the normal proliferation of germ cells, and later, the accumulation of abnormal annulate lamellae in the cytoplasm of the germ cells. By 4 d postirradiation, only the males experienced a decrease in the number of germ cells. When Ijiri (1977) exposed embryos to γ -rays (2000 rad) at 4 d postfertilization, he reported a significant number of germ cells with enlarged nuclei within 48 h postirradiation. The number of large nuclei reached a maximum by 112 h postirradiation, and decreased to normal thereafter. When embryos at three different stages of development (1, 3, and 5 d postfertilization) were exposed to γ -rays (0 - 20 Gy or 0 - 2000 rad) at two different dose-rates (0.14 and 2.5 Gy/min), a decrease in the number of primordial germ cells was seen with increased γ -ray dose, but no differences were seen between the two exposure rates (Shimada and Egami, 1984b). Shimada and Egami (1984b) also reported the occurrence of resistance if embryos were irradiated at high temperatures

(40°C for 120 min), and theorized that this may be a result of heat shock proteins. In a later study Shimada et al. (1985a) determined that the most sensitive embryonic stages occur prior to 3 d postfertilization, and that if embryos are exposed to γ -rays during that sensitive period, postirradiation heating could be effective in preventing radiation effects.

Shimada, et al., 1985b continued research into the phenomenon of heat shock resistance in the irradiated embryos as measured by the number of primordial germ cells. These researchers reported that while heat alone reduced the number of primordial germ cells, heat (41°C) following γ -irradiation (10 Gy) resulted in a relative increase in the number of primordial germ cells at temperatures up to 42°C. This study also included investigations into the effects of several other factors on the primordial germ cell number following γ -irradiation, including hypoxia and the release from hypoxia, and exposure to cadmium and arsenite (Shimada, et al., 1985b). Hypoxic conditions were created by incubating the embryos in sealed vials (at 28°C) in which nitrogen filled the air space. Hypoxia appeared to confer some resistance on γ -irradiated embryos (as measured by the survival of the primordial germ cells). When released from hypoxic conditions, the embryos no longer showed radioresistance. In tests using cadmium and arsenite, the embryos were exposed to either 500 ppm cadmium or 10 mM arsenite. These concentrations did not alter the hatching success of the embryos, and exposure to the chemicals prior to irradiation (4 - 24 h) failed to affect the survival of the primordial germ cells (Shimada, et al., 1985b).

Medaka embryos have also been the subject of tests designed to determine the effects of ultraviolet (UV) irradiation to developing tissues (Ijiri, 1980; Hyodo-Taguchi, 1983). Ijiri (1980) found that embryos begin to become resistant to UV irradiation within 30 minutes postfertilization. Exposed embryos responded to UV irradiation in a dose-dependent manner, however, even when one-cell-stage embryos were exposed to UV irradiation and then visible light, the embryos exhibited photoreactivation and increased survival rates. In a similar study (Hyodo-Taguchi, 1983), three different inbred strains and one substrain of medaka embryos were exposed to varying concentrations of UV irradiation in an attempt to determine the LC_{50} values. The four groups tested did not respond identically to the UV irradiation, with LC_{50} values ranging from 50 J/m² to 150 J/m², however all four groups did exhibit some degree of recovery with photoreactivation.

Suyama, et al. (1981) also studied the effects of several different β -radiation sources on the numbers of chromosomal bridges in developing medaka. These sources included tritiated water, a solution of ⁹⁰Sr and ⁹⁰Y. When embryos were exposed to tritiated water (3.7 - 370 rad), researchers observed a concurrent increase in the number of chromosomal bridges, with significantly higher numbers occurring with doses at and above 18.5 rad. Similar results were found in embryos exposed to ⁹⁰Sr and ⁹⁰Y, with doses ranging from 5.8 to 580 rad.

Medaka fry have been used to determine the effects of X-rays on the germ cells, also. Hamaguchi and Egami (1975) report decreases in the number of germ cells, oogonia, and oocytes in fry exposed to X-rays (50 R/min).

While numerous studies with various different endpoints have utilized adult medaka in tests designed to determine the effects of X-rays, only a brief overview will be presented in this discussion (Egami, 1955b; M. Yamamoto, 1972; M. Yamamoto and Egami, 1974b; Hama, et al., 1976). Egami (1955b) exposed adult male and female medaka to sublethal doses of X-rays (4000 r) and noted the sterilization effects on both sexes, and the ability of the germ cells to regenerate and recover from the radiation damage. However, doses as high as 16 kR have been found to cause permanent sterility in female medaka (M. Yamamoto, 1972). Vitellogenic oocytes appeared to be most sensitive to X-ray damage (M. Yamamoto, 1972), although damage has also been noted in the liver hepatocytes and pituitary gonadotrophic cells (M. Yamamoto and Egami, 1974b). Chromosomal abnormalities, reported in gill epithelium in response to X-rays, also apparently disappeared from the tissue and cells were regenerated from undamaged cells within 10 d following X-irradiation (2000 R) (Hama, et al., 1976).

The effects of γ -irradiation on the adult medaka have also been investigated (Hama and Egami, 1977; Ghoneum, et al., 1979; Ijiri, 1983). Hama and Egami (1977) observed the changes in regenerating tail fin cells of adult medaka exposed to γ -irradiation (at rates of either 0.8 R/min or 540 R/d). Irradiation (2 kR) prior to cutting the tail fin was found to inhibit the natural regeneration process in the tail fin. This effect decreases as the time between irradiation and the operation increases. Ghoneum, et al. (1979) determined the radiosensitivity of the adult medaka thymus to γ -irradiation, through histological

observations and measurements following exposure to 200 and 500 R (^{137}Cs). Initial changes (two days following irradiation) included a reduction in overall organ size, degenerated lymphocytes, and the appearance of numerous cysts. The thymus appeared to recover by eleven days after irradiation. Ijiri (1983) reported the occurrence of the Hertwig effect in medaka. In this phenomenon first reported by Hertwig (1911) in frogs, embryos fertilized with irradiated sperm exhibit high mortality at low doses and a lower mortality at higher doses. It is thought to be a result of photoreactivation and repair (Ijiri, 1980). Ijiri (1983) demonstrated this with both UV and γ -irradiation of sperm. UV-irradiation of intact oocytes and sperm were also done by Ijiri (1980), and included fertilization of non-irradiated oocytes by irradiated sperm and fertilization of irradiated oocytes by non-irradiated sperm. Both experimental groups exhibited a decreased survival rate compared to controls.

Sperm of the adult medaka has also been demonstrated to be sensitive to β -particle irradiation (Hyodo-Taguchi and Egami, 1977). Adult males were exposed to tritiated water (5×10^{-3} - 5×10^{-1} Ci/L), for three to thirty days. Maximum uptake into the body and the gonads was reached within one day and remained constant thereafter. The overall weight of the testis decreased by day 30 of the exposures, and histopathological degenerations were noted at the cellular level. These changes included a decrease in the number of spermatogonia (not statistically significant), the occurrence of dying, pyknotic cells, and damaged spermatocytes

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**PART II: REPORT ON ATTEMPTS TO PRODUCE ACCEPTABLE
CONTROLS FOR A MEDAKA EMBRYO ASSAY (*ORYZIAS LATIPES*)
THROUGH THE EVALUATION OF SEVERAL WATER SOURCES AND
INCUBATION PROCEDURES.**

1. ABSTRACT

Although medaka embryos (*Oryzias latipes*) have been used in many types of toxicity tests, investigators have not critically evaluated the effects that different types of incubation conditions, different water sources used for incubation medium, or varying medium exchange regimes have on control embryos. This study attempts to determine the advantages and disadvantages associated with incubations of both individual embryos and groups of embryos, and to determine the quality of water and types of medium exchange regimes needed to establish adequate control data for individually incubated medaka embryos. It is necessary to know such information before proceeding to the level of toxicity testing with this assay. The endpoints evaluated are hatching success, mortality rate, time-to-hatch, and the period of time over which hatching occurs. Three different methods of incubation are compared (Experiments A and B). These include two methods of incubating groups of embryos (Bowers III, et al., 1980; Marty, et al., 1991), and the incubation of single embryos by a method developed in this laboratory. Among the advantages associated with the incubation of individual embryos are:

- (1) control of the spread of external fungi, bacteria, and/or parasites without the addition of antibacterial agents such as methylene blue,
- (2) ability of researchers to observe and follow development in individual embryos, as well as the progression of anomalies, and

(3) ability of researchers to work with smaller sample sizes (i.e. 2350 individual embryos, as opposed to 3 - 5 groups of 10 embryos for each exposure).

Both types of group incubations are less labor intensive than individual incubations. However, due to both the spread of fungi and/or parasites within groups and the inability to view abnormalities as well, the incubation of individual embryos appears to be the most useful method of the three tested. When various laboratory water supplies were tested as sources for rearing medium (Experiment C), the results indicate that sterile water (distilled Millipore water) that is free of residual chlorine provides excellent control data. The results also indicate that medium exchanges increase the hatching success (Experiment D) , however, little difference in hatching success or mortality rate was found between media exchanges done every 24 h and those done every 48 h (Experiments E, F and G). While embryos incubated in aerated medium did show shorter, more discrete hatching periods, aeration of media would not always be a recommended approach for toxicity testing due to the possible loss of volatile compounds. In the interest of time and labor conservation, the less laborious 48 h, non-aerated exchange regime is recommended for the production of adequate control data.

2. INTRODUCTION

Toxicologists use embryo and embryo-larval assays to assess potential lethal and sublethal effects of aquatic pollutants. The embryo is widely considered to be

the most sensitive stage of the life-cycle (McKim, 1977), thus toxicologists can use embryo and embryo-larval tests to by-pass more costly and time consuming whole-life-cycle tests. One species of fish that is gaining popularity in this field is the Japanese medaka (*Oryzias latipes*). Various medaka embryo and embryo-larval assays have been used in toxicity testing, both to replace or augment current tests that use embryos of other non-mammalian species (i.e. Briggs and Wilson, 1959; Dial, 1978a; Hiraoka and Okuda, 1983; Marty, et al., 1990; Marty, et al., 1991). The use of the medaka embryos in experiments offers several advantages:

- (1) Adults are small, easy to maintain, and require less space than larger species.
- (2) Adults are very hardy and can be induced to spawn with temperature and photoperiod cues.
- (3) Approximately 10 - 30 embryos can be collected daily from each female.
- (4) Medaka embryos have a clear chorion that allows for non-invasive observation of the developing embryos.
- (5) There is a large volume of literature examining the basic biology, reproduction, and development of the Japanese medaka.

While researchers continue to accumulate data on the sensitivity of medaka embryos to various pollutants, little work has been directed at developing techniques that can eventually lead the EPA or ASTM to recommend or accept a medaka embryo assay. For example, in previously reported medaka embryo

studies, researchers have incubated embryos individually (Wisk and Cooper, 1990) or as groups of embryos in a single container (Heisinger and Green, 1975; Schreiweis and Murray, 1976; Dial, 1978a, b; Leung and Bulkley, 1979; Stoss and Haines, 1979; Solomon and Weis, 1979; Bowers III, et. al., 1980; Michibata, 1981a, b; Hiraoka and Okuda, 1983; Michibata et. al. 1986; Shi and Faustman, 1989; Marty et. al., 1991). Another variable between studies has been the exchange of the rearing medium. In some studies the medium was not exchanged during incubation (Schreiweis and Murray, 1976; Stoss and Haines, 1979; Solomon and Weis, 1979; Marty, et. al., 1991), while others have exchanged the medium on some regular basis (Ishida, 1951; Smithberg, 1962; Heisinger and Green, 1975; Dial, 1978a, b; Leung and Bulkley, 1979; Bowers III, et. al., 1980; Sakaizumi, 1980; Michibata, 1981a, b; Hiraoka and Okuda, 1984a; Michibata, et. al., 1986; Michibata, et. al., 1987).

Although researchers have also used varied chemical formulations for medaka rearing medium, the medium used by Kirchen and West (1976) is most commonly used and accepted. Due to this widespread use, it has been selected for use in this study, although without the antibacterial agent methylene blue. Other incubation media that have been used include various other high-salt compounds (e.g. Heisinger and Green, 1975; Dial, 1978a, b; Cloud, 1981; Cameron and Hunter, 1984; Cameron, et. al., 1985a, b; Chong and Vielkind, 1989; Belanger, et. al., 1990), distilled water (Michibata, 1981a; Cameron and Hunter, 1984), dechlorinated water (e.g. Hiraoka and Okuda, 1983, 1984a, b),

and well water (e.g. Akiyama, 1970). Such wide variation is possible because medaka embryos will develop normally and hatch in (1) concentrated sea water, (2) sea water, (3) fresh water, or (4) distilled water (Yamamoto, 1934, 1941).

In the development of alternative species assays, one of the necessary areas of research is the establishment of refined protocols, as outlined by Goss and Sabourin (1985). The experiments reported here are part of a larger project, the overall goal of which is to provide techniques for a medaka embryo assay that is flexible enough to test laboratory-derived solutions, effluent samples, and soil extracts. Whenever possible, the methods used should not be labor-intensive and should consistently provide good control data.

The primary objective of these experiments is to examine the effects of different culturing techniques on medaka embryos. This study compares several different culturing techniques to determine if these methods themselves produce varying results. Four factors are specifically investigated:

- (1) the advantages and disadvantages associated with incubation of both individual embryos and groups of embryos in media without methylene blue,
- (2) the quality of water needed to make up the embryo culture medium,
- (3) the effects of several different culture-medium exchange regimes, and

(4) the effects of using either aerated or non-aerated culture medium.

3. MATERIALS AND METHODS

3.1 Fish Care and Culture

Japanese medaka (*Oryzias latipes*) fry were obtained from the Gulf Coast Research Laboratory in Ocean Springs, Mississippi. New fish stocks arrived at 7 - 8 d posthatch and were maintained at 23 - 25°C under a 16 h light to 8 h dark photoperiod until adulthood. Fish were fed Stress Flakes (Aqua-Vet, Kordon Oasis, Haywood, Ca.) 2 to 3 times daily and freshly hatched brine shrimp once daily. Breeding stocks were maintained in 15-gal flow-through glass aquaria at a ratio of 3 females to 2 males, for a total of 60 fish per 15 gal of water.

Dechlorinated tap water for the breeding aquaria was passed through an activated carbon filter, a UV sterilizer (H-O-H Bio Industries, Model UV25), and a series of 3 - 25 μm filters (Balston 54/50 filter system with LP-20 cartridges), and then into a collecting tank, where it was heated to $25 \pm 1^\circ\text{C}$ prior to entering the aquaria.

3.2 Embryo Collection

Beginning 2 - 3 h after the onset of the light cycle, females were removed from the aquaria with soft nylon nets. Embryos were gently collected from the

outer ventral area with either gloved fingers or blunt-ended forceps, then placed into petri dishes containing embryo-rearing medium (ERM) (modified from Kirchen and West, 1976), consisting of 1 g NaCl, 30 mg KCl, 40 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and 163 mg $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in 1000 ml of water. Clutches were gently separated and chorionic filaments removed from the surface of the embryos with the aid of a binocular dissecting microscope and fine-tipped forceps. Damaged embryos, unfertilized eggs, and any embryos remaining from previous spawns were discarded.

For experiments comparing individual and group incubations (see Experiments A and B, Table 2.1), embryos were either incubated in groups of 8 or 10. Groups of 8 embryos were incubated in 20 ml scintillation vials (Kimble) containing 2 ml of rearing medium. Groups of 10 embryos were incubated in 60 x 15 mm disposable petri dishes containing 20 - 25 ml of rearing medium. These experiments used both ERM (Kirchen and West, 1976) and sterile water (Millipore-filtered water) as rearing media. Within each test (A and B), replicate experiments were run.

For individual incubations, viable embryos were transferred with wide-tipped, sterile, plastic pipets from petri dishes to 7 ml vials (Kimble) that contained 1 ml of rearing medium and 6 ml of air space (medium varied with experiments, see Table 2.1 and section 3.4). Damaged embryos were usually dead within the first 8 h of the experiment. These were removed and replaced with spare embryos that had been identically treated up to this point. Embryos were

Table 2.1 List of experiments with treatment groups and respective variables tested in each experiment. Primary variables are the type of incubation media used, the exchange regime for the media and the use of either aerated or non-aerated media.

Experiment	N	Treatment groups	Incubation media	Media exchange regimes (h)	Media aeration (+/-)
Experiment A ^a					
individual	50 ^b	1 embryo/6 ml vial	ERM ^c (1 ml)	every 48 h	(-)
groups of 10	50 ^b	10 embryos/petri dish	ERM ^c (20 - 25 ml)	every 48 h	(-)
groups of 8	48 ^b	8 embryos/20 ml vial	ERM ^c (2 ml)	none	(-)
Experiment B ^a					
individual	50 ^b	1 embryo/6ml vial	sterile water ^d (1 ml)	every 48 h	(-)
groups of 10	50 ^b	10 embryos/petri dish	sterile water ^d (20 - 25 ml)	every 48 h	(-)
groups of 8	48 ^b	8 embryos/20 ml vial	sterile water ^d (2 ml)	none	(-)
Experiment C	40	1. ERM1	1. sterile water ^c 2. dechlorinated tap water 3. UV-sterilized dechlorinated tap water	1. every 48 h	1. (-)
	49	2. ERM2		2. every 48 h	2. (-)
	49	3. ERM3		3. every 48 h	3. (-)
Experiment D	15	1. Non-renewal A	1. ERM ^d 2. ERM ^d	1. none	1. NA
	40	2. Non-renewal B		2. none	2. NA

Experiment	N	Treatment groups	Incubation media	Media exchange regimes (h)	Media aeration (+/-)
Experiment E	100	1. 24H-N	1. ERM ^d	1. every 24 h	1. (-)
	100	2. 48H-N	2. ERM ^d	2. every 48 h	2. (-)
Experiment F	100	1. 24H-A	1. ERM ^d	1. every 24 h	1. (+)
	100	2. 48H-A	2. ERM ^d	2. every 48 h	2. (+)
Experiment G	50	1. 48H-A2	1. ERM ^d	1. every 48 h	1. (+)
	50	2. 24H-N2	2. ERM ^d	2. every 24 h	2. (-)

^aExperiments A and B were each done in replicate.

^b(N) denotes the sample size in one replicate of these experiments.

^cERM was made with sterile water (as was ERM1 in Experiment C).

^d"Sterile water" is distilled water that is passed through series of Millipore filters.

incubated in a $25 \pm 1^{\circ}\text{C}$ water bath for up to 28 d. Vials remained closed except during changes of the rearing media. Sterile ERM-exchange regimes, which varied with the individual experiments, were carried out until 72 h posthatch.

3.3 Observations

Embryos were observed every 24 h using a binocular dissection microscope, regardless of ERM-exchange regime. The following observations were recorded:

- (1) descriptions of any visible lesions,
- (2) mortalities (with descriptions of the embryo and chorion),
- (3) presence of fungal growth,
- (4) hatching,
- (5) time of hatch,
- (6) ability of the newly hatched fry to swim, and
- (7) ability of newly hatched fry to inflate their swim bladders.

Fry were observed for 72 h posthatch.

3.4 Experimental Design

Experiments comparing the two different group incubations and individual incubations were run in duplicate (Experiments A and B). A series of five experiments (C through G) were conducted to determine the better method for

the incubation of individual medaka embryos. The sample groups within each experiment are listed in Table 2.1, including the variables tested.

In both A and B, two methods were used for group incubations. These were taken from previously reported uses of medaka embryos (Bowers III, et al., 1980 and Marty, et al., 1991), in an attempt to determine the better method for group incubations. Groups of embryos were either incubated in 60 x 15 mm disposable petri dishes (10 embryos per dish) or in 20 ml scintillation vials (8 embryos per vial). The petri dishes contained 20 - 25 ml of rearing medium, and received media exchanges every 48 h (Bowers III, et al., 1980). Embryos in the scintillation vials were incubated in 2 ml of rearing medium and did not receive media exchanges during the first 8 d postfertilization (Marty, et al., 1991). Simultaneously with the group incubations, other embryos were individually incubated, receiving media exchanges every 48 h with nonaerated rearing media. All three groups of embryos were incubated (Fisher Model 146 Low Temperature Incubator) at $25 \pm 1^{\circ}\text{C}$. Replicate tests within Experiment A used ERM (Kirchen and West, 1976) for rearing medium, whereas replicate tests within Experiment B used sterile water. Replicate tests in Experiment A were run on 03/07/92 and 05/01/92, respectively. Replicate tests in Experiment B were run on 07/18/91 and 09/10/91, respectively.

In Experiment C, three water sources were selected based on their common availability in laboratories. The water source for ERM1 was sterile

water, and the water source for ERM2 was dechlorinated tap water (routinely used to maintain non-breeding fish cultures within the laboratory). ERM3 was made with dechlorinated tap water that was passed over an activated carbon filter, a UV sterilizer, and 3 - 25 μm filters. Salts for the rearing medium were then added to 1 L of water from each source. Each group of embryos underwent complete 1 ml exchanges of the respective non-aerated ERM (1, 2, or 3) at 24 - 25°C every 48 h.

Experiment D was carried out in two sample sets (non-renewal A and non-renewal B) using embryos collected on different days from the same group of adults. These embryos were incubated in embryo rearing medium made with sterile water and did not receive any medium exchanges throughout the incubation period.

Experiment E was conducted to determine if either a 24 or 48 h exchange regime produced (1) better hatching or time-to-hatch, (2) decreased mortality, or (3) a decreased percentage of developmental abnormalities. ERM was made using sterile water. The two groups of embryos (24H-N and 48H-N) received ERM exchanges every 24 h and 48 h respectively.

Experiment F was designed to evaluate the effects of using freshly aerated ERM for both the 24 h and 48 h exchange regimes. Aliquots of 100 ml of ERM (made up in sterile water) were warmed to 24 - 25°C, with constant aeration (by laboratory-supplied air) for at least 30 min before exchange. Two groups of

embryos (24H-A and 48H-A) underwent ERM exchanges (every 24 and 48 h, respectively) with the freshly aerated ERM until 72 h posthatch.

To have a direct comparison between the aerated and non-aerated techniques, in Experiment G the 48 h aerated exchange regime was compared to the 24 h non-aerated exchange regime. The 24 h non-aerated regime was chosen because embryos in this group had previously demonstrated the best hatching results. Embryos were incubated in ERM (made using sterile water) and received either aerated or non-aerated ERM during media exchanges. The first group of embryos (48H-A2) received aerated ERM exchanges every 48 h, and the second group (24H-N2) received non-aerated ERM exchanges every 24 hours.

3.5 Statistics

Hatch and mortality data from individually incubated embryos were compared using Chi-square analysis with a 0.05 significance level. When only two groups were compared by Chi-square, a correction for continuity was used (Zar, 1984). For individually incubated embryos, the standard error of the proportion was calculated according to Zar (1984). For embryos incubated in groups of 8 or 10 in either petri dishes or 20 ml scintillation vials, statistical evaluation was done using PC-SAS (SAS Institute, Cary, NC). When only two groups were compared (i.e. for pooling replicates), a Student's t-Test was used (SAS); for comparisons of

more than two groups, Tukey tests were used. In both cases, data was arcsine-transformed, and the predetermined significance level was 0.05.

Embryos that died as an obvious result of technical errors during ERM-exchange were removed from the sample and disregarded in the statistical analysis. Embryos that were still alive at 28 d, but that failed to hatch, were counted as mortalities.

4. RESULTS

4.1 Experiments A and B: Comparison of Group and Individual Incubation Methods

The results of comparative tests are included in Table 2.2. No significant differences were found between replicate groups, thus the two replicate groups were pooled for t-Test determinations and means. When the two types of group incubations were compared by Tukey test, no statistical differences were found, either within or between types of incubation media. Growth (either bacterial or fungal) was noted in both petri dishes and 20 ml scintillation vials. Rotifers were also noted in both types of group incubations. Neither rotifers nor fungal/bacterial groups were noted in individually incubated embryos. In embryos incubated in 20 ml scintillation vials, the water often became filled with debris and whitish flocculents. This tended to obscure observations somewhat. Also, incubation of

Table 2.2 Comparison of data for Experiments A and B. Experiments compared three different embryo incubation methods.

	Individual vials	20 ml Scintillation vials	Petri Dishes
Experiment A (ERM)			
N=(number of embryos tested)	100	80	100
Number of embryos/dish	1	8	10
Average percent hatch	98.00%	91.25%	87.0%
± Standard Error	±1.4% ^a	±4.5%	±7.3%
Experiment B (Sterile Water)			
N=(number of embryos tested)	100	78	89
Number of embryos/dish	1	8	10
Average percent hatch	98.95%	86.25%	86.50%
± Standard Error	±1.02% ^a	±5.7%	±2.3%

^aFor individually incubated embryos, ± standard error reflects the standard error of the proportion (Zar, 1984).

individual embryos allowed the observer to follow the developmental progression of each individual embryo; this was not possible in group incubations.

4.2 Experiment C: Laboratory Water Sources

Embryos were incubated in ERM (Kirchen and West, 1976) made with three different water sources of varying quality: ERM1 = sterile water, ERM2 = dechlorinated tap water, and ERM3 = UV-sterilized, dechlorinated tap water. A summary of the data from Experiment I is presented in Table 2.3. Compared to ERM2 and ERM3, a significantly higher percentage of embryos incubated in ERM1 hatched. Embryos incubated in ERM1 also exhibited a significantly lower percentage of mortalities than ERM2 and ERM3 (Table 2.3). Compared to ERM1, significantly more fry from ERM2 and ERM3 incubation groups failed both to inflate their swim bladders and to swim actively within 48 h posthatch (Table 2.3). The time-to-hatch for each group varied as well. Embryos incubated in ERM1 completed hatching within 192 hours of the first successful hatch, whereas embryos incubated in ERM2 and ERM3 required 11.1% and 20% longer, respectively, to complete hatching (data not shown), despite the fact that embryos in ERM2 and ERM3 began hatching earlier than did those in ERM1. Based on these results, ERM1 (Kirchen and West [1976] medium, made in sterile water) was used as the standard hatching medium in Experiments C-2 through C-5. Further investigation into the experimental water sources revealed that ERM2 contained 0.0208 ± 0.0069 mg/L total residual chlorine. This was probably the

result of sub-optimal functioning of the dechlorination system (activated carbon bed) caused by increased water usage throughout the laboratory. Chlorine concentrations were unavailable for the water used to make ERM3; however, additional carbon filters were subsequently added to this system.

Table 2.3 Experiment C: Effects of various laboratory water sources on four different endpoints.

	ERM1 (sterile H ₂ O)	ERM2 (dechlorinated tap H ₂ O)	ERM3 (UV-sterilized, dechlorinated tap H ₂ O)
Sample size	40	49	49
Percent mortality ^a	22.5±6.7	63.3±7.1 ^e	71.4±6.5 ^e
Percent hatch ^b	77.5±6.7	36.7±7.1 ^f	28.6±6.5 ^f
Percent without swimbladder ^c	0	64.3±13.3	61.1±11.8
Percent failure to swim ^d	0	71.4±12.5	55.6±12.1

^aIncludes embryos that either died prior to hatch or that failed to hatch.

^bPercent of embryos that hatched and survived at least 48 h posthatch.

^cPercent of hatched embryos that failed to inflate swimbladder within 48 h post-hatch.

^dPercent of hatched embryos that failed to swim within 48 h posthatch

^eSignificantly different from ERM1, $P < 0.001$.

^fSignificantly different from ERM1, $0.001 < P < 0.005$.

4.3 Experiment D: Static, Non-renewal Incubation with ERM

In an attempt to determine the effect of static, non-renewal incubation, two groups of embryos were incubated throughout development in ERM and did not receive any media exchanges. The two groups of embryos (non-renewal A and non-renewal B) were from different collection days, but from the same population of adult medaka. Both groups exhibited low hatching success, but could not be pooled due to considerable variation between groups (Table 2.4). The fry in the non-renewal B group contained significant proportions that failed to inflate their swim bladders and failed to swim within 48 h posthatch. These effects were not observed in the non-renewal A group because only one of these embryos actually hatched. The time period during which hatching occurred in non-renewal B extended from 288 h postfertilization until 648 h postfertilization. This period was longer than any other group tested.

4.4 Experiment E: 24 h and 48 h Exchange Regime with Non-aerated ERM

Two groups of embryos were incubated in ERM throughout development, and received complete media exchanges either every 24 or 48 h with non-aerated ERM (24H-N and 48H-N). No significant differences were seen in either hatching success or mortality rates for the two groups of embryos (Table 2.5). Both groups showed relatively high hatching success and low mortality. Also, both groups of

Table 2.4 Experiment D: Results of two replicate groups of embryos incubated in ERM without medium exchange.

	Non-renewal A	Non-renewal B
Sample size	15	40
Percent mortality ^a	93.3±6.7	62.5±7.8
Percent hatch ^b	6.7±6.7	37.5±7.8
Percent without swimbladder ^c	NA ^e	53.3±13.3
Percent failure to swim ^d	NA ^e	33.3±12.6

^aIncludes embryos that either died prior to hatch or that failed to hatch.

^bPercent of embryos that hatched and survived to 48 h posthatch.

^cPercent of hatched embryos that failed to inflate their swimbladder within 48 h posthatch.

^dPercent of hatched embryos that failed to swim within 48 h posthatch.

^eNA= Not available, since only one embryo hatched.

Table 2.5 Experiment E: Comparison of 24 h and 48 h exchange regimes using non-aerated ERM.

	24H-N	48H-N
Sample size	100	100
Percent mortality ^a	8.0±2.7	6.0±2.4
Percent hatch ^b	92.0±2.7	94.0±2.4
Percent without swimbladder ^c	6.5±2.6	3.2±1.8
Percent failure to swim ^d	23.9±4.5	11.7±3.3

^aIncludes both embryos that died prior to hatch and embryos that failed to hatch.

^bPercent of embryos that hatched and survived at least 48 h posthatch.

^cPercent of hatched embryos that failed to inflate swimbladder within 48 h post-hatch.

^dPercent of hatched embryos that failed to swim within 48 h posthatch

embryos required 312 hours to complete hatching. Hatching was initiated in the 24H-N and 48H-N groups at 240 and 264 h and continued until 552 and 576 h, respectively. Some fry in both groups failed to inflate their swim bladders, although there was no statistically significant difference between the two groups. A difference was found in the ability of fry to swim within 48 h posthatch, with more fry in the 48H-N group swimming. Embryos in 48H-N group received identical treatment to ERM1 embryos in Experiment C. The disparity between the data for the two groups may be due to the fact that the embryos were collected from different groups of breeding stock, with ERM1 embryos being collected from older adults (1 - 1.5 y old) just prior to their retirement from the breeding tanks. This data appears to indicate that the age of the adults from which embryos are collected may affect the overall condition of the embryos.

4.5 Experiment F: 24h and 48 h Exchange Regime with Aerated ERM

In experiment F, the conditions were identical to those of Experiment E, except that media exchanges were done with freshly aerated ERM (24H-A and 48H-A). When ERM was aerated prior to the solution exchange, there was again no difference between the 24 h and 48 h regimes in mortality and hatching success (Table 2.6). The embryos in 24H-A group required longer to hatch than the 48H group, but both are within the time period required for embryos in ERM1 for Experiment Ic. One difference, however is that this method produced only swimming fry with inflated swim bladders.

Table 2.6 Experiment F: Comparison of 24 h and 48 h exchange regimes using aerated ERM.

	24H-A	48H-A
Sample size	100	100
Percent mortality ^a	7.0±2.6	6.0±2.4
Percent hatch ^b	93.0±2.6	94.0±2.4
Percent without swimbladder ^c	0	0
Percent failure to swim ^d	0	0

^aIncludes embryos that either died prior to hatch or failed to hatch.

^bPercent of embryos that hatched and survived at least 48 h posthatch.

^cPercent of hatched embryos that failed to inflate swimbladder within 48 h post-hatch.

^dPercent of hatched embryos that failed to swim within 48 h posthatch

4.6 Experiment G: Comparison of 48 h Aerated and 24 h Non-aerated ERM Exchange Regimes

Experiment V was a comparison between two media exchange regimes: 24 h exchanges with non-aerated ERM (24H-N2) and 48 h exchanges with aerated ERM (48H-A2). The two methods were comparable in both hatching success and mortality (Table 2.7). However, the 24H-N2 group contained both fry that failed to inflate their swim bladder and fry that were unable to swim. The 48H-A2 group contained a low percentage of fry that failed to inflate their swim bladders, but none that could not swim.

Table 2.7 Experiment G: Comparison of 24 h non-aerated and 48 h aerated ERM-exchange regimes.

	24H-A2	48H-N2
Sample size	50	50
Percent mortality ^a	4.0±2.8	12.0±4.6
Percent hatch ^b	96.0±2.8	88.0±4.6
Percent without swimbladder ^c	8.3±4.0	2.3±2.3
Percent failure to swim ^d	4.2±2.9	0

^aIncludes embryos that either died prior to hatch or failed to hatch.

^bPercent of embryos that hatched and survived at least 48 h posthatch.

^cPercent of hatched embryos that failed to inflate swimbladder within 48 h post-hatch.

^dPercent of hatched embryos that failed to swim within 48 h posthatch

5. DISCUSSION

The overall goal of this project is to develop a method of incubating individual medaka embryos for utilization in a medaka embryo-larval assay.

Previous medaka embryo studies have used various methods, including groups of embryos in single incubation vials (e.g. Heisinger and Green, 1975) and single embryos in individual vials (Wisk and Cooper, 1990). The incubation of individual embryos used in this study offers several advantages over the incubation of groups:

- (1) It allows researchers to observe the developmental progression of each embryo on daily basis.

- (2) It allows researchers to observe both progressive and transient abnormalities and to note the subsequent outcomes of those particular embryos.
- (3) It eliminates the spread of fungi and/or parasites within groups of embryos, and the spread of these agents from dead embryos to otherwise healthy embryos in the same incubation medium and container. This advantage allows researchers to eliminate the use of antibacterial agents that may have adverse affects on the embryos.
- (4) It allows researchers to work with smaller numbers of embryos within each exposure group.
- (5) The use of individual vials with screw caps prevents accidental loss or spillage of the rearing medium that often occurs with Petri dishes. This, in turn, decreases the potential exposure to the researchers.

The only advantage noted in both types of group incubations is the time and labor involved. The incubation of groups of 8 embryos in 20 ml scintillation vials with rearing media exchanges only on incubation day 8 is less labor intensive (Marty, et al., 1991); however, in this study, there was noticeable accumulation of debris (white flocculent) in the vials by incubation days 4 and 5. The use of Petri dishes involved somewhat more labor, receiving rearing media exchanges every 48 h; however, a group of 50 embryos would be incubated in only 5 dishes, as opposed to 50 vials for individually incubated embryos. Petri dishes appeared to

be more susceptible to external influences, such as fungi, parasites, and bacteria. This may be due to the fact that the lids do not form a seal, and thus allow for air exchange in the incubator. In several dishes, embryos which were apparently healthy and viable would become infected with fungi from other, less healthy embryos, leading to an inflated mortality rate for that particular dish. Large numbers of rotifers (10 - 20) were commonly seen in both types of group incubations; however, these were rarely seen in individual vials, and then only 1 or 2 in a vial. The advantages of using individual embryo incubations outweigh the advantages of either type of group incubation used in this study.

No studies have been found that attempt to determine the best incubation procedure for single embryos, or that consider the effects of different water sources or ERM-exchange regimes on the hatching success and time-to-hatch for the embryos. The results from Experiments C through G indicate that there may be considerable variation in some endpoints based on the methods of incubation used. Most notably, the time-to-hatch for the individual embryos varies with ERM-exchange regime and with aerated or non-aerated ERM. When used as a toxicity test, the extension of the time-to-hatch may then result from the treatment (e.g. Leung and Bulkley, 1979) or from variation in procedures.

When various laboratory water supplies were tested as sources for rearing medium (Experiment C), the results indicate that sterile water that is free of residual chlorine provides excellent control data. The results (Experiment D) also indicate that medium exchanges increase the hatching success, however, little

difference in hatching success or mortality rate was found between media exchanges done every 24 h and those done every 48 h (Experiments E, F and G). While embryos incubated in aerated medium did show shorter, more discrete hatching periods, aeration of media would not always be a recommended approach for toxicity testing due to the possible loss of volatile compounds. In the interest of time and labor conservation, the less laborious, 48 h, non-aerated exchange regime is recommended for the production of adequate control data.

Another variable which may have affected the hatching success of the embryos used in some of these experiments was the original age of the adult fish from which the embryos were collected. When embryos were collected from adults that were more than 1 year old, lower hatching rates were seen (Section 4.4: Experiment E). On the other hand, while the embryos used in Experiments A and B were collected at four different times from younger fish (6 - 8 months old), little variation was found in the hatching success of embryos from the four different collection dates (see Section 4.1). This may indicate that older females produce sub-optimal oocytes, possibly incorporating fewer nutrients into each oocyte. Although some correlation was noted between the age of the adult fish and the survivability of the offspring, this study does not adequately test the correlation. Researchers have investigated the correlation between the size of the medaka embryo and the size and age of the parents, but failed to substantiate any firm linkages (Lindsey and Ali, 1973).

While results may be comparable within a laboratory, the variation in techniques hinder comparison with studies done in different laboratories. The need for inter-laboratory comparisons of toxicity data should be met with a standard procedure for medaka embryo-larval assays. The procedures outlined in this paper offer researchers the advantages of single embryo incubation and smaller sample numbers (20 - 50 single embryos as opposed to 3 - 5 groups of 10 - 50 embryos). The data indicate that the methods outlined in this paper can provide a framework for a medaka embryo-larval assay that will produce adequate control data with water sources typically available in laboratories (i.e. Millipore filtration systems).

The data reported here indicate that the methods for the incubation of individual embryos can be used for in-laboratory toxicity testing. If the assay is to be used to evaluate the effects of environmental water sources, additional investigations will be needed. Among these should be an investigation into the types of sterilization treatment that would be adequate for environmental water sources, the type of controls, and a comparison between different incubation methods. The next steps to be taken to investigate the types of uses for which these procedures may be acceptable include using these procedures to :

- (1) test for developmental toxicity in single compound solutions,
- (2) test for developmental toxicity in soil extracts, and
- (3) test for developmental toxicity in environmental water samples.

This information will provide further substantiation and validation that the assay needs for standardization and overall acceptance.

5.1 Conclusions

Goss and Sabourin (1985) list five major criteria that should be filled by both mammalian and alternative species toxicity tests. The tests should be sensitive, specific, reproducible between laboratories, and reliable, and should accommodate extrapolation. The results reported here are intended to be a step toward this goal for the medaka embryo-larval assay. In addition, these procedures allow for close observation and tracking of an individuals' development, whereas group incubations do not. Thus, the individual incubations provide increased flexibility in endpoints.

Future tests should investigate the use of this assay in actual toxicity tests. Ideally, group and individual incubations would be run simultaneously to determine the advantages and disadvantages associated with each type of incubation.

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**PART III: JAPANESE MEDAKA EMBRYO ASSAY FOR
DEVELOPMENTAL TOXICITY: EFFECTS OBSERVED FOLLOWING
EXPOSURE OF EMBRYOS TO BENZO[A]PYRENE.**

1. ABSTRACT

In an effort to further document the use of a medaka embryo assay for developmental toxicity testing, medaka embryos (*Oryzias latipes*) were exposed to benzo[a]pyrene (BaP) in three separate experiments. Microparticulate suspensions of BaP were used in all experiments. The first experiment was a preliminary exposure in which individual medaka embryos were exposed to 10 ppb BaP in embryo rearing medium (ERM), and received medium exchanges either every 24 or every 48 h. Percent mortalities and abnormalities, as well as the time-to-hatch for exposed embryos were compared to untreated, identically incubated embryos. A significantly higher percent mortality was seen in one exposure group (48 h exchanges). Significantly more embryos exhibited circulatory abnormalities and extended time-to-hatch in both BaP-exposure groups. In the second experiment, three different incubation methods were compared. Two different group incubation methods taken from the literature (Bowers III, et al., 1980; Marty, et al., 1991) were compared to the individual incubation method used in the first experiment (48 h media exchange). This experiment was designed to determine the advantages and disadvantages associated with each incubation method when used for embryos exposed to a toxic chemical in a laboratory-prepared solution. Previous tests investigated the use of these methods for control data (see Part II of this document). BaP-exposed embryos (10 ppb) in all three incubation groups showed embryo survival rates that were significantly lower than control rates (54, 59, and 67.5% survival for exposed groups, compared to 96, 98

and 100% for control groups). Numerous disadvantages were associated with both types of group incubations, including accumulation of fungi, bacterial growth, and the inability to observe abnormalities *in situ* or to follow transient abnormalities. In the third experiment, individually incubated embryos were exposed to one of four different concentrations of BaP (0, 4, 6, and 12 ppb), and received media exchanges every 48 h. The appearance of severe abnormalities and increased mortality appeared to be dose-related, with significantly higher mortality ($21.0\% \pm 4.9$) observed in the group exposed to 12 ppb BaP. The time-to-hatch was significantly extended in all three exposure groups. These three experiments demonstrated (1) the lethal effects of BaP on the developing medaka embryo, (2) the ability to monitor various types of abnormalities in individually incubated medaka embryos, and (3) the disadvantages associated with incubating and evaluating groups of medaka embryos for toxicity tests in lab-generated solutions. The results of these experiments indicate that the individual incubation of medaka embryos for developmental toxicity tests may produce more accurate data than group incubations.

2. INTRODUCTION

The Japanese medaka (*Oryzias latipes*) is a fish that is commonly used for embryo toxicity and developmental tests. Medaka embryo assays have been utilized in a number of attempts to determine the toxicity (TL_m 's and LC_{50} 's) and teratogenicity of various xenobiotics. However, there is currently neither an EPA

nor an ASTM protocol for the assay and there has been little consistency in its application, between labs and researchers. For example, initial exposures of medaka embryos to tested xenobiotics have varied from 2 hours postfertilization (Dial, 1978; Solomon and Weis, 1979) to organogenesis (Waterman, 1939; Solomon and Faustman, 1987; Shi and Faustman, 1989); and mode of exposure has varied from short-term pulses (Leung and Bulkley, 1979; Hiraoka, et al. 1989; Marty, et al. 1990) to exposure throughout the incubation period (Heisinger and Green, 1975; Schreiweis and Murray, 1976; Stoss and Haines, 1979; Hiraoka and Okuda, 1983; Michibata, et al. 1987).

This lab is currently investigating various methods of conducting developmental toxicity tests utilizing the medaka, and it is our goal to develop a standard method for the embryo assay which is not labor intensive and can be easily adapted for various classes of chemicals. Ongoing experiments are designed to determine and contrast the effects of exposure of embryos to xenobiotics at several reproductive and developmental levels, including exposure to the parents, at the point of fertilization and during embryogenesis. Data from BaP-exposures will complement existing carcinogenesis and adult toxicity data utilizing the Japanese medaka (Hatanaka et al., 1982; Kyono and Egami, 1977; Hawkins et al., 1985, 1988a, b; Maccubbin, et al., 1987).

BaP has been shown to cause various developmental abnormalities in steelhead trout embryos after 24 h pulse exposure (2 and 20 $\mu\text{g/ml}$ in DMSO) at specific developmental stages (Kocan and Landolt, 1984). This study

demonstrated that the trout embryos were permeable to BaP, could metabolize the parent compound, and excreted some of the metabolites. Embryos exposed earlier in development were seen to both accumulate more and excrete less [^{14}C]BaP. This experiment describes teratogenic effects, including cephalic and spinal deformities, ocular abnormalities, and fin reductions or eliminations.

Others have shown that lesser amounts of BaP could both reduce the hatching success of sand sole (*Psettichthys melanostichus*) embryos (0.10 $\mu\text{g/L}$) and produce developmental abnormalities (Hose, et al., 1982). Kocan and Landolt (1984) observed an extension of the hatching period of the steelhead trout as a result of BaP exposure (2 - 20 $\mu\text{g/ml}$ on either day 15 or 25 postfertilization). BaP was found to produce similar effects in Rainbow trout (Hannah et al., 1982), where aqueous doses of BaP (as low as 0.08 ppb) in the hatching medium extended the hatching time of the embryos. Such results are not necessarily consistent in all species of fishes. For example, Hose et al. (1982) reported that the survival of flathead and English sole was unaffected by exposure to BaP.

The object of the work described here was to determine if the polycyclic aromatic hydrocarbon, benzo[a]pyrene (BaP), could cause measurable developmental or lethal effects in medaka embryos exposed from 4 hours postfertilization through the yolk sac absorption stage. Other data on adult medaka BaP exposures done in this lab have indicated that this species may have some resistance to BaP (unpublished data), thus this experiment will also provide data on the sensitivity to another life-stage of the medaka.

3. MATERIALS AND METHODS

3.1 Adult Care and Maintenance

Japanese medaka fry (*Oryzias latipes*) obtained from the Gulf Coast Research Laboratory, Ocean Springs, MS, were raised to adulthood in 100 gal flow-through aquaria supplied with dechlorinated tap water. Adult fish were maintained at 25°C under a 16 h light to 8 h dark photoperiod. Medaka were offered Stress Flakes twice daily and 24-h-old brine shrimp once daily. Breeding stocks were maintained at a ratio of 3 females to 2 males, at a density of 5 medaka per gal. Breeding tanks were supplied with dechlorinated tap water that was purified by passage through an activated carbon filter cartridge, a UV sterilizer and a series of three 25- μ m filters (Balston 54/50 LP-20 cartridge filters).

3.2 Embryo Collection

Embryos were collected beginning 1 h after the initiation of the light cycle. Females were removed from breeding tanks with soft, nylon nets. Embryos were gently removed from the outer abdomen with gloved fingers or blunt forceps and placed into a (150 x 10 mm) Petri dish containing embryo rearing medium (ERM). ERM was modified from Kirchen and West (1976), and contained 1 g NaCl, 30 mg KCl, 40 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 163 mg $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in 1 L of sterile water (distilled Millipore-filtered). Embryos taken from different breeding tanks were placed into the Petri dish and mixed thoroughly in an attempt to randomize the

embryos prior to separation into exposure groups. After removal from the female, embryos remain in clutches of 3 - 50 each, held together by chorionic filaments. Clutch separations and removal of these filaments were done under a binocular dissection microscope using fine-tipped watchmakers' forceps. Damaged or unfertilized eggs, and any embryos which were obviously remnants of a previous day's spawns, were discarded.

3.3 BaP Exposures

3.3.1 Experiment 1: Initial exposure of embryos to BaP

Treatment with BaP was initiated within 3.5 to 4 h following embryo collection. There were three treatment groups in Experiment 1: a) controls, embryos that were incubated in ERM alone and received exchanges of aerated ERM every 48 h until hatch, at which time they received 48 h exchanges of aerated, distilled water, b) 24 h BaP, embryos that were incubated in ERM + 10 ppb BaP and received exchanges of this aerated solution every 24 h until hatch, at which time they received 24 h exchanges of 10 ppb BaP in distilled water, and c) 48 h BaP, embryos that were incubated in ERM + 10 ppb BaP and received exchanges of this aerated solution every 48 h until hatch, at which time they received 48 h exchanges of 10 ppb BaP in sterile water.

Individual embryos were placed into 7 ml scintillation vials containing 1 ml of either ERM or ERM + 10 ppb BaP. Incubations were carried out at 24.5 -

25°C under low light conditions with a 16 h light, 8 h dark photoperiod. Medaka embryos have a clear chorion, making microscopic observation of development easy. Embryos were observed, until 72 h posthatch, at 24 h intervals for abnormalities, deaths, or hatching. Once hatched, fry were observed daily for survival, swimbladder inflation, development of swimming ability, and posthatch manifestation of any other obvious developmental abnormalities.

3.3.2 Preparation of exposure solutions for Experiment 1

ERM + 10 ppb BaP for exposures was made using BaP-coated glass beads. The glass beads (10 - 20) that had been previously coated with BaP were placed into a glass jar and ~250 ml of ERM was added. The mixture was stirred for 24 h, after which a 10 ml aliquot was recovered for analysis on HPLC. This procedure was repeated every 48 h. Beads were replaced when chemical analysis showed any changes in BaP concentration of the ERM medium. BaP concentrations in the stock solution did not fall below 6 ppb throughout the experiment.

3.3.3 Experiment 2: Comparison of three different incubation techniques

In Experiment 2, we compared the incubation techniques used in two reported studies to the incubation techniques developed in this laboratory (see Part II of this document, and section 3.3.3 of Part III). In one reported method, groups of 8 embryos were incubated in 20 ml scintillation vials containing 2 ml of

incubation medium (Marty, et al., 1991). These embryos did not receive any medium exchanges during the first 8 - 9 d following fertilization, but received one complete medium change at the onset of hatching. At this time, the incubation medium was replaced with 2 ml of sterile water. In the second method, groups of 10 embryos were incubated in 60 x 15 mm petri dishes containing 20 - 25 ml of incubation medium (Bowers III, et al., 1980). These embryos received complete medium exchanges every 48 h throughout development. The third set of embryos was incubated individually in 7 ml scintillation vials, each containing 1 ml of medium. These received complete exchanges every 48 h. There were both control and exposed groups (12 pb BaP) within each incubation test type. All three test groups were observed every 24 h for abnormalities, mortalities, and hatching. Dead embryos and fry were removed daily.

3.3.4 Experiment 3: Dose-response determination

In Experiment 3, embryos were exposed to one of three concentrations of BaP in ERM: a) 12 (stock) ppb BaP, b) 6 ppb BaP, or c) 4 ppb BaP. There was also a control group that was incubated in ERM. Treatment of embryos with BaP was initiated within 3.5 - 4 h following embryo collection.

As in Experiment 1, individual embryos were placed into 7 ml scintillation vials containing 1 ml of exposure medium. Incubation conditions and observations were identical to those in Experiment 1, except that embryos received complete media changes every 48 h.

3.3.5 Preparation of exposure solutions for Experiments 2 and 3

A generator column was used to produce microparticulate suspensions of BaP. Sterile water was pumped through a glass container filled with BaP-coated beads, and into an amber glass jar. This water was allowed to recirculate through the column for 24 h prior to use. Dilutions were made from this stock water for final concentrations of 12 (stock), 6, and 4 ppb. ERM salts were then added to the final products.

3.4 Chemical Analysis of BaP Solutions

A 10 ml aliquot was removed from test solutions and passed over an equilibrated C18 Sep Pac for chemical analysis every 48 h (corresponding to media exchanges). The collection vial was extracted twice with 2 ml of 20% HPLC-grade methanol. This eluent was then passed through the Sep Pac. All BaP was removed from the Sep Pac with 3 ml of 100% methanol. The 100% methanol eluent was then taken to dryness under air at 85°C and resuspended in 100 μ l of 100% methanol. The sample was injected onto an HPLC C18 column and eluted isocratically with 100% methanol. BaP quantities were calculated by comparison of peaks to known standards. Solutions that contained BaP in concentrations exceeding the solubility of BaP in water (4 ppb) were stirred (in amber flasks) for 48 h prior to use and rigorous sampling was done to insure a thorough mixture of the microparticulate suspension.

3.5 Statistics

Data for mortality and hatching success for individually incubated embryos were subjected to Chi-square analysis with a predesignated 0.05 significance level. When two-by-two contingency tables were analyzed, corrections for continuity were included. Standard error of the proportion (S_p) was calculated according to Zar, 1984. Mortality and hatching success data for embryos incubated in groups were analyzed using PC SAS (SAS Institute, Cary, NC). Comparisons of groups of data were done using a Tukey test. Data was arcsine-transformed and the predetermined significance level was 0.05.

4. RESULTS

4.1 Experiment 1

4.1.1 Abnormalities in embryo and fry

Both 24 h BaP and 48 h BaP groups showed significantly more developmental abnormalities during embryogenesis than the control group. The most commonly observed abnormalities were pooling of the blood along the veins of the yolk sac and within the developing embryo. Pooling generally occurred in the fore- and hindbrain and in the tail region. Pooling in the brain was most obvious at 168 - 192 h postfertilization; thereafter, increased pigmentation made continued observation difficult. Incidence of circulatory problems were $14.29\% \pm$

3.55 and 12.24% $\pm$ 3.33 in the 24 h BaP and 48 h BaP groups, respectively, as compared with only 4.55% $\pm$ 2.22 of the control embryos (Table 3.1).

Several embryos in the BaP-treated group exhibited multiple abnormalities. There was one case of twinning in the 24 h BaP group, with the second embryo apparent only as a heart and associated areas of pooled blood along with a urinary bladder. The heart of this smaller twin ceased beating prior to hatch, but the larger twin hatched and survived to yolk sac absorption. The remains of the second twin appeared to be incorporated into the abdominal area of the larger twin during yolk sac absorption. Also in this group, one embryo exhibited marked unilateral exophthalmia, severe circulatory problems and an overall yellow-green coloration. This embryo did hatch, but was never observed swimming and failed to inflate its swimbladder. In the 48 h BaP group, one embryo had a large mass of tissue within the yolk sac adjacent to the abdomen. The mass was gray and opaque in coloration. The embryo failed to hatch and was observed dead within the chorion at 456 h postfertilization.

Although not quantitated, it was observed that many of the BaP-exposed embryos and fry had the previously mentioned yellow-green coloration along the dorsal surface and around the outer edges of the mouth. The altered coloration was easily distinguished from the golden-brown coloration of the controls. These embryos did hatch and suffered no apparent problems through yolk sac absorption. No abnormalities other than previously mentioned circulatory problems were observed in the control embryos and fry.

Table 3.1 Percent hatching success and mortality ($\pm$ standard error of the proportion) in the three BaP treatment groups in Experiment 1. Treated embryos were exposed to 10 ppb BaP.

	Control	24HBaP (%)	48HBaP (%)
N=	88	98	98
Embryo mortality	5.68 $\pm$ 2.49	5.10 $\pm$ 2.23 ^a	13.27 $\pm$ 3.40 ^b
Embryo hatch	94.32 $\pm$ 2.48	94.70 $\pm$ 2.23	86.73 $\pm$ 3.40
Fry that failed to inflate swimbladder within 48 h posthatch	2.41 $\pm$ 1.69	2.30 $\pm$ 1.62	3.70 $\pm$ 2.11
Embryos and fry with circulatory problems	4.55 $\pm$ 2.22	14.29 $\pm$ 3.55	12.24 $\pm$ 3.33

^a2 embryos failed to hatch by 648 h postfertilization

^b1 embryo failed to hatch by 648 h postfertilization

4.1.2 Mortality, hatching success and swimbladder inflation

The group of 48 h BaP embryos exhibited a significantly higher percent mortality than the 24 h BaP or the control embryos. No significant differences were seen in the ability of the fry to inflate their swimbladders (Table 3.1). All control embryos that survived to 288 h postfertilization hatched successfully; however, both 24 h BaP and 48 h BaP groups contained embryos which failed to hatch within 840 h postfertilization. Unhatched embryos were treated as mortalities for statistical purposes (Table 3.1). There were distinct differences in

the time-to-hatch for the various treatment groups. BaP-exposed embryos did not complete hatching during the time period required for the controls. BaP-exposed embryos continued to hatch for 192 h after the last control embryo had successfully hatched. Control embryos hatched in 46.5% of the time required for the exposed embryos, leaving 15.3% of 24 h BaP and 13.3% of 48 h BaP embryos still unhatched. Another way to express time-to-hatch is the time at which 50% of the embryos have hatched (Leung and Bulkley, 1979). Embryos in BaP-treatment groups required 48 to 72 h longer for 50% hatch.

No further abnormalities were observed in the fry and there was no significant difference in the time during which the fry were able to actively swim. This parameter was determined by noting the time period during which each fry was observed swimming and the time at which swimming ceased. Without the addition of food, fry generally live 2 to 4 d following yolk sac absorption. Cessation of swimming usually preceded death by 48 to 96 h.

4.2 Experiment 2

4.2.1 Comparison of hatching success and mortality rates with three different types of incubations

The hatching success data for the three types of incubations are reported in Table 3.2. In each case, the lethal effects of BaP in a laboratory-prepared

Table 3.2 Percent hatching success in Experiment 2, comparing two types of group embryo incubations and a single embryo incubation.

Treatment	Individually Incubated Embryos		Groups of 8 Embryos		Groups of 10 Embryos	
	Control	12 ppb BaP	Control	12 ppb BaP	Control	12 ppb BaP
N=	50	50	5 ^a	5 ^a	5 ^b	5 ^b
Average Percent Hatch	98%	54%*	100%	67.5%**	96%	59%**
± Standard Error			0.00	5.00%	4.00%	8.86%

^aN= 5 groups of 8 embryos

^bN= 5 groups of 10 embryos

*Data are statistically different from controls when analyzed by Chi-square, significance level is 0.05.

**Data are statistically different from controls when analyzed by Tukey test, with a significance level of 0.05.

solution compared to controls is evident. However, consistent documentation of transient abnormalities and developmental delays was not possible in group incubations. Also, consistent with data presented in Part II of this document, the presence of fungi and/or bacteria was noted in both types of group incubations.

4.3 Experiment 3

4.3.1 Abnormalities seen in embryos and fry

Embryos exposed to varying concentrations of BaP exhibited both developmental abnormalities and mortalities (See Figures 3.1 and 3.2) In Figure 3.1, these are presented as general, retinal, and lethal abnormalities. For discussion purposes, general abnormalities includes all observed abnormalities, including severe, minor, or transient abnormalities. Abnormal retinal development occurred in all three groups of exposed embryos. This was most commonly seen as diffuse or light retinal pigmentation. Although these embryos continued to develop and hatch, they failed to live longer than 48 h posthatch. The third group of abnormalities includes those considered to be lethal abnormalities. These were embryos that died prior to hatch and were observed with severe abnormalities. Severe abnormalities include large areas of pooled blood, irregularly or incompletely enveloped hearts, and gross morphological abnormalities. The occurrence of lethal abnormalities appears to be dose-related (Figure 3.1).

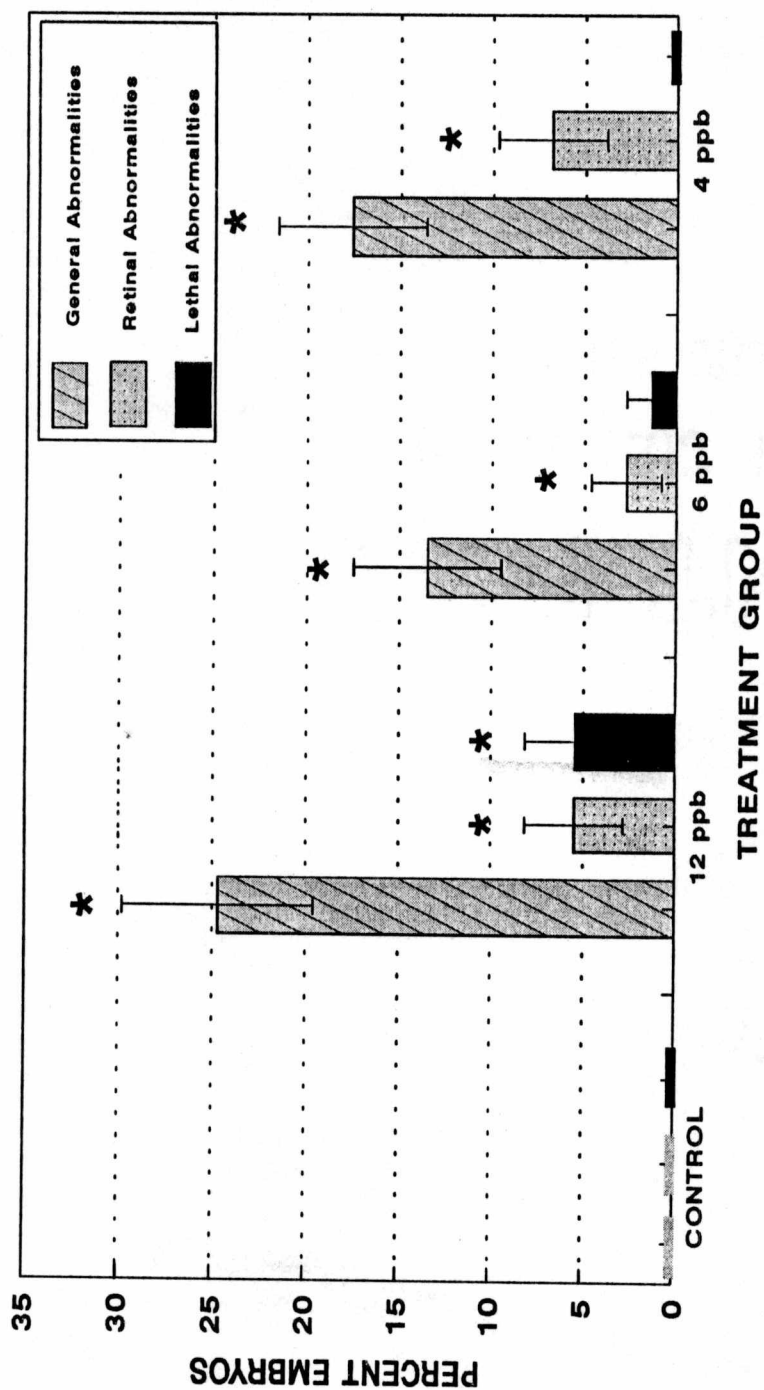


Figure 3.1 Abnormalities seen in BaP-exposed embryos. General abnormalities include all observed abnormalities, regardless of severity. Retinal abnormalities include only those embryos with abnormally developed eyes, and lethal abnormalities include those embryos that had severe abnormalities and died prior to hatch. Error bars indicate the standard error of the proportion (Zar, 1984). (*) denotes significant difference from controls at $P < 0.001$ by Chi-square analysis.

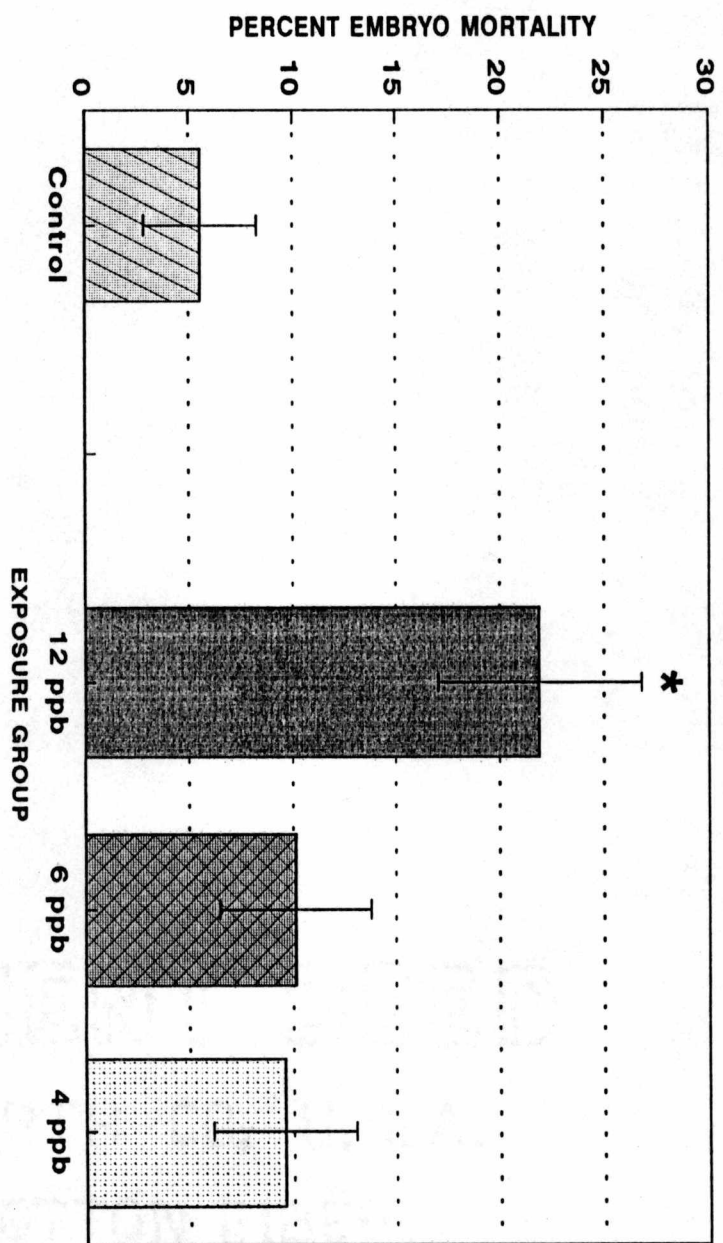


Figure 3.2 Percent mortality in BaP-exposed embryos. Error bars indicate the standard error of the proportion. (*) denotes significant difference from controls at $P < 0.001$ by Chi-square analysis.

4.3.2 Mortality, hatching success and time-to-hatch

Only those embryos exposed to 12 ppb BaP experienced a significantly higher mortality rate than the controls ($21.9\% \pm 4.9$ -v- $5.6\% \pm 2.7$). While mortality appears to be dose-related, the trend is not statistically significant (Figure 3.2). There is also an increased time-to-hatch in exposed embryos in a threshold, not dose-dependent type response. All three exposure groups exhibited a significantly later mean hatch time than controls ($P < 0.0001$) and continued hatching for a longer period of time (see Figure 3.3).

5. DISCUSSION

The primary goal of this study was to determine if BaP could produce an effect on medaka embryos when exposure was initiated within 4 h postfertilization and continued through yolk sac absorption. Marty, et al. (1990) documented the uptake of labeled BaP into medaka embryos over time. The increased incidence of developmental abnormalities and prevalence of circulatory problems in both BaP-exposure groups provides evidence that the compound is entering the embryo and affecting development. Not only did the hatching success and mortality of the 48 h BaP embryos differ significantly from the controls, both exposure groups exhibited extended hatching times. The extended hatching time corresponds with the data presented in Kocan and Landolt (1984) where the time to hatching for steelhead trout embryos was also extended when these embryos were exposed to BaP. These researchers theorized that BaP could interfere with the function of

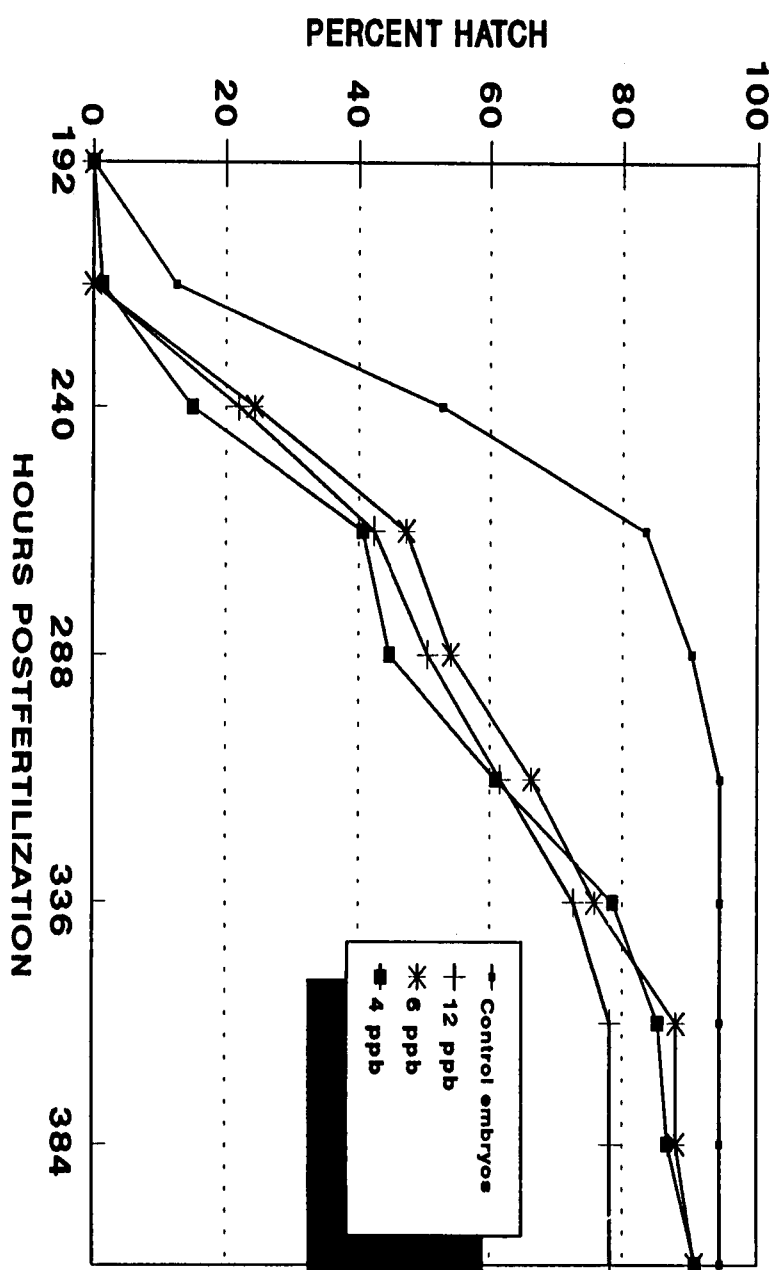


Figure 3.3 Time-to-hatch profile for BaP-exposed embryos. Arrows indicate the time at which the last embryo had successfully hatched. When analyzed by Tukey test, the time-to-hatch for the three exposure groups differed significantly from the controls ($P < 0.001$).

the hatching enzyme, thereby altering the hatching dynamics of the treated embryos. It has been established that such enzymes exist and control hatching, both in the medaka and in other fish (Ishida, 1944; Blaxter, 1969; Yamagami, 1981). Interference with the ability of the enzyme to function would account for both extended time to hatch and failure to hatch. Leung and Bulkley (1979) found that varying concentrations of benzene could alter the ability of medaka embryos to hatch, with premature hatching occurring at lower concentrations and delayed hatching occurring at higher concentrations.

The mortality and hatching success reported in this study is counter to other data reported for BaP-exposed embryos, including Hannah, et al. (1982) and Kocan and Landolt (1984). These reported no significant decrease in hatching success or increase in mortality. One might expect fry survival time to be altered (compared to embryonic survival) by the continued exposure to BaP, however, fry may develop resistance to some chemicals. For instance, Nebeker, et al. (1974) found that, in the case of one PCB mixture, fry produced by fathead minnow adults aqueously exposed to the commercial PCB Aroclor 1242 during spawning and subsequent embryonic development, showed excellent survival if kept at the same concentrations of the PCB as the adults and embryos. This could indicate a resistance developed toward specific compounds or chemical classes that the parents confer on the offspring or which is acquired via exposure during embryonic development. Exposure of fry which undergo embryonic development

in control ERM would begin to elucidate the sensitive stage and possibility of resistance.

Additionally, this laboratory is in the process of documenting the effects of different culturing techniques on medaka. Thus far, we have developed the previously described protocol, which enables us to incubate individual embryos without the use of the antibacterial agent methylene blue. This type of incubation procedure has several advantages for the researchers. First, it allows the observer to follow the progress of individual embryos on a day to day basis, where culturing several embryos in a petri dish or vial does not. Second, it prevents possible interactions between embryos, such as stimulating hatching, and, thirdly it prevents the spread of bacteria or algae between embryos. This becomes very important when environmental water sources are tested. This water is generally sterile filtered (0.1 μm Nalgene filters), but problems still occur with infection in the embryos (see Part IV of this document). Since embryos may contain some microbes from the parents and the water in which they were spawned, it is possible that some water sources are providing nutrients for these endogenous infections. When distilled, sterile water is used, there is no evidence of contamination in the embryos.

While lack of aeration has been shown to extend the time-to-hatch in control embryos, the time extension observed as a result of BaP exposure was significantly greater than that of the non-aerated control. Thus, this aspect of the incubation procedure did not affect the end results seen with BaP exposures.

Further studies should be two-fold:

(1) the verification of altered time-to-hatch, increased abnormalities and mortality in BaP-exposed medaka embryos, and

(2) exposure during other reproductive and developmental times

(a) fry through yolk sac absorption,

(b) gametes during fertilization, and

(c) parental exposure prior to spawning.

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**PART IV: JAPANESE MEDAKA EMBRYO ASSAY FOR
DEVELOPMENTAL TOXICITY: EVALUATION OF THE TOXICITY OF
TWO TCLP EXTRACTS FROM U.S. ARMY KWAJALEIN ATOLL,
REPUBLIC OF THE MARSHALL ISLANDS**

1. ABSTRACT

Three different incubation methods were used to observe lethal and sublethal effects in medaka embryos (*Oryzias latipes*) exposed to contaminated soil extracts throughout embryonic development. Soil extracts were made from soil samples taken on the U.S. Army Kwajalein Atoll, in the Republic of the Marshall Islands, during February, 1991. Embryos incubated in groups experienced more embryonic mortality and reduced hatching success, whereas individually incubated embryos exhibited a higher frequency of abnormalities. Embryos exposed to the two soil extracts exhibited toxic and teratogenic effects. Group incubations may provide an increased stress on the embryos, causing a higher or amplified mortality in those samples. Abnormalities were more easily observed and followed in individually incubated embryos; this may account for the increased abnormality rate reported for this group.

2. INTRODUCTION

Variations of the medaka embryo assay have been used to determine the toxicity and teratogenicity of single compounds and complex mixtures for many years. Recently, the assay has been used to evaluate the toxicity of soil extracts (Marty, et. al., 1991). This use of medaka embryos opens the door for further and extended uses in bioremediation.

In the experiments reported here, two types of group embryo incubation methods are compared to a method incorporating individual embryo incubation.

Embryos were exposed to soil extracts from contaminated pits on the US Army Kwajalein Atoll (USAKA), in the Republic of the Marshall Islands. Embryos were exposed throughout embryonic development. In one method (Marty, et al., 1991), groups of 8 embryos were incubated in 20 ml scintillation vials containing 2 ml of solution. In the second method (Bowers III, et al., 1980), groups of 10 embryo were incubated in 60 x 10 mm petri dishes containing 20 - 25 ml of solutions. In the third method, individual embryos were incubated in 7 ml scintillation vials, each containing 1 ml of extract. These experiments are part of a larger project aimed toward developing a method for single egg incubations which can be used to evaluate the toxicity of effluents and leachates. It is important to develop a method of incubation for individual embryos that eliminates any influences one embryo may have upon other embryos, produces adequate control data, and eliminates the use of antifungal agents in the incubation media (e.g. methylene blue).

The endpoints evaluated were hatching success, time required for embryos to achieve 50% hatch, and occurrence of developmental abnormalities. It is assumed that one method will not prove to be ideal for the evaluation of each endpoint, but instead, each method will show advantages and disadvantages. The goal is to determine whether or not these methods are equivalent, and to determine the advantages and disadvantages of using either method.

The two soil samples were taken from test pits from the USAKA in Feb. 1991 by researchers from the Oak Ridge National Laboratory, Oak Ridge, TN

(Siegrist, et al., 1991). One sediment was known to be contaminated with diesel fuel (TP-04-65), while the second sample (TP-01-56) lacked diesel fuel contamination, but was potentially contaminated with other hydrocarbons (Table 4.1).

3. MATERIALS AND METHODS

3.1 Soil Collection

The two soil samples used to produce extracts in this experiment were taken from a group of samples collected at USAKA in February, 1991 by researchers from the Oak Ridge National Laboratory, Oak Ridge, TN (Siegrist, et al., 1991). A summary of the contaminants found in the two soil samples is given in Table 4.1. The identification codes for the two samples are TP-01-56 and TP-04-65, respectively. These designate the test pit (numbers 1 and 4) and the depth at which the sample was taken (i. e. 56 and 65 inches, respectively). Chemical analysis were overseen by Siegrist, et al (1991). The soil sample TP-04-65 was heavily contaminated with diesel fuel, and heavy metals were detected in both samples. TP-01-56 failed to show hydrocarbon contamination at the ppm detection level, but a soil sample from the same test pit (48 inches above TP-01-56) showed ppm contamination of several other hydrocarbons. Few other differences were detected in contaminant presence or concentration between the two soil samples.

Table 4.1 Summary of the physical and chemical analysis for the two Kwajalein soil samples. Table was condensed from Siegrist, et al., 1991.

I. Onsite analysis of saturated soil extracts and basic physical/chemical properties of soil samples.

TEST	UNITS	TP-01-56	TP-04-65
Water content	% DRY WT.	25.30	27.20
pH of soil		9.29	9.16
pH of extract		8.23	8.04
Conductance	μ mhos/cm	525.	470.
NO ₃ -N	ppm	0	0
PO ₄ -P	ppm	2.75	4.75

II. Nutrient content in soil samples

TEST	UNITS	TP-01-56	TP-04-65
TKN	mg-N/kg	110	150
NO ₃ -N	mg-N/L	< 0.1	< 0.1
PO ₄ -P	mg-P/L	< 0.1	< 0.1
SO ₄ -S	mg-S/kg	< 0.1	< 0.1

III. Exchangeable cation content in soil samples

TEST	UNITS	TP-01-56	TP-04-65
Ba	mg/L	< 0.3	< 0.3
Ca	mg/L	440	440
Fe	mg/L	< 0.2	< 0.2
K	mg/L	0.16	0.16
Mg	mg/L	17	20
Mn	mg/L	0.015	0.004
Mo	mg/L	0.16	0.16
Na	mg/L	3.2	3.2
Zn	mg/L	< 0.02	< 0.02

IV. Total hydrocarbons measured in soil

TEST	UNITS	TP-01-56	TP-04-65
Friedel crafts acylation	ppm	< 10	< 10
Infrared spectroscopy	ppm	< 10	4235

V. Concentrations of total petroleum hydrocarbons, semivolatile hydrocarbons, and volatile hydrocarbons in soil

TEST	UNITS	TP-01-56	TP-04-65
Total concentration of hydrocarbons	ppm	< 10	8920 (diesel)
Total concentration of semivolatile organic compounds	mg/kg or mg/L	< 0.03 ^a	0.07 (4,6-dinitro-2-methyphenol)
Total concentration of volatile organic compounds	µg/kg	< 5	< 5

^aTP-01-08: detection of phenanthrene, fluorathene, di-n-butyl phthalate, pyrene, benzo(a)anthracene, chrysene, and BaP, with 4.69 mg/kg SVOC.

VI. Concentrations of heavy metals in soil

TEST	UNITS	TP-01-56	TP-04-65
Silver	mg/kg	1.7	< 1.6
Arsenic	mg/kg	< 16	< 16
Barium	mg/kg	5.7	5.1
Cadmium	mg/kg	< 1.6	< 1.6
Chromium	mg/kg	11	8.8
Mercury	mg/kg	< 0.01	< 0.01
Lead	mg/kg	< 16	< 16
Selenium	mg/kg	< 16	< 16

3.2 Preparation of Extracts

Extracts were produced using a modification of the EPA TCLP procedures (Federal Register, 1990). Both soil samples were extracted into sterile water (Millipore-filtered) to prevent the possibility of toxic effects from the standard extraction buffers. A blank, consisting of sterile water, was treated identically during the extraction process, and served as the control in the embryo assays. When 10% dilutions were made, the diluent was sterile water. Chemical analysis of the extracts was not done due to the limited quantity of extract and preliminary nature of the test. However, the extract from TP-04-65 did smell of diesel. All solutions were sterile filtered (20 μ m) prior to use.

3.3 Animal Care and Embryo Collection

Japanese medaka (*Oryzias latipes*) fry were obtained from the Gulf Coast Research Laboratory in Ocean Springs, Mississippi. New fish stocks arrived at 7 - 8 d posthatch and were maintained at 23 - 25°C under a 16 h light to 8 h dark photoperiod until adulthood. Fish were fed Stress Flakes (Aqua-Vet, Kordon Oasis, Hayward, Ca.) 2 - 3 times daily and freshly hatched brine shrimp once daily. Breeding stocks were maintained in 15-gal flow-through glass aquaria at a ratio of 3 females to 2 males, for a total of 60 fish per 15 gal of water. Dechlorinated tap water for the breeding tank aquaria was passed through an activated carbon filter, a UV sterilizer (H-O-H Bio Industries, Model UV25) and

a series of 3 25 μ m filters (Balston 54/50 filter system with LP-20 cartridges), then into a head tank where it was heated to $25 \pm 1^\circ\text{C}$ prior to entering the aquaria.

Females were removed from the tank with a soft nylon net beginning 2 - 3 h after the onset of the light cycle. Embryos were gently collected from the outer ventral area with either gloved fingers or blunt-ended forceps, then placed into petri dishes containing sterile water. Clutches were gently separated and chorionic filaments removed from the surface of the embryos with the aid of a binocular dissecting microscope and watchmakers' forceps. Damaged or unfertilized embryos and any embryos remaining from previous day's spawns were discarded. Embryos damaged by handling were usually obvious as mortalities within the first 8 h of the experiment. At this time, these embryos were removed and replaced with spare embryos that had been identically treated up to that point. Embryos were observed every 24 h using a binocular dissection microscope, regardless of incubation method. Data recorded included developmental staging, descriptions of any visible lesions, notation of mortalities with descriptions of the embryo and chorion, and presence of fungal growth, hatching, time to hatch and ability of the newly hatched fry to swim and inflate their swim bladders. Fry were observed for 72 h posthatch.

The exposure groups for all three incubation methods were as follows: controls (blank extract), TP-04-65 100% strength, TP-01-56 100% strength, TP-04-65 10% strength, and TP-01-56 10% strength. All dilutions were made with sterile water.

3.4 Embryo Exposures

3.4.1 Method I

An attempt was made to duplicate the incubation procedures used by Marty, et. al., 1991. Groups of eight embryos were placed into 20 ml scintillation vials, each containing 2 ml of test medium. The vials were incubated at 25°C and did not receive medium exchanges during the first 8 - 9 d. Vials remained closed with screw-top lids throughout the experiment. The test solutions were replaced with fresh sterile water when the first embryo hatched, and did not receive any other exchanges. Each exposure group contained 5 - 10 vials, each with 7 - 8 embryos. Dead embryos and fry were removed during daily observations to prevent the spread of fungal growth.

3.4.2 Method II

An attempt was made to duplicate the incubation procedures of Bowers III, et. al., (1980). Groups of 10 embryos were transferred to 60 x 15 mm glass petri dishes containing 20 - 25 ml of test medium. The petri dishes were incubated at 25°C throughout embryonic development. Medium exchanges were done every 48 h and observations every 24 h. Exposure groups contained 3 - 9 petri dishes, each with 9 - 10 embryos. Dead embryos and fry were removed during daily observations to prevent fungal growth from spreading within dishes.

3.4.3 Method III

Individual embryos were transferred with wide-tipped sterile plastic pipets from petri dishes to 7 ml scintillation vials (Kimble) containing 1 ml of experimental medium and 6 ml of air space. Each group consisted of 48 - 50 individually incubated embryos. Embryos were incubated in a 25°C water bath for up to 28 d. Vials remained closed with screw-top lids except during changes in the rearing medium. Medium exchanges were carried out under sterile conditions every 48 h until 72 h posthatch.

3.5 Statistical Analysis

Hatching success data for methods 1 and 2 were analyzed using PC SAS (SAS Institute, Cary, NC). Two-way comparisons were done with SAS ANOVA, and analysis of more than two groups of data were done using a Tukey test. In both cases, data was arcsine-square root transformed and the predetermined significance level was 0.05. Hatching success data for method 3 were analyzed by Chi-square with a 0.05 significance level.

Time-to-hatch data were analyzed by comparing the time required for complete hatching of the embryos treated in each method. All vials in method 1 were pooled for comparison, as were petri dishes in method 2. Since method 1 utilized individual vials, pooling was unnecessary. Fifty-percent hatch time was determined as the 24 h observation period during which 50% of all embryos had hatched (e.g. Leung and Bulkley, 1979).

The percent abnormalities was determined by pooling all petri dish data for method 1 and pooling all vial data for method 2. Percent abnormalities reflects the number of abnormal embryos divided by the number of embryos incubated in each exposure group. Embryos which possessed more than one obvious developmental abnormality were counted only once, thus the percent developmental abnormalities also reflects the percentage of abnormal embryos.

4. RESULTS

4.1 Hatching Success

Control embryos in the three different tests showed no significant difference between incubation methods when analyzed by Chi Square ($0.10 > P > 0.25$). As can be seen in Figure 4.1 - 4.3, the control values for percent hatch ranged from 98% to 86%. Single embryo incubations (method 3, Figure 4.3) resulted in the highest percent hatch.

Figure 4.1 shows the percent hatch for groups of 8 embryos incubated in 2 ml of the two concentrations of the extract (method 1). While only embryos exposed to TP-04-65 at 100% strength exhibited significantly reduced hatching success ($P < 0.05$), 10% TP-04-65-exposed embryos also showed a trend toward

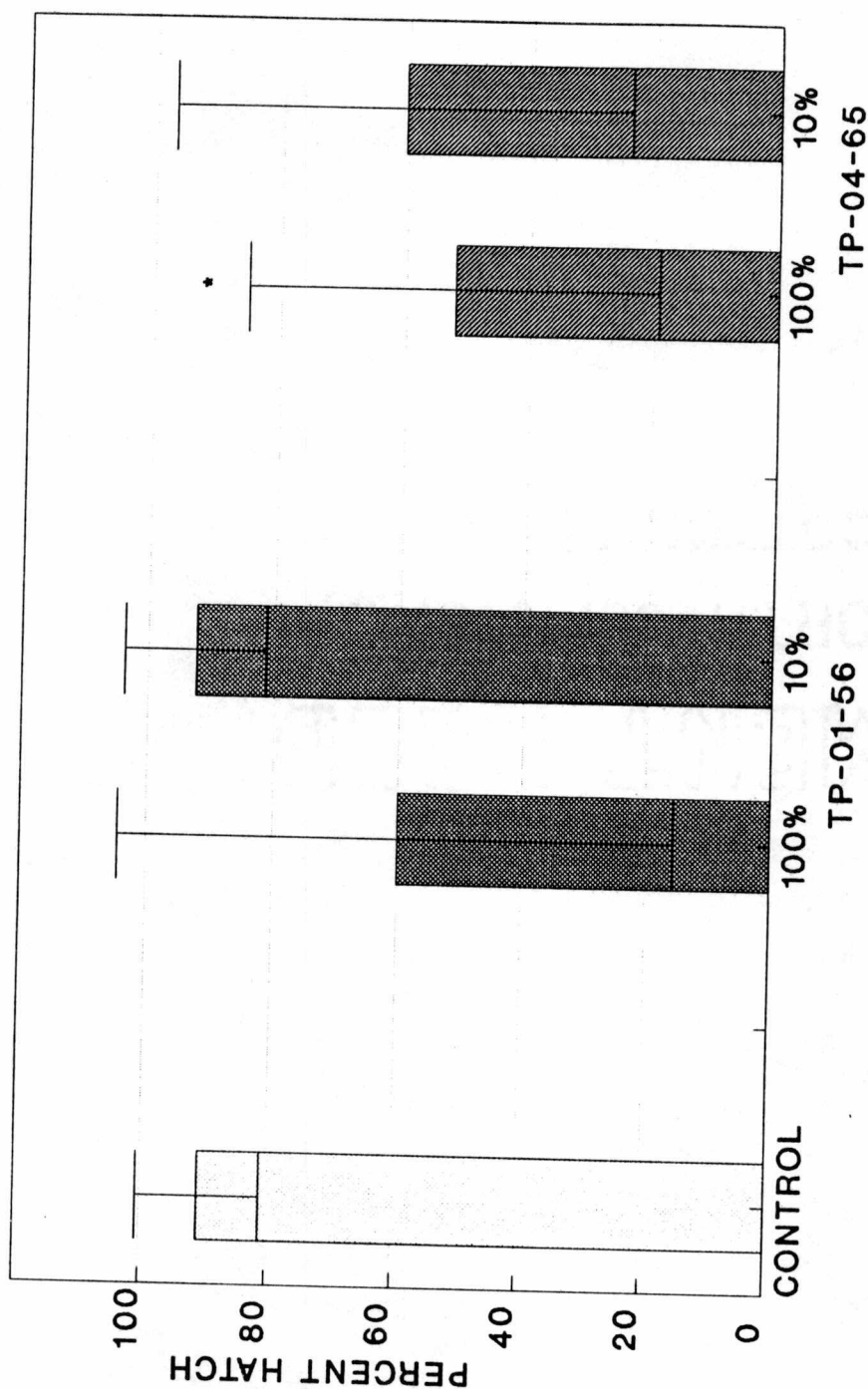


Figure 4.1 Method 1: Percent hatch for embryos incubated in TP-01-56 and TP-04-65 soil extracts using incubation method 1 ($\pm$ standard error of the mean). Groups of 8 embryos were incubated in 20 ml scintillation vials, each containing 2 ml of medium. Arcsine-square root transformed data were analyzed by SAS ANOVA with a 0.05 significance level. (*) denotes groups differing significantly from controls.

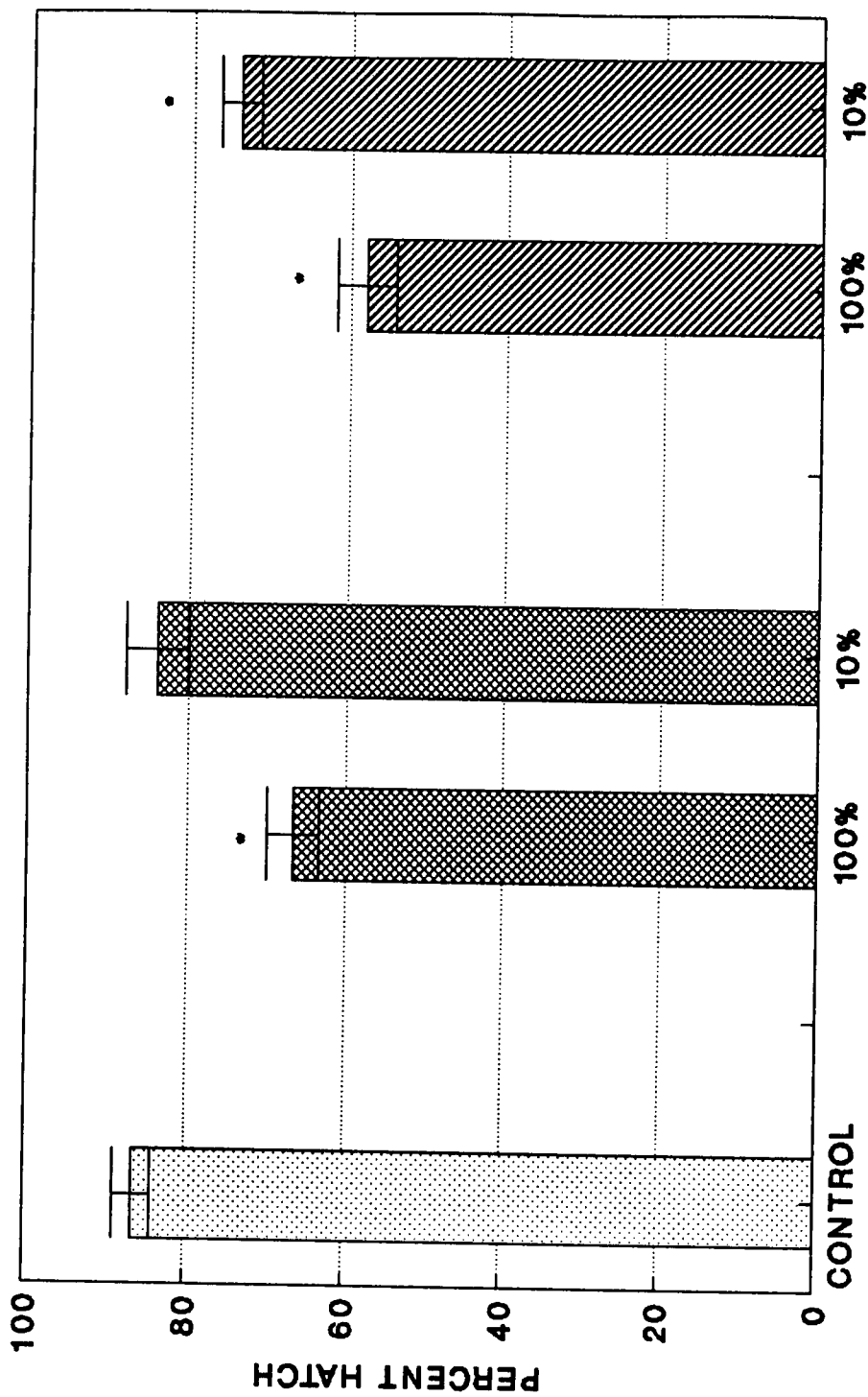


Figure 4.2 Method 2: Percent hatch for embryos incubated in TP-01-56 and TP-04-65 soil extracts using incubation method 2 ($\pm$ standard error of the mean). Groups of 10 embryos were incubated in Petri dishes containing 20-25 ml of medium each. Arcsine-square root transformed data were analyzed by SAS ANOVA with a 0.05 significance level. (*) denotes groups differing significantly from controls.

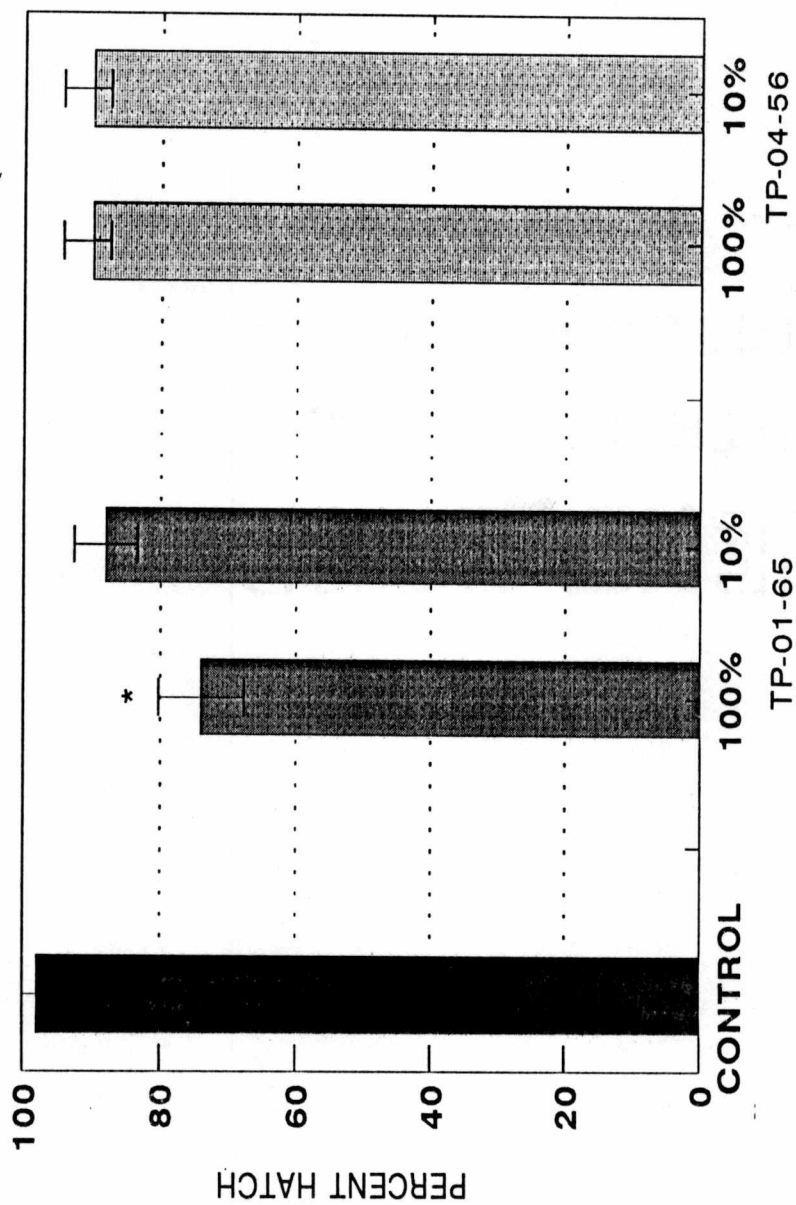


Figure 4.3 Method 3: Percent hatch for embryos incubated in TP-01-56 and TP-04-65 soil extracts using incubation method 3 ($\pm$ standard error of the proportion). Individual embryos were incubated in 7 ml scintillation vials containing 1 ml of solution each. Data were analyzed by Chi Square with a 0.05 significance level. (*) denotes data that differs from the controls (significance level 0.05).

reduced hatching success. Embryos exposed to TP-01-56 100% extract showed a trend toward reduced hatching success. Embryos exposed to 10% TP-01-56 did not show reduced hatching success and had a variance comparable to controls.

The hatching success of embryos incubated according to method 2 is shown in Figure 4.2. Groups of 10 embryos were incubated in 60 x 15 mm petri dishes containing 20 - 25 ml of test solution. While the variability seen in these samples was less than in embryos incubated by method 1, three groups of embryos showed significantly reduced hatching success compared to controls ($P < 0.05$). These are the three groups that were either significantly different in Figure 4.1, or showed a trend in that direction. The mean percent hatches for the extract-exposed embryos were somewhat higher in method 2 compared to method 1, possibly due to the decreased variability.

Embryos which were incubated in individual vials (Figure 4.3) showed significantly decreased hatching only in TP-01-56 at 100% strength, with the 10% strength of the same extract being significant only at the $0.05 > P > 0.10$ level.

4.2 Time-to-Hatch

Time-to-hatch data for control embryos incubated by the three different methods is shown in Figure 4.4. While the mean percent hatching success for the three groups of controls did not differ significantly by Chi Square analysis, the

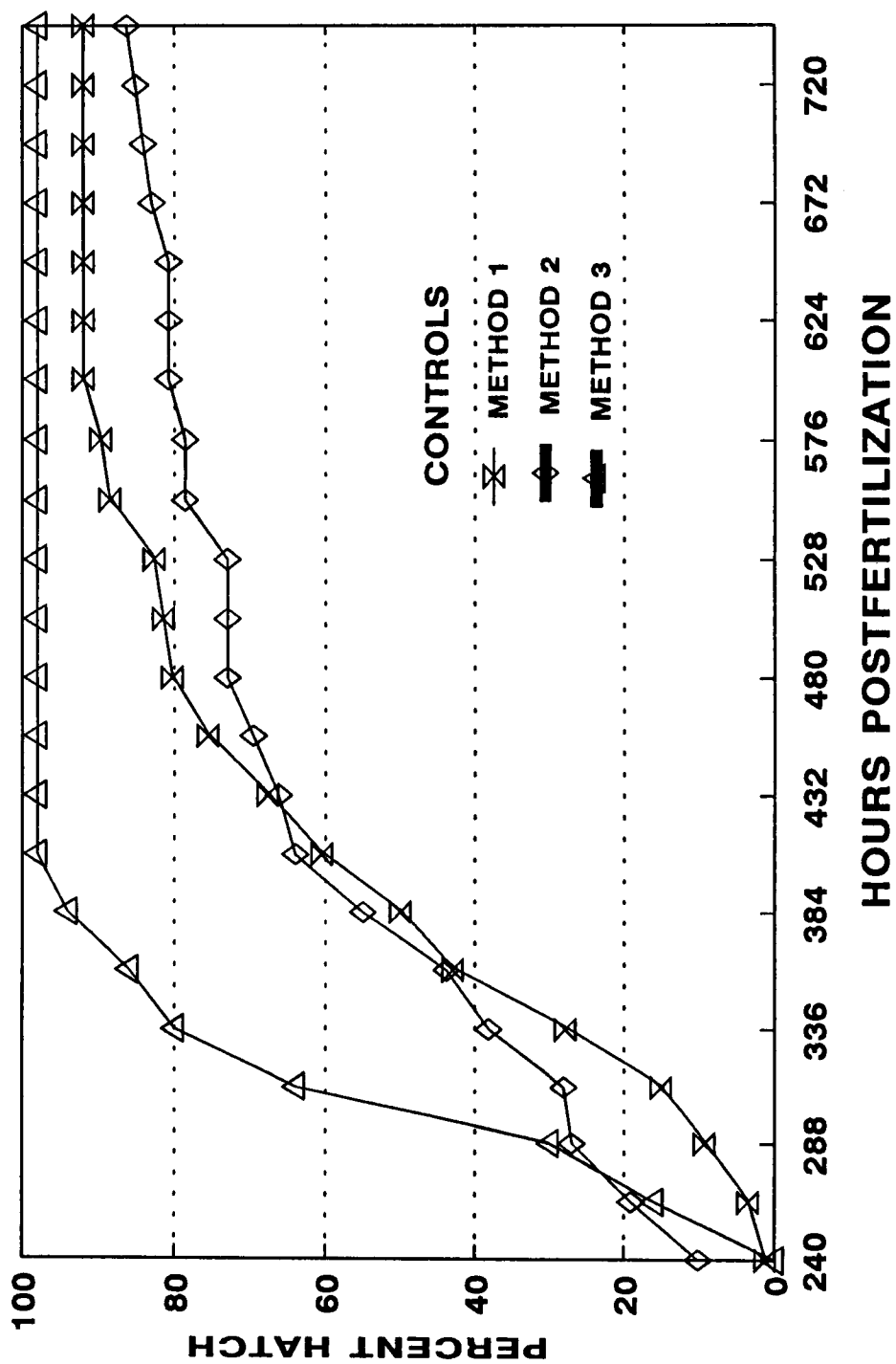


Figure 4.4 Hatching profile for control embryos incubated according to methods 1, 2, and 3. For embryos used in methods 1 and 2, all vials and petri dishes were pooled and cumulative percent hatches calculated for each day.

time-to-hatch profile for the three groups did differ. Note that control embryos incubated by method 1 and 2 continued to hatch long after hatching of embryos incubated by method 1 was complete.

Results of time-to-hatch data for embryos exposed to the two extracts and incubated by method 1 are presented in Table 4.2. The 50% hatching times indicate the first 24 h observation period during which at least 50% of the embryos had successfully hatched. Embryos incubated by method 1 in TP-01-56 at 100% and 10% strengths have longer 50% hatching times than controls, and exhibit dose-dependency in the response. Embryos exposed to both the 100% strength and 10% dilution of TP-04-65 required much longer to complete 50% hatching than method 1 controls or TP-01-56-exposed, but exhibit a threshold-type response.

Time-to-hatch data for embryos incubated by method 2 shows that embryos incubated in 100% strength TP-01-56 and TP-04-65 had longer 50% hatch times than controls and 10% dilutions of the extracts. The response for TP-01-56 100% and 10% -exposed embryos appears dose-dependent, with the 10% -extract exposed group returning to below control values. Embryos exposed to both concentrations of TP-04-65 have larger 50% hatch times than controls, but show a less marked trend toward dose-dependency. All extract-exposed embryos incubated by method 3 have larger 50% hatch times than controls, but neither group exhibits dose-dependency in that response.

Table 4.2 50% hatch times for experimental groups. 50% hatch time indicates the first 24 h observation period during which at least 50% of the embryos had hatched.

Method	Control (h)	TP-01-56 100% (h)	TP-01-56 10% (h)	TP-04-65 100% (h)	TP-04-65 10% (h)
Method 1	384	456	360	600	600
Method 2	384	552	336	432	408
Method 3	312	336	336	360	360

4.3 Developmental Abnormalities

Developmental abnormalities were found in all groups of embryos exposed to 100% strength of both soil extracts, and both groups consistently responded in a dose-dependent manner (Figure 4.5 and 4.6). For TP-01-56, the percent of developmental abnormalities found in the 100%-extract-exposed embryos remained at or just above 10%, regardless of the incubation method. Embryos exposed to 10% dilutions of TP-01-56 showed 0 - 5% abnormal embryos, with little variation between methods.

Embryos exposed to TP-04-65 extract showed an increase in percent abnormal embryos (Figure 4.5) in all incubation methods, compared to TP-01-56. The magnitude of the response differed, depending upon the incubation method, with method 3 showing an amplified response. The response was consistently greater than the controls and, again, maintained a dose-dependent response regardless of incubation method.

The types of developmental abnormalities found in embryos exposed to TP-04-65 extract included: dwarfed embryos lacking external morphological definition in the eye and head region, incomplete or diffuse retinal pigmentation, stunted or tailless embryos, and extensive regions of pooled blood in the brain, eyes, tail, and yolk sac. Embryos that failed to incorporate the heart into the body and embryos with incompletely developed hearts (tube hearts) were also seen in these embryo groups. Similar abnormalities were seen in TP-01-56-exposed embryos, but on a smaller scale. The developmental abnormalities found in the two groups did not

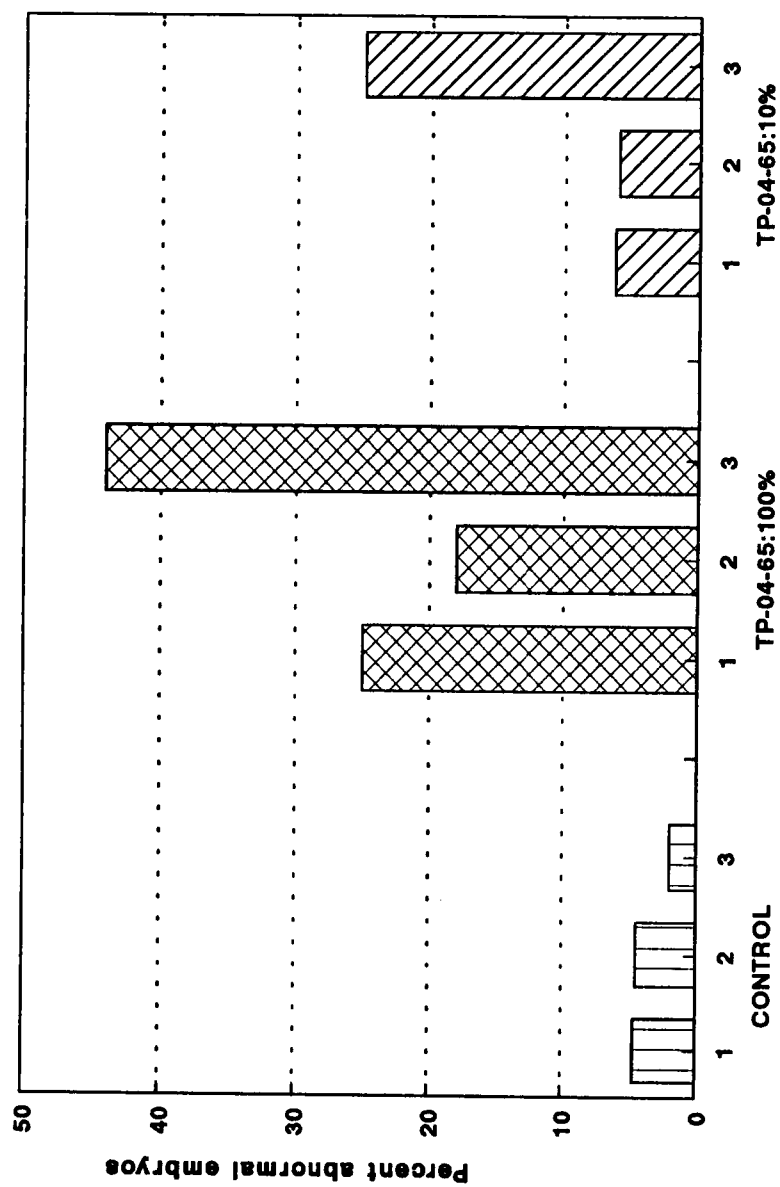


Figure 4.5 Percent abnormal embryos in each incubation method for 100% and 10% exposures to TP-04-65 soil extract. One, 2, and 3 on X axis represent the incubation methods.

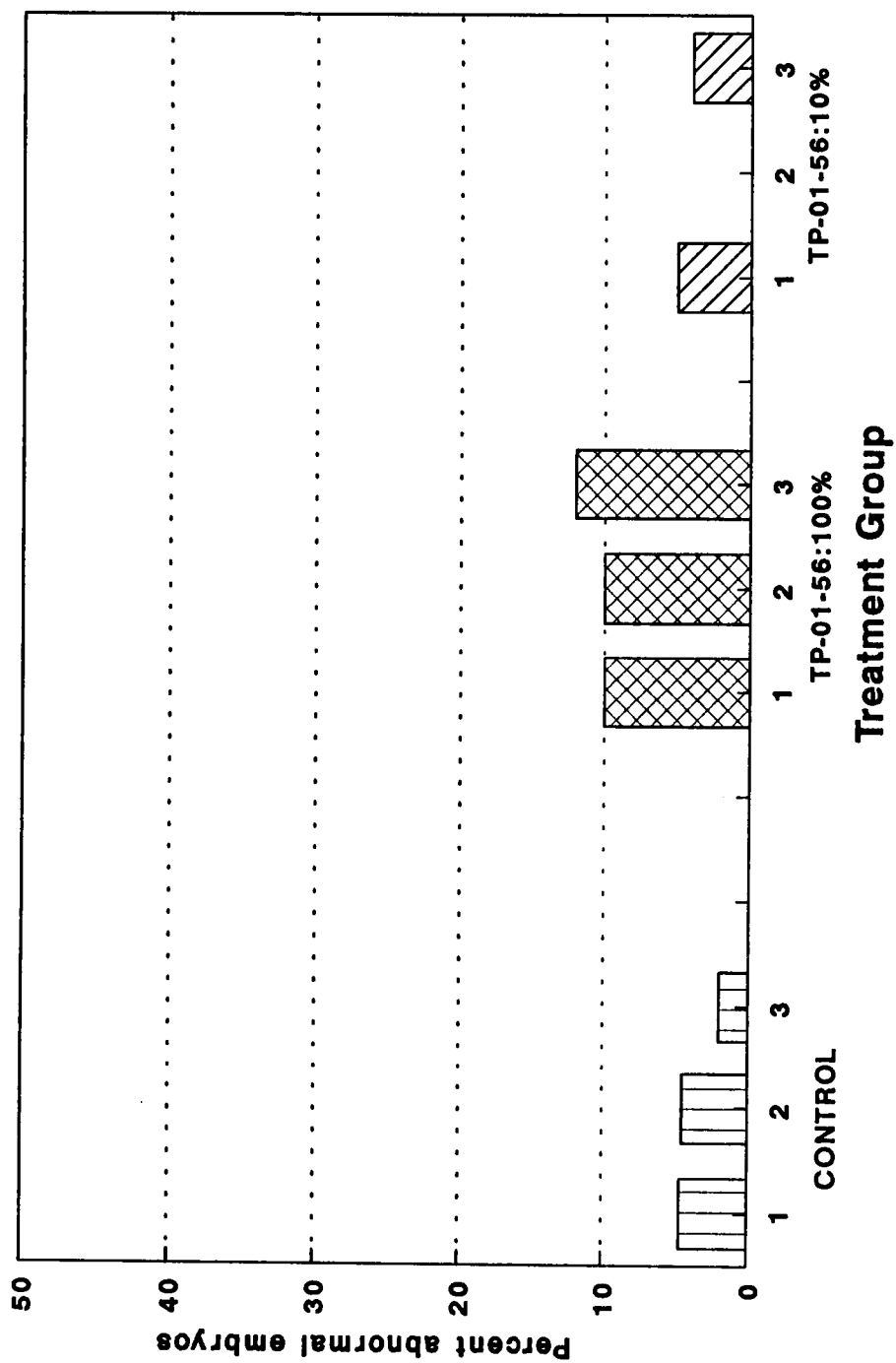


Figure 4.6 Percent abnormal embryos in each incubation method for 100% and 10% exposures to TP-01-56 soil extract. One, 2 and 3 on X axis represent the incubation methods.

appear to be target-organ related, as no common abnormalities appeared consistently. Most abnormalities found were evident by 48 - 72 h postfertilization.

Embryos incubated in 100% TP-04-65 experienced developmental delays in the formation of the spleen. According to Kirchen and West (1976), the spleen appears at 200 h postfertilization (PF). We have found that our control embryos incubated by method 3 begin exhibiting tiny spleens as early as 168 h PF. We monitored the appearance of spleens from 168 - 216 h PF in control and TP-04-65 exposed embryos. At 192 h PF, 6% of control and 48% of the exposed embryos lacked spleens ($P < 0.001$). At 216 h PF, when only 4% of the controls still lacked spleens, 21% of the exposed embryos had no sign of spleens. This disparity in developmental timing was apparent in the exposed embryos until 264 h PF, when they all had begun to develop spleens. The control embryos initiated hatching at 240 h PF, whereas the exposed embryos initiated hatching at 264 h PF.

5. DISCUSSION

While the long-range goal of this project is to better understand the effects of varying incubation conditions and methods on the medaka embryo assay for toxicity testing, the data reported herein also provides information on the potential toxic effects of the two soil extracts tested.

5.1 Overall Toxic Effects of the Two Soil Extracts

The most significant effects seen in embryos exposed to the 100% or 10% TP-04-65 were (a) the increase in morphological abnormalities, (b) the developmental delay in spleen formation, and (c) the extended time-to-hatch. TP-04-65 extract failed to demonstrate a toxic effect on the embryos as measured by hatching success. Soil sample TP-04-65 was known to be contaminated with diesel fuel; the resulting extract also had the characteristic odor of diesel fuel.

While the soil sample TP-01-54 did not show any contamination from diesel fuel, several heavy metals were present (Table 4.1). Embryos exposed to the extract of TP-01-54 did show toxic effects as measured by lower hatching success. The level of developmental abnormalities was greater than control levels, although not statistically significant at the $P < 0.05$ level. However, considering that the detection limits for hydrocarbon analyses were ppm, and we have seen increased toxicity (as measured by reduced hatching success) in the medaka at 6 - 12 ppb benzo[a]pyrene (Hull, 1991; see Part III of this document), it is felt that there must be a toxic component in this soil extract. The difference between the types of responses seen in the two samples may indicate that the types of contamination differ between the two soil samples.

No consistent morphological abnormalities were found in exposed embryos, most probably indicating that the contaminants have non-specific modes of action. Also, almost all morphological abnormalities were observable within 48 - 72 h postfertilization. Thus, the compounds are affecting development during the

formation of the embryonic axis and retinal pigmentation (Kirchen and West, 1976). Pooling of blood in the body of the embryo and yolk sac rarely appeared as an isolated abnormality, but, instead, occurred in conjunction with other abnormalities.

The developmental delay in spleen formation, on the other hand, was not observable until later in development (168 - 216 h postfertilization), and appeared only in the group of embryos exposed to the 100% TP-04-65. The effect was identified and separated from the observed morphological abnormalities because it did not appear to result in a morphologically abnormal organ. Developmental delays and asynchronous development associated with diesel fuel exposure have been reported in the embryos of parentally exposed sea urchins (Vashchenko, et al., 1988; Vashchenko and Najdenko, 1989).

Groups exposed to 100% strengths of both extracts showed an increased time to successfully complete 50% hatching. Alterations in the time to required to complete hatching (increase or decrease in time required) have been demonstrated with the exposure of embryos to BaP (Hull, 1991; Part III of this document), and by exposure of embryos to crude oil fractions (Leung and Bulkley, 1979).

5.2 Comparison of Three Incubation Methods

Methods 1 and 2 showed decreased hatching success in groups of embryos incubated in the 100% and 10% extract, while embryos treated in method 3 failed

to show any decrease in hatching success. Methods 1 and 2 are the conventional methods for running medaka embryo assays, however, the data between the three methods is not comparable. The incubation of groups of embryos in contaminated solutions may provide a stress beyond that of individual embryos in the same solution. This stress would most probably include the spread of fungi or other pathogens from recently dead embryos to healthy or borderline healthy embryos. Where this "dead embryo effect" is not a problem in controls, due to the low percentage of mortality, it does present a problem when the baseline hatching success or health of the embryos is lower.

The disparity between the results of hatching success and abnormal embryos between methods 1 and 2 and method 3 in TP-04-65-treated embryos is probably a result of increased stress on embryos in methods 1 and 2, resulting in earlier and higher mortality. The failure to detect as many abnormalities in these embryos would be a result of earlier death of affected embryos. Embryos in method 2 would be under less stress from possible "dead embryo effects" than those in method 1 because these embryos received an exchange of medium every 48 h. While it does renew the dose of the extract, it also provides a fresh, sterile incubation medium. This could account for the higher variability seen between vials incubated in method 1 compared to petri dishes in method 2.

Individual incubations in method 3 completely eliminated the stress one embryo may cause another, while providing a fresh, sterile medium exchange every 48 h. In this situation, abnormal or borderline unhealthy embryos could

survive to hatching, accounting for the twofold to threefold rise in abnormal embryos seen in this method.

Again, the variability in hatching success data between vials in method 1 is likely the result of continual incubation in the same medium. The assumption, then, is that this medium is not only contaminated with hydrocarbons, but also may contain fungi and/or pathogens from other dying embryos. Embryos exposed to this extract at 100% strength continue to show decreased hatching success in all three methods and the effect does not appear to be attenuated by the reduction of stress in method 3. The level of developmental abnormalities remains above control levels in all three methods, but does not differ between methods. These data further indicate that the types of contamination may differ between the two soil samples.

Data for 50% hatch times are difficult to compare between incubation methods; however, within each method, samples exposed to 100% extracts showed an increased time to successfully complete 50% hatching. This was most evident in methods 1 and 2 and least evident in method 3--supporting the theory that method 3 produces less stress on the individuals and provides more optimal conditions for development.

In summary, the three variations of the medaka embryo assay employed in this study provided information on the toxicity and teratogenicity of the two soil extracts. Embryos exposed to both extracts demonstrated reduced hatching success, increased time-to-hatch, and increased percentages of abnormal embryos.

Further studies would ideally include tests of extracts from non-remediated and remediated test pits. Such tests could again encompass all three methods of incubation; however, it is felt that method 3 provides advantages over methods 1 and 2. Although the embryos may not show direct toxicity to all contaminants at USAKA, this method allows for better observation of abnormal embryos, without the potential for embryo-embryo interactions.

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**PART V: JAPANESE MEDAKA EMBRYO ASSAY FOR
DEVELOPMENTAL TOXICITY: EVALUATION OF THE TOXICITY OF
WATER SAMPLES DOWNSTREAM OF AN INDUSTRIAL FACILITY.**

1. ABSTRACT

A medaka embryo assay was used to evaluate the toxicity of water samples collected from a stream receiving industrial discharges, in East Tennessee. The headwaters of this stream, East Fork Poplar Creek (EFPC), lie within the Department of Energy Y-12 Plant, in Oak Ridge, TN. EFPC receives industrial discharges from the Y-12 facility, most notably chlorine and ammonia. Historic contamination of the stream and sediment included radioisotopes, PCB's, PAH's and numerous heavy metals, including mercury and nickel (1983 Mercury Task Force, 1983; Van Winkle, et al., 1984; Theodorakis, et al., 1992).

Experiments were conducted from January through May, 1992. Individual medaka embryos were incubated in water collected from 10 different stream sites in early January, 1992. High mortality (100%) was observed in embryos exposed to all EFPC water samples (sites 1 - 10). Subsequent experiments were limited to a subset of the original 10 sites. Several follow-up experiments were conducted to determine the nature of the toxic effect and to eliminate the possibility of pathogenic contaminants in the water. A site on the nearby Clinch River was selected as a reference site. To further determine the nature of the toxicity seen in the earlier sites, serial dilutions (1:10, 1:100, 1:1000) were made from water at 4 different sites. Water samples from the reference site were used as both a control and as a diluent. Toxicity in the 1:10 dilutions was reduced at all 4 sites tested, and appeared to reflect a downstream gradient from the Y-12 Facility. Medaka embryos are more sensitive than fry to the effects of EFPC water samples. When

fry (less than 24 h old) were exposed to water from EFPC sites, no mortality was observed for the first 96 h. A study was also conducted to compare group incubations (Bowers III, et al., 1980; Marty, et al., 1991) to individual embryo incubations. Mortality results for groups of embryos exposed to water from EFPC were similar to those of individually exposed embryos (high mortality). However, while embryos incubated individually in water from the reference site produced data comparable to that of other controls (embryo rearing medium and sterilized water), group-incubated embryos exposed to water from the reference site experienced high mortality. Fungal, bacterial, and rotifer contamination were problems in all group incubations with environmental water samples.

This set of experiments demonstrated several important points about the toxicity seen in EFPC: (1) the high medaka embryo mortality observed in EFPC samples was probably not the result of a waterborne pathogen, (2) the high medaka embryo mortality observed in 100% strength EFPC samples can be reduced by diluting with reference stream water, (3) water samples diluted with reference water produce a down-stream gradient in mortality, with the higher mortality rates occurring in embryos exposed to water samples from sites nearest the Y-12 Plant, and (4) the medaka embryo is more sensitive to the toxic component in EFPC than is the fry life-stage.

This set of experiments also demonstrated several important points about the use of medaka in this capacity: (1) individually incubated medaka embryos are sensitive to industrial pollutants, (2) 96-h fry survival appears to be a less

sensitive indicator than embryo mortality, and (3) group incubations of medaka embryos are not applicable to environmental water samples (experimental and reference sites), possibly due to the presence of nutrients.

2. INTRODUCTION

Various types of medaka embryo assays have been used by researchers in attempts to evaluate the toxicity of xenobiotics. The most traditional use of the assay has been in the evaluation of laboratory-prepared solutions (i.e. Heisinger and Green, 1975; Dial, 1978a, b; Bowers III, et. al., 1980; Sakaizumi, 1980; Michibata, 1981a, b). While this application is useful for testing pure chemicals and solutions of known constitution, the evaluation of undefined solutions and mixtures (such as environmental effluents) is perhaps more applicable to real world situations. Affected reservoirs, streams, lakes, and sediments are rarely found to be contaminated with single, pure chemicals--instead, representatives of various classes of chemicals may be present. In addition, these chemicals interact with the physical and chemical properties of the natural water (i.e. pH, temperature, conductivity), further complicating the process of determining the toxicity of the water sample.

In this study, a medaka embryo assay has been used to evaluate the toxicity of water samples collected from a stream receiving industrial discharges. The goal of these experiments was to determine if this medaka embryo assay could be successfully applied to environmental water samples. Individual embryo

incubation and sterilization of water samples (0.1 μm filtration) were used to reduce the fungal and bacterial contamination often seen with the use of environmental water samples. The incubation of individual embryos has provided adequate control data (see Part II of this document), and proved useful in the evaluation of both single compounds (see Part III of this document), and soil extracts (see Part IV of this document). Useful endpoints have included observations of mortality, developmental abnormalities, and altered time-to-hatch.

The elimination of group incubations and anti-fungal agents (i.e. methylene blue), coupled with the use of sterile filtration, has provided a medaka embryo assay that can be used to evaluate the developmental toxicity of environmental water samples. While further research needs to be done on the effects of natural variations in factors such as pH and conductivity, these experiments provide the data for initial validation of a single-embryo medaka assay for developmental toxicity utilizing environmental effluents.

The stream chosen for this study was EFPC in Oak Ridge, TN. The headwaters for EFPC lie within the Department of Energy's (DOE) Y-12 Plant. The stream sediment is contaminated with PAH's, PCB's, radionuclides, and heavy metals from historic releases (1983 Mercury Task Force, 1983; Theodorakis, et al., 1992), as well as ongoing releases of industrial pollutants such as chlorine (Ham and Peterson, 1992) and ammonia (Stewart and Kszos, personal communication, 1992). This stream is the site of an ongoing Biological Monitoring

and Abatement Program (Loar, et al., 1989), and thus several concurrent studies will be discussed where applicable.

3. MATERIALS AND METHODS

3.1 Adult Care and Maintenance

Japanese medaka fry (*Oryzias latipes*) obtained from the Gulf Coast Research Laboratory, Ocean Springs, MS., were raised to adulthood in 100-gal flow-through aquaria supplied with dechlorinated tap water. Adult fish were maintained at 25°C under a 16 hour light to 8 hour dark photoperiod. Medaka were offered Stress Flakes twice daily and 24 h brine shrimp once daily. Breeding stocks were maintained in a ratio of 3 females to 2 males at a density of 5 medaka per gal.

3.2 Embryo Collection

Embryos were collected beginning 1 h after the initiation of the light cycle. Females were removed from breeding tanks with soft, nylon nets. Embryos were gently removed from the outer abdomen with gloved fingers or blunt forceps and placed into a (150 x 10 mm) Petri dish containing sterile water (Millipore-filtered). Embryos taken from different breeding tanks were placed into the Petri dish and mixed thoroughly in an attempt to randomize the embryos prior to separation into exposure groups. After removal from the female, embryos remain in clutches of 3

- 50 each, held together by chorionic filaments. Clutch separations and removal of these filaments were done under a binocular dissection microscope using fine-tipped watchmakers' forceps. Damaged or unfertilized eggs, and any embryos which were obviously remnants of a previous day's spawns, were discarded.

Although several different types of control solutions were used during the course of this series of experiments, embryo rearing medium (ERM) was consistently used for each experiment. ERM is generally used as a control for medaka embryo assays testing laboratory-generated solutions. ERM was modified from Kirchen and West (1976), and contained 1 g NaCl, 30 mg KCl, 40 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and 163 mg $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in 1 L of sterile water. This solution was then sterile filtered ($0.1 \mu\text{m}$). Water samples from an environmental reference site (Clinch River, site 11) also served as control incubation media. Embryos were introduced into exposure media within 3 h of collection.

3.3 Water Sampling Sites

This series of experiments was conducted during the first part of 1992 (January through May), and involved the collection of water samples from various sites on East Fork Poplar Creek, in Oak Ridge, TN (Table 5.1). East Fork Poplar Creek (EFPC) originates within the Department of Energy's Oak Ridge Y-12 Nuclear Weapons Facility. Numerous industrial pipes empty into the stream as it passes through the facility. An oil separator and a lined lake (Lake Reality) have been built to help reduce the

Table 5.1 Water collection sites along East Fork Poplar Creek.

Site Number	Location
1.	At the North/South Pipe (NSP): inside Y-12 Facility; just outside the security fence
2.	At Area Source Site #8: approximately 500 m downstream from NSP
3.	Above Lake Reality: below oil separator and above the inflow to Lake Reality
4.	Below Lake Reality: just at lake's weir (approximately 23.4 km above the confluence with Poplar Creek)
5.	Behind Dean Stallings Ford Dealership (approximately 1 km below Lake Reality, and 22 km above the confluence with Poplar Creek)
6.	Behind Rocky Top Store on HWY 95: stream has already passed through the town of Oak Ridge (approximately 18.2 km above confluence with Poplar Creek)
7.	On Jackson Farm: just prior to the Oak Ridge Sewage Treatment Plant (approximately 13.5 km above confluence with Poplar Creek)
8.	On Gum Hollow Road: adjacent to Oak Ridge Country Club (10 km above confluence with Poplar Creek)
9.	USGS Gauging Station (6.3 km above confluence with Poplar Creek)
10.	After the confluence with Bear Creek and prior to the confluence with Poplar Creek (less than .5 km above the confluence with Poplar Creek)
11.	Reference site: Clinch River, upstream of the input from Bear or Poplar Creeks: just below the Melton Hill Dam (not shown in Figure 5.1)

chances of any accidental fish kills resulting from chemical releases within the facility. After exiting Lake Reality, EFPC runs approximately 23 km through the city of Oak Ridge. The stream passes the Oak Ridge Sewage Treatment Plant at 13.8 km prior to the confluence with a larger stream, Poplar Creek. Bear Creek, which drains the western side of the Y-12 facility, empties into EFPC prior to the confluence with Poplar Creek. Poplar Creek is a tributary of the Clinch River.

Sampling sites for this study were selected based upon several factors. Ongoing population, toxicity, and biomarker studies done by the Environmental Sciences Division of ORNL have well-documented and established sampling sites that are readily accessible. These sites are routinely monitored for compliance with the Y-12 NPDES permit. In addition, due to these other studies, much collaborative data were already available. Study sites and locations are listed in Table 5.1.

3.3.1 Water sample collection and analysis

Water samples were collected for medaka embryo assays on the morning of the first day of each test. Samples were collected in clear plastic water bladders and transported back to the laboratory for use. With the exception of chlorine analysis, water chemistry analysis was done within 5 h of sample collection. Water for chlorine analysis was collected in amber plastic containers, separately from water to be used in the assays. Chlorine analysis was done immediately upon returning to the lab (never longer than 1.5 h following collection). The following

tests were done on all water samples collected: pH, conductivity, hardness, alkalinity, and chlorine content.

Some specific water samples were analyzed for ammonia and nitrates. When possible, pH, temperature, dissolved oxygen, and conductivity were analyzed in the stream by use of an Horiba U-7 Water Quality Checker (Horiba, Co.) at the water collection sites. For in-lab measurements, the pH meter (Orion 811 meter with a Fisher 13-639-80 probe), conductivity meter (hand-held Cole Palmer PCM1), and chlorine titrator (Wallace and Tiernan) were used and calibrated daily.

Alkalinity and hardness measurements were done using the Environmental Science Division's Aquatic Toxicology Laboratory Quality Assurance Manual (SOP-11). Alkalinity was determined by titrating water samples (50 ml) with 0.01 N HCL to $\text{pH } 4.50 \pm 0.04$ and is expressed as $\text{mg CaCO}_3/\text{L}$. Hardness, also expressed as $\text{mg CaCO}_3/\text{L}$, was determined by titrating a 50 ml water sample to a colorimetric endpoint using Eriochrome Black T. Total residual chlorine and free chlorine measurements were done using phenylarsene oxide as a titrant and were measured in mg/L . Examples of the water chemistry parameters from a sample collection are provided in Table 5.2.

Table 5.2 Results of analytical tests on water samples collected from EFPC on 02/05/92. These numbers are typical of those consistently measured from sites/samples.

Water Collection Site

Analysis	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Site 7	Site 8	Site 9	Site 10	Site 11 ^a	ERM ^b
Stream Temp. (°C)	20.1	17.9	14.9	13.5	12.5	10.4	9.0	10.6	9.6	9.3	9.5	26
Stream pH	8.5	8.0	8.4	8.2	8.5	8.3	8.6	8.1	8.5	8.5	8.3	7.3
Water Sample pH	8.1	8.3	8.5	8.2	8.5	8.3	8.6	8.1	8.5	8.6	8.3	7.0
Stream Conductivity (mS/cm)	0.88	0.72	0.63	0.41	0.36	0.32	0.27	0.31	0.24	0.22	0.33	NA
Water Sample Conductivity (mS/cm)	0.86	0.74	0.71	0.50	0.44	0.43	0.38	0.42	0.31	0.30	0.28	2300.
Hardness (mg CaCO ₃ /L)	328	280	264	210	204	210	198	184	468	468	426	98
Alkalinity (mg CaCO ₃ /L)/pH	98/ 4.48	102/ 4.53	110/ 4.48	117/ 4.45	120/ 4.48	135/ 4.45	136/ 4.51	140/ 4.45	130/ 4.51	132/ 4.53	117/ 4.54	5/ 4.45
NH ₃ (mg/L)	0.07	0.03	0.39	0.42	0.17	0.10	0.10	0.66	0.13	0.17	0.02	0.01
NO ₃ (mg/L)	4.19	6.61	6.14	5.62	5.10	3.55	2.55	5.37	3.85	3.20	4.82	0.83

^aMeasurements at site 11 (reference site) were made 2 d after the other measurements.

^bERM = embryo rearing medium

^cAlkalinity is expressed as milligrams of CaCO₃/L as titrated to the pH endpoint in parentheses.

3.4 Experimental Design

The tests were broken down into discrete experiments, and each experiment was conducted independently of the others. For convenience, each experiment has been assigned a number (EFPC 1 - EFPC 6). This also denotes the sequence in which the experiments were conducted. Unless otherwise stated, all embryo assays involved the incubation of individual embryos in 7 ml scintillation vials. Each embryo was incubated in 1 ml of incubation media at 24 - 25°C for the duration of the incubation. Embryos received complete incubation media exchanges every 48 h. Observations under a binocular dissection microscope were done every 24 h to note mortalities, abnormalities, or hatches. Water samples were collected on the first day of each experiment. Once in the laboratory, these were filtered with 0.1 μ m disposable Nalgene filters. The filtered water samples were then used for all incubation media exchanges throughout that test. When not in use, the water samples were stored at 20°C.

3.4.1 EFPC 1

EFPC 1 began on 01/20/92. This was a preliminary test designed to determine the toxicity at all ten selected sites along EFPC. ERM served as the control. A spill (exact chemical content unknown) occurred within the Y-12 plant during the previous week. In an attempt to isolate the spilled mixture following observation of a fish kill, Lake Reality was closed and the stream bypassed the lake. Lake Reality was re-opened at least 24 h prior to sample collection.

3.4.2 EFPC 2

EFPC 2 began on 01/31/92. This study concentrated on upstream sites 2 and 4 in an attempt to eliminate some of the possible causes for the acute toxicity seen in EFPC 1. Filtration, formalin rinsing, and UV-sterilization have all been shown to remove pathogens from water sources. Groups and treatments are provided in Table 5.3.

3.4.3 EFPC 3 and 4

In EFPC experiments 3 and 4, water samples from sites 2, 4, 6, and 7 were used in experiments designed to determine the nature of the toxic effects seen to medaka embryos in EFPC. A reference site (11) was selected on the Clinch River, below the Melton Hill Dam. This site should be relatively free of any influence from either Y-12 or the Oak Ridge National Laboratory. Water samples were collected from sites 2, 4, and 11 on 03/18/92 (EFPC 3) and from 6, 7, and 11 on 03/26/92. Water from site 11 was used to make serial dilutions with the EFPC water samples (100%, 1:10, 1:100, 1:1000). Embryos incubated in ERM were also run simultaneously as controls.

3.4.4 EFPC 5

Medaka fry (less than 24 h old), were exposed to water samples from sites 2, 4, and 11 in an attempt to determine if the toxic effects seen in developing medaka embryos would also occur in newly hatched fry.

Table 5.3 Treatment groups for EPFC 2 embryo toxicity test, initiated on 01/31/92.

Treatment site (N)	Treatment
Control	Sterile filtered (0.1 μm)
Control-FR	Embryos incubated in sterile filtered (0.1 μm) ERM following 10 rinses in 0.01% formalin
Unfiltered #2	Embryos incubated in unfiltered water sample from site 2
Unfiltered-FR #2	Embryos incubated in unfiltered water from site 2 following 10 rinses in 0.01% formalin
Filtered #2	Embryos incubated in sterile filtered (0.1 μm) water sample from site 1
Unfiltered #4	Embryos incubated in unfiltered water sample from site 4
Unfiltered-FR #4	Embryos incubated in unfiltered water sample from site 4 following 10 rinses in 0.01% formalin
Filtered #4	Embryos incubated in sterile filtered (0.1 μm) water sample from site 4
Unfiltered-UV #4	Embryos incubated in unfiltered, UV-treated water sample from site 4

Embryos were exposed to the same water samples. All tests were initiated on 04/03/92 and were allowed to run for 168 h. All data will be reported as the 96 h mortality rates. The tests were allowed to run the additional time to insure that no latent toxic effects would be seen in the fry. Embryos were not carried through to hatching, only to 168 h as well. No exogenous food sources were provided to the fry.

3.4.5 EFPC 6

In EFPC 6, medaka embryos were exposed to water samples from sites 4 and 11 in three different incubation methods. Two types of group incubations, taken from the literature (Bowers III, et al., 1980; Marty, et al., 1991) were compared to the single embryo incubation method used in EFPC 1 - 5. One type of group incubation (Bowers III, et al., 1980) involved the incubation of groups of 10 embryos in 60 x 15 mm Petri dishes containing 20 - 25 ml of incubation media. These embryos received complete media exchanges every 48 h. In the second type of group incubation (Marty, et al., 1991), groups of 8 embryos were incubated in 20 ml scintillation vials (Kimble) containing 2 ml of incubation media. These embryos received no media exchanges until day 9 of incubation (1st day of hatching), at which time 2 ml of clean water replaced the test water sample. This test was initiated 05/01/92.

4. RESULTS:

4.1 EFPC 1

The cumulative mortality percentages for embryos exposed to EFPC water samples in EFPC 1 are provided in Figures 5.1 and 5.2. The data for 50% lethality time (the first 24 h interval during which at least 50% of the embryos were observed dead) for the groups are given in Table 5.4.

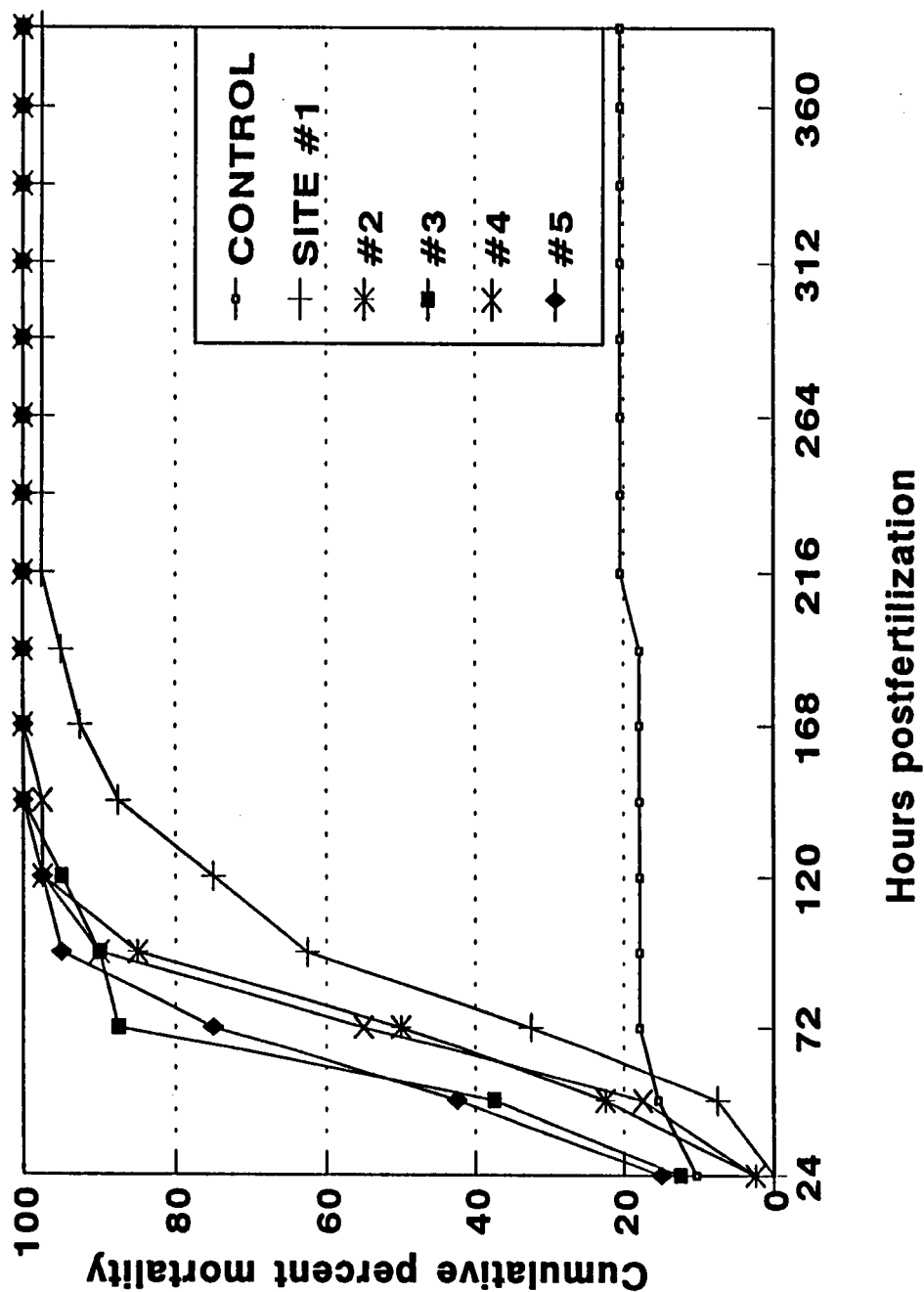


Figure 5.1 EFPC 1: Cumulative percent mortality for medaka embryos exposed to water samples collected at sites 1 - 5. Control embryos were incubated in ERM. Cumulative mortality is expressed as percent of total mortality occurring at each 24 h interval postfertilization. Experiment was initiated on 01/20/92.

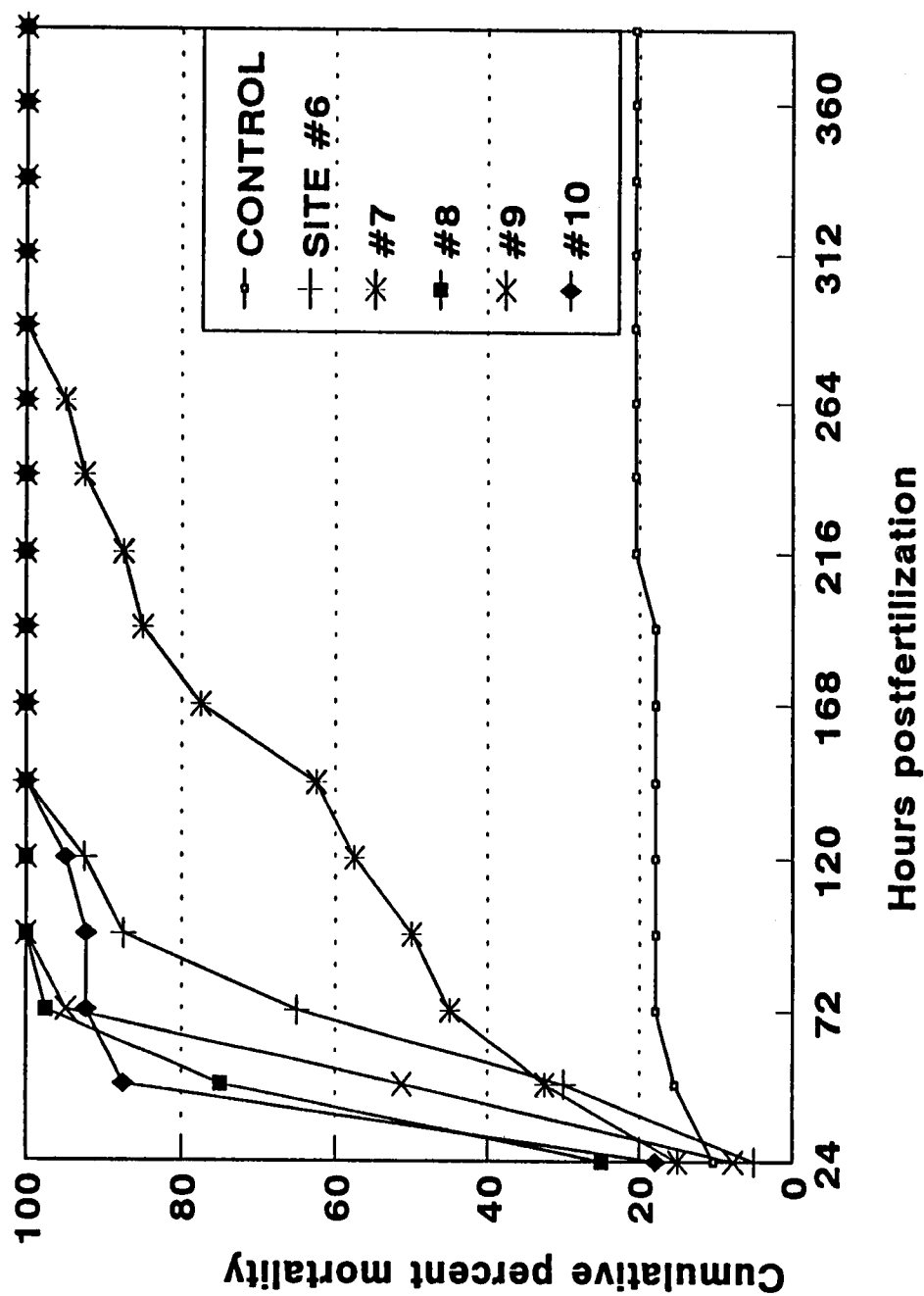


Figure 5.2 EFPC 1: Cumulative percent mortality for medaka embryos exposed to water samples collected at sites 6 - 10. Control embryos were incubated in ERM. Cumulative mortality is expressed as percent of total mortality occurring at each 24 h interval postfertilization. Experiment was initiated on 01/20/92.

Table 5.4 50% lethality time (50% LT) for medaka embryos exposed to water samples in EFPC 1, initiated on 01/20/92. (50% lethality time indicates the first 24 h-observation period during which at least 50% of the embryos were recorded as dead)

Site (N)	50% LT (h)	Site (N)	50% LT (h)
ERM	NA ^a	6	72
1	96	7	96
2	72	8	48
3	72	9	48
4	72	10	48
5	72		

^a Mortality rates did not reach 50%.

4.2 EFPC 2

Cumulative mortality for embryos exposed to water samples from sites 2 and 4 are provided in Figures 5.3 and 5.4. Neither sterile filtering nor formalin rinsing reduced the acute toxicity seen in embryos exposed to these water samples. UV-sterilization, used on water samples from site 4, also failed to reduce the toxicity. Formalin treatment in ERM-incubated controls did not have any affect on the mortality rate.

4.3 EFPC 3 and 4

In EFPC 3 and 4, embryos were exposed to serial dilutions of water samples from sites 2, 4, 6 and 7. The cumulative mortalities for these groups are provided in Figures 5.5, 5.6, 5.7, and 5.8, respectively. Water from the control site

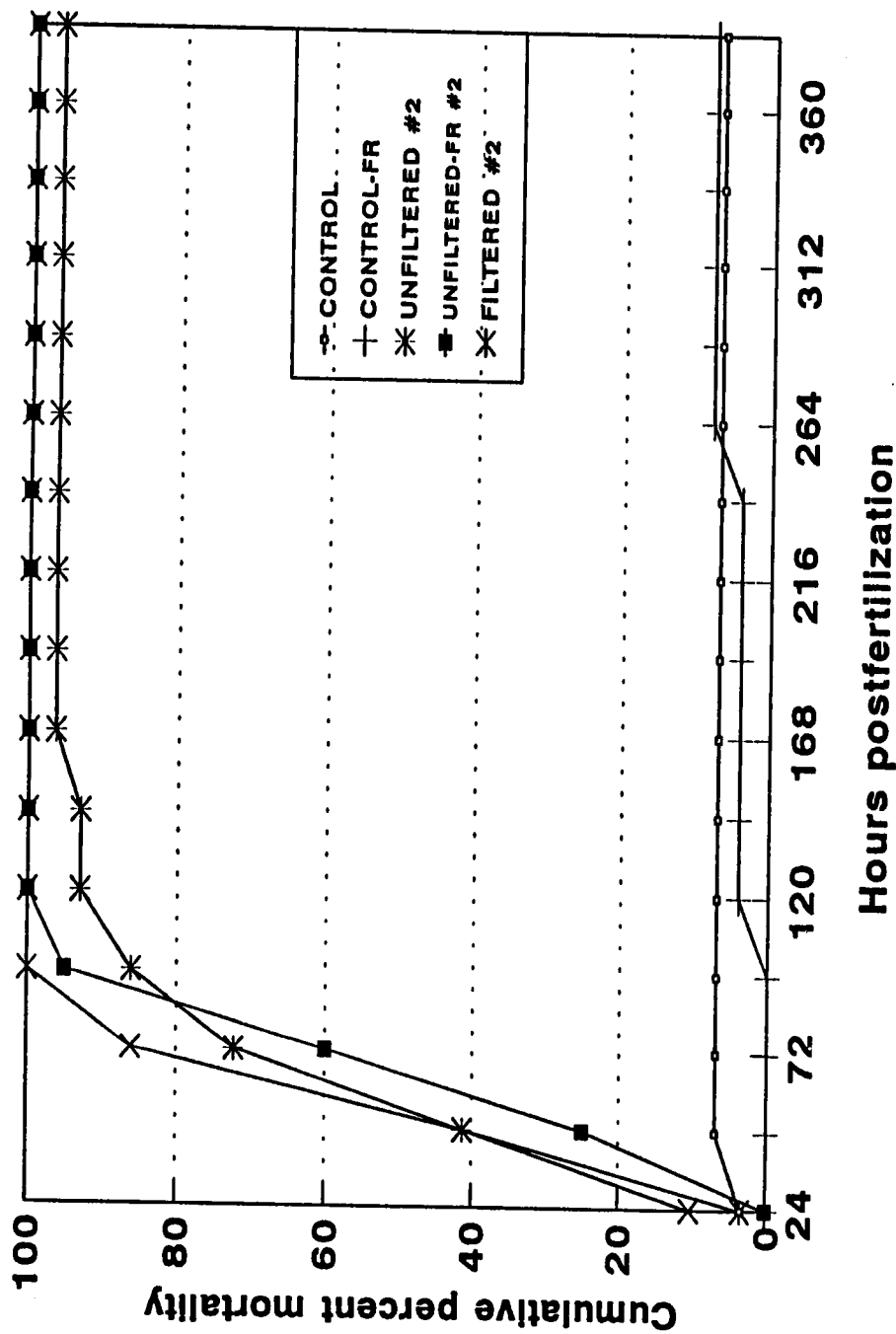


Figure 5.3 EFPC 2: Cumulative percent mortality for embryos exposed to differently treated water samples collected from site 2. Water samples were unfiltered, filtered (0.1 μ m Nalgene filter). One group of embryos exposed to unfiltered water from site 2 was rinsed in 0.1% formalin prior to being placed into the water samples (Unfiltered-FR). Experiment was initiated on 01/31/92.

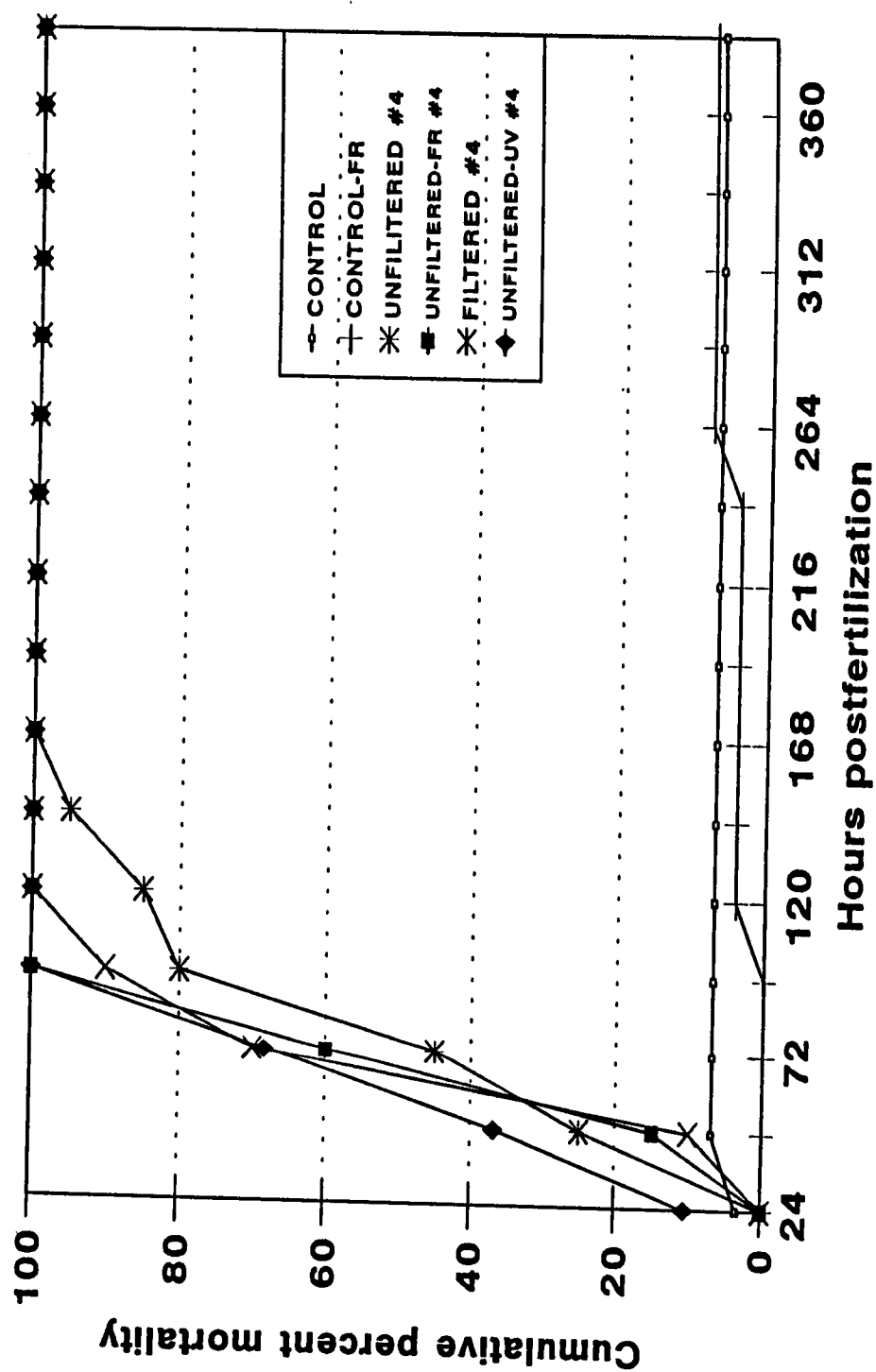


Figure 5.4 EFPC 2: Cumulative percent mortality for embryos exposed to differently treated water samples collected from site 4. Water samples were unfiltered, filtered ($0.1 \mu\text{m}$ Nalgene filter). One group of embryos exposed to unfiltered water from site 4 was rinsed in 0.1% formalin prior to being placed into the water samples (Unfiltered-FR). Water from site 4 was also sterilized by UV light prior to use (Unfiltered-UV). Experiment was initiated on 01/31/92.

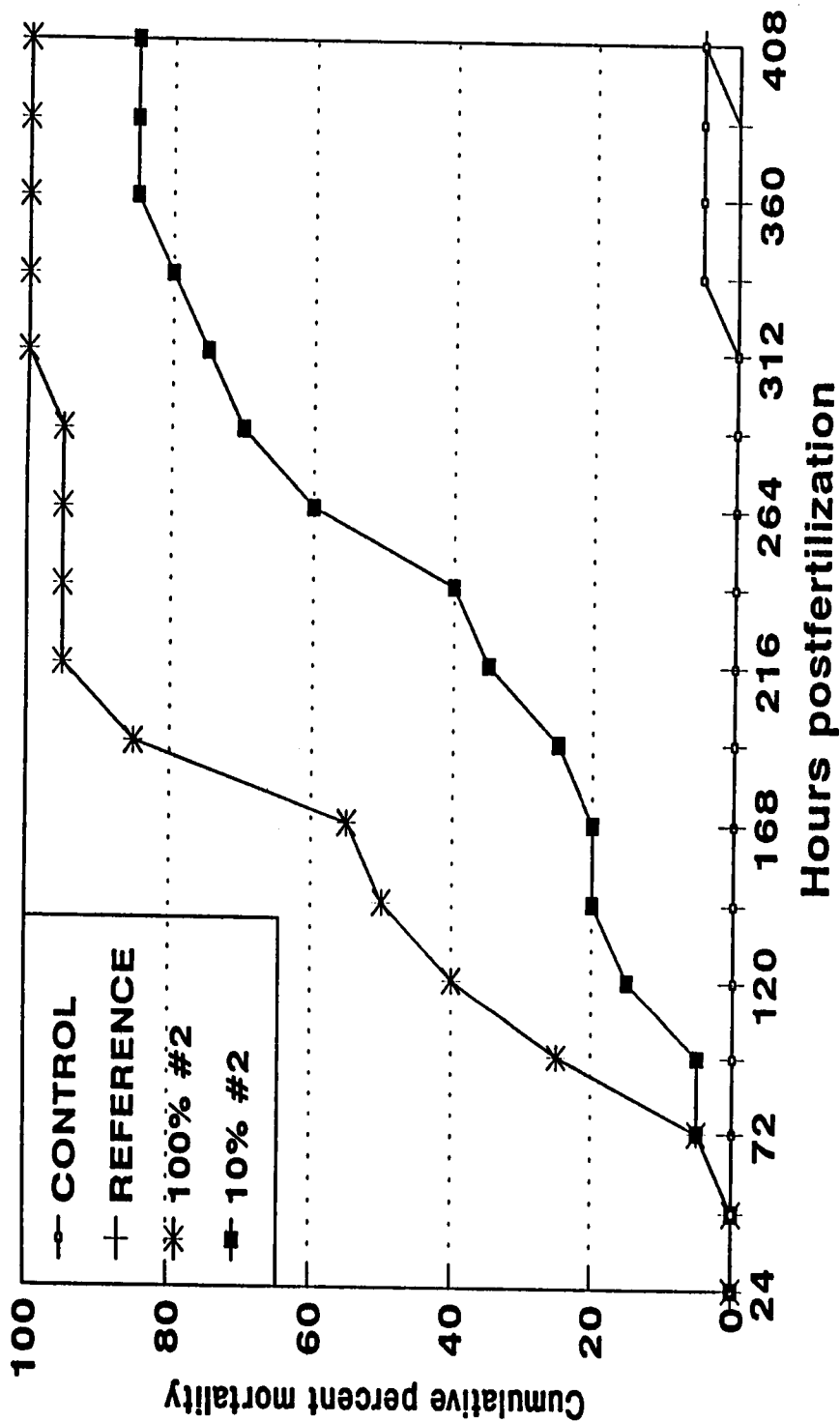


Figure 5.5 EFPC 3: Cumulative percent mortality for embryos exposed to 100% and 10% strengths of water collected from site 2. Control embryos were exposed to ERM and reference embryos were exposed to water collected at site 11. Water collected at site 11 was also used as the diluent. Experiment was initiated on 03/18/92.

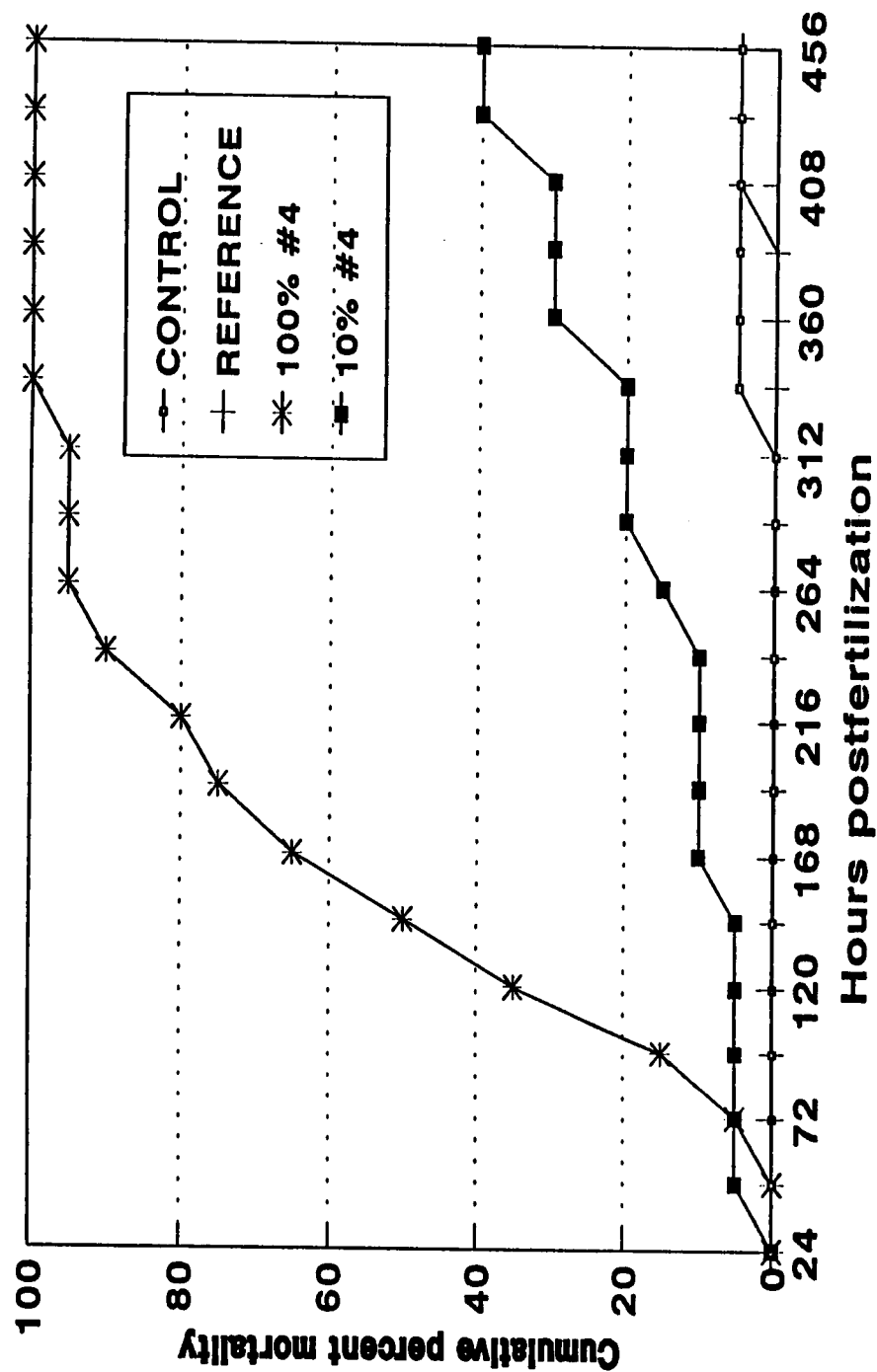


Figure 5.6 EFPC 3: Cumulative percent mortality for embryos exposed to 100% and 10% strengths of water collected from site 4. Control embryos were exposed to ERM and reference embryos were exposed to water collected at site 11. Water collected at site 11 also served as the diluent. Experiment was initiated on 03/18/92.

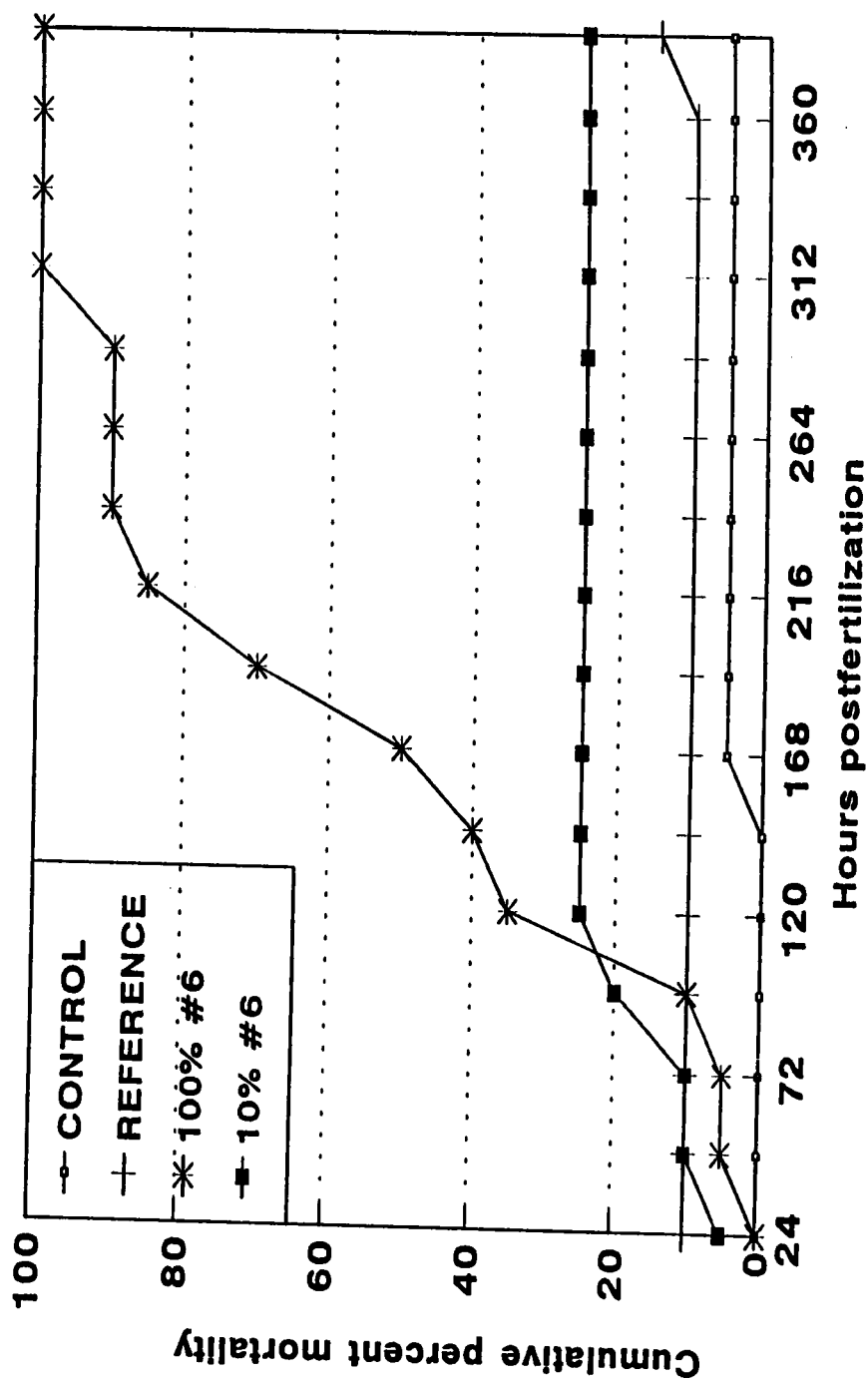


Figure 5.7 EFPC 4: Cumulative percent mortality for embryos exposed to 100% and 10% strengths of water collected from site 6. Control embryos were exposed to ERM and reference embryos were exposed to water collected at site 11. Water collected at site 11 was also used as the diluent. Experiment was initiated on 03/26/92.

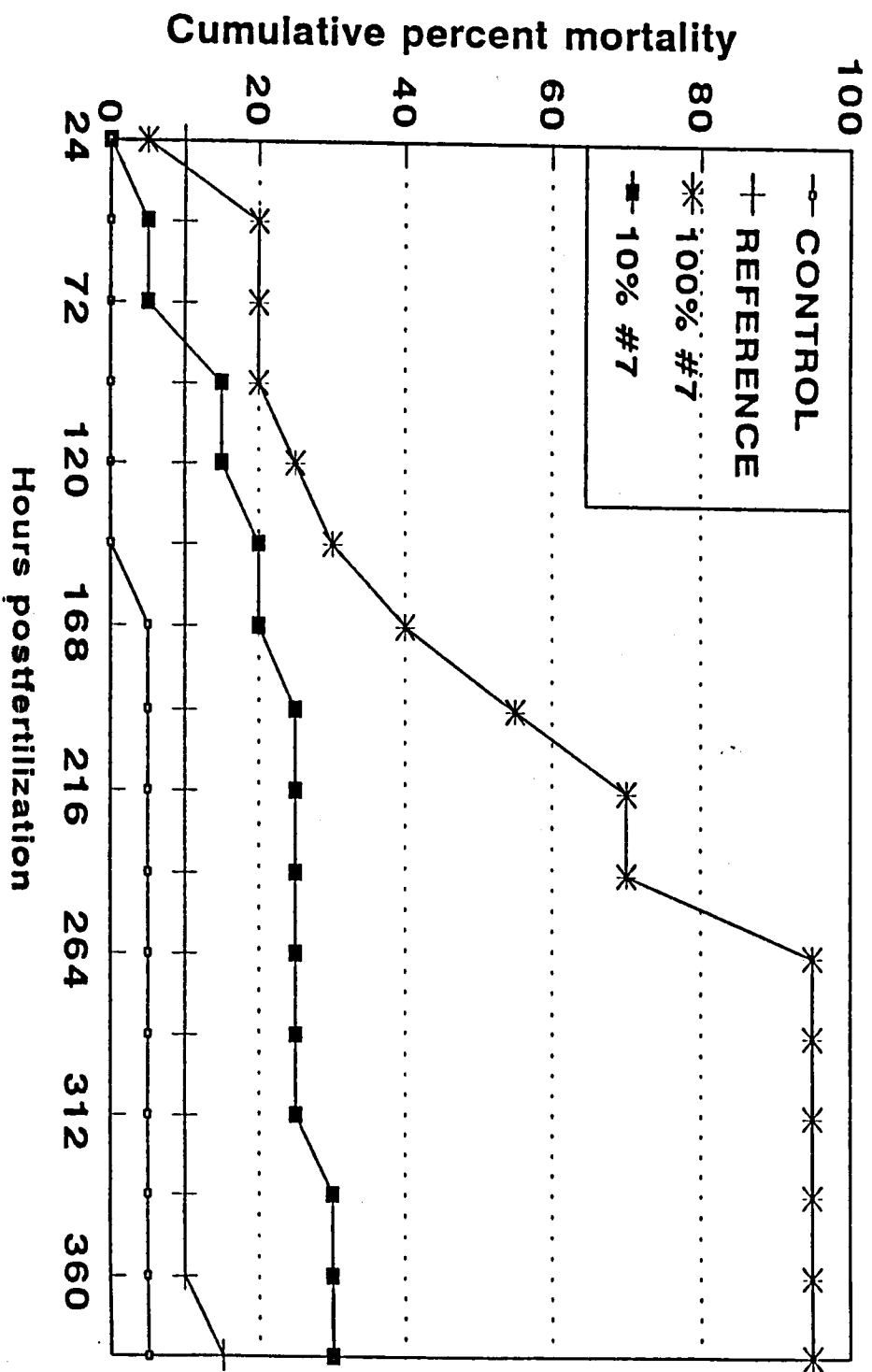


Figure 5.8 EFPC 4: Cumulative percent mortality for embryos exposed to 100% and 10% strengths of water collected from site 7. Control embryos were exposed to ERM and reference embryos were exposed to water collected at site 11. Water collected at site 11 was also used as the diluent. Experiment was initiated on 03/26/92

(11) did not appear to affect the hatching success of the medaka embryos (5% mortality in site 11 embryos and 0% mortality in ERM embryos). A dose-dependent relationship is evident in the higher concentrations, as seen in the total percent mortalities and 50% lethality times given in Table 5.5.

4.4 EFPC 5

Results from fry and embryo exposures in EFPC 5 are given in Table 5.6. Data is reported as 96 h mortality rate. However, experiment was terminated at 168 h. Embryos continued to show very high mortality rates, although fry mortality rates did not change. No changes in swimming ability or behavior were observed. Fry appeared unaffected by the EFPC water samples.

4.5 EFPC 6

In EFPC 6, two different types of group embryo incubations were compared to the single embryo incubations used in the previous experiments. Control (ERM) data (Table 5.7) for the three test groups showed very little difference from former experiments (Experiments 1 - 5 of Part VI). Single embryos exposed in reference site 11 also exhibited mortality rates similar to those in previous experiments. However, none of the embryos incubated in environmental water samples (experimental and reference sites), in groups, survived past 192 h.

Table 5.5 Total mortality and 50% lethality times (50% LT) for embryos exposed to serial dilutions of water samples from sites 2 and 4 in EFPC 3, initiated on 03/18/92, and sites 6 and 7 in EFPC 4, initiated on 03/26/92. Diluent water was from site 11. 50% LT is defined as the 24 h time-interval during which 50% unfiltered was observed in exposure groups.

Site/ Concentration	Total Mortality (%)	50% LT (h)	Site/ Concentration	Total Mortality (%)	50% LT (h)
EFPC 3			EFPC 4		
ERM	5	NA ^a	ERM	5	NA ^a
Site 11/100% ^b	5	NA ^a	Site 11/100% ^b	15	NA ^a
Site 2/100%	100	144	Site 6/100%	100	168
Site 2/10%	85	264	Site 6/10%	25	NA ^a
Site 2/1%	10	NA ^a	Site 6/1%	25	NA ^a
Site 2/0.1%	5	NA ^a	Site 6/0.1%	25	NA ^a
EFPC 3			EFPC 4		
ERM	5	NA ^a	ERM	5	NA ^a
Site 11/100%	5	NA ^a	Site 11/100%	15	NA ^a
Site 4/100%	100	144	Site 7/100%	95	192
Site 4/10%	40	NA ^a	Site 7/10%	30	NA ^a
Site 4/1%	15	NA ^a	Site 7/1%	30	NA ^a
Site 4/0.1%	10	NA ^a	Site 7/0.1%	20	NA ^a

^aMortality rates did not reach 50%.

^bSite 11 is a reference site.

Table 5.6 96 h mortality data for fry and embryos, from EFPC 5, initiated on 04/03/92.

Site (N)	Embryo Mortality (%)	Fry Mortality (%)
ERM (40)	2.5	2.5
Site 11 (40)	5	0
Site 2 (40)	25	2.5
Site 4 (40)	27.5	0
Site 6 (40)	40	0
Site 8 (40)	27.5	0

Table 5.7 Percentage mortality for three test groups of embryos in EFPC 6. Experiment was initiated on 05/01/92.

Site	Single embryo incubation rate (N)	Groups of 10 embryos: percent mortality (N)	Groups of 8 embryos: percent mortality (N)
ERM	2 (50)	60 (10)	0 (8)
		0 (10)	37.5 (8)
		50 (10)	0 (8)
		0 (10)	12.5 (8)
		0 (10)	25 (8)
Site 11	4 (50)	100 (10)	100 (8)
		100 (10)	100 (8)
		100 (10)	100 (8)
		100 (10)	100 (8)
		100 (10)	100 (8)
Site 4	100 (50)	100 (10)	100 (8)
		100 (10)	100 (8)
		100 (10)	100 (8)
		100 (10)	100 (8)
		100 (10)	100 (8)

5. DISCUSSION

5.1 Overall Toxic Effects of EFPC to Japanese Medaka Embryos

We reached several conclusions from the data obtained from the incubation of medaka embryos in water samples collected from EFPC:

- (1) medaka embryos are sensitive to a component of the water in EFPC, and experience high toxicity in 100% strength water samples,
- (2) no such toxic effect is seen in medaka embryos individually incubated in water from a reference site (#11),
- (3) the toxic effect does not appear to be due to the presence of a pathogen in the stream,
- (4) medaka embryos are apparently more sensitive to this toxic component than are 24 - 96-h-old medaka fry,
- (5) toxicity for medaka embryos (incubated in diluted water samples) was greatest at sites closest to the DOE facility, and decreased in water samples collected further downstream from the same facility, and
- (6) the observed toxicity varied with individual water samples and sample collection times, indicating a dynamic effluent (See Figures 5.9 and 5.10).

The high toxicity observed in these experiments is not found when fathead minnow larvae are exposed to effluents collected during the same time periods (Stewart and Kszos, personal communication, 1992). This information may be

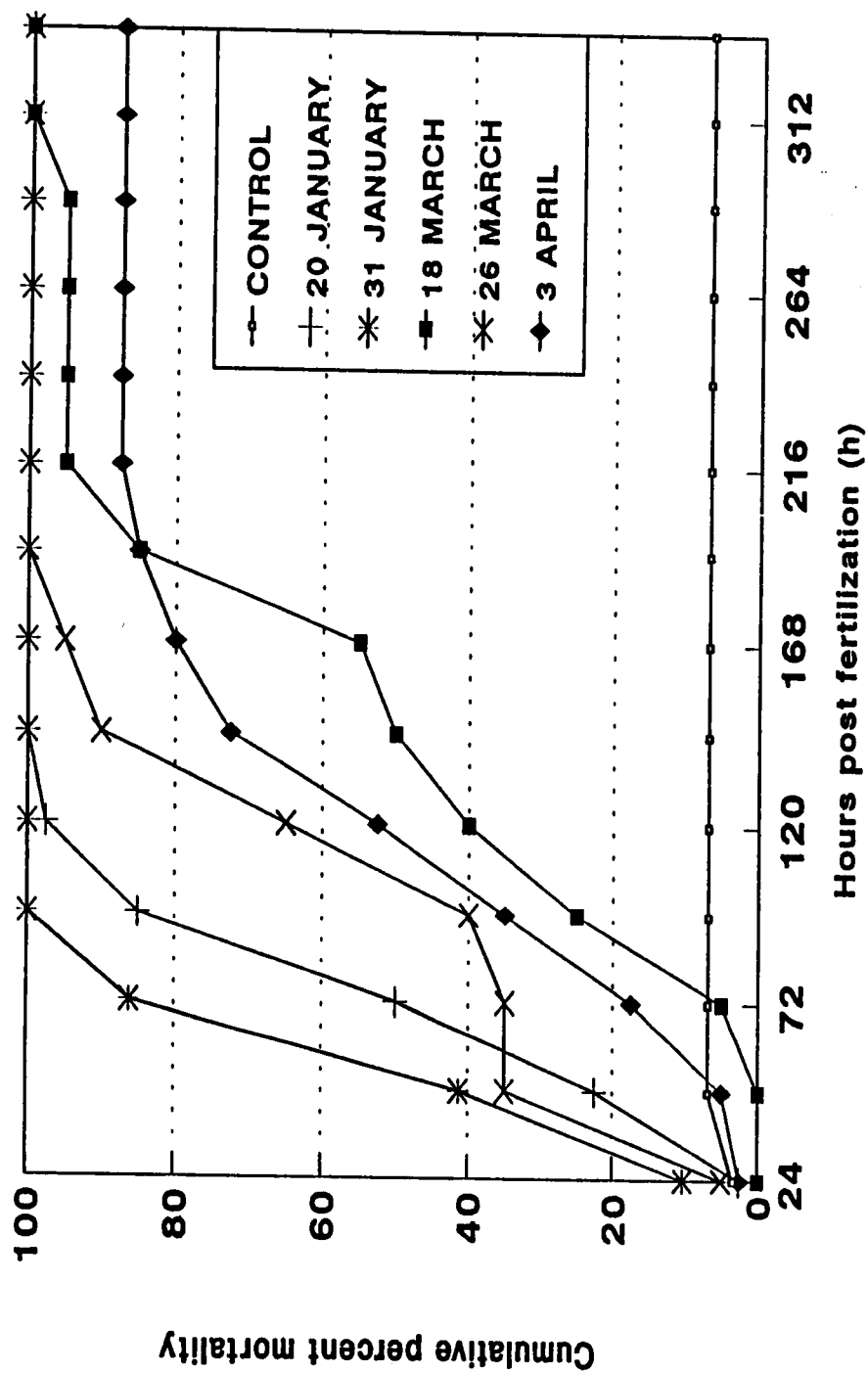


Figure 5.9 Changes in the percentage mortality in medaka embryos exposed to EFPC effluent (Site 2) over time.

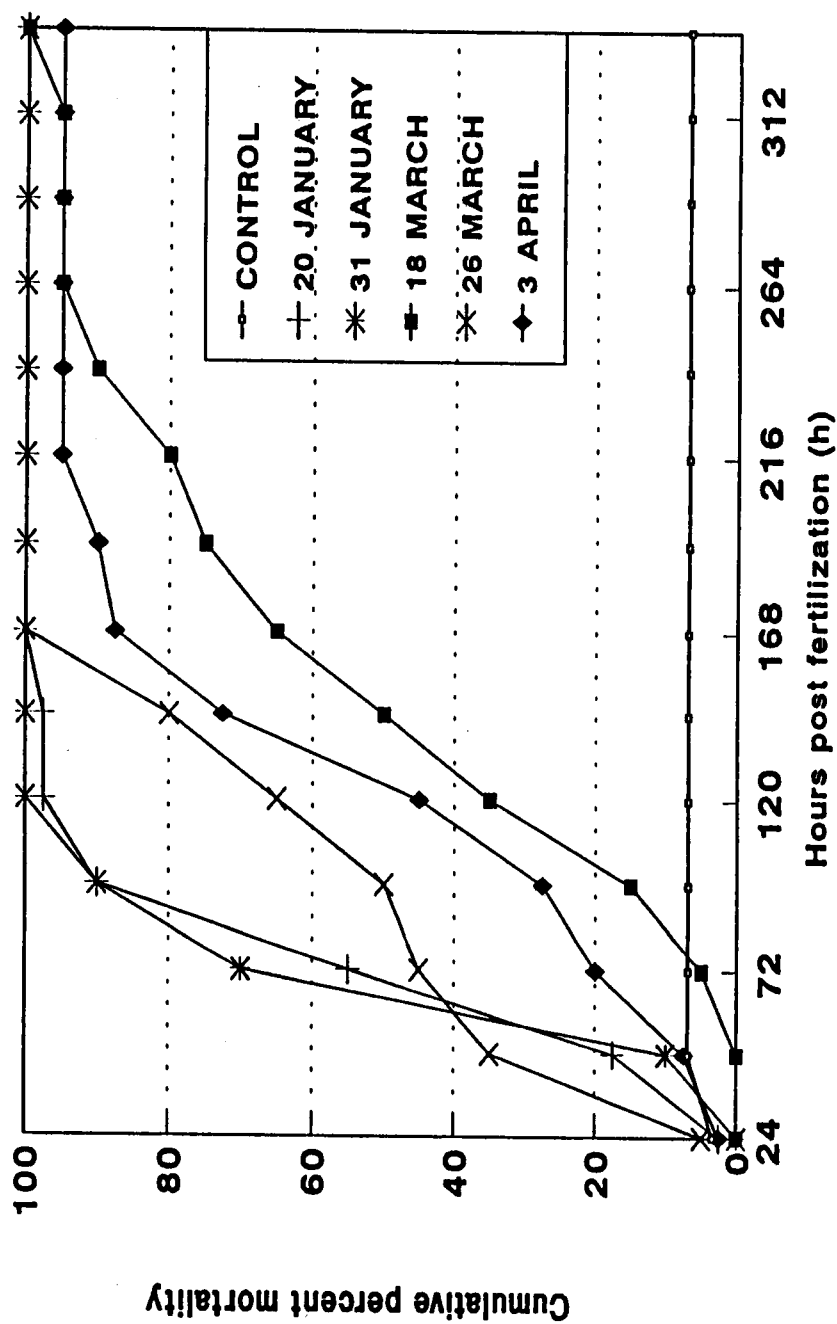


Figure 5.10 Changes in the percentage mortality in medaka embryos exposed to EFPC effluent (Site 4) over time.

evaluated from two perspectives: (1) either the medaka embryos are too sensitive to be used in monitoring some streams, or (2) the fathead minnow larval stage, like the 24-h-old medaka fry, is not sensitive to the toxic component of the effluent. These two perspectives are not mutually exclusive. There may be a toxic component to the EFPC effluent to which the medaka embryo is hypersensitive, but also to which the medaka fry and fathead larvae are less sensitive.

Unfortunately, due to health problems in the fathead minnow culture, we were unable to conduct side-by-side medaka/fathead embryo tests. Ongoing biomonitoring studies of the indigenous populations in EFPC appear to indicate that there may be effects of chronic toxicity in EFPC, as well as acute effects (fish kills) as a result of occasional industrial releases from the facility (Adams, et al., 1989, 1990; Shugart, 1990; Kornegay, 1991) .

The historic information available on EFPC and the Y-12 facility indicate that the stream has been contaminated with various heavy metals (mercury, lead and nickel), PCB's, PAH's, and radionuclides, as well as the typical industrial pollutants (1983 Mercury Task Force, 1983; Van Winkle, et al., 1984; Theodorakis, et al., 1991), chlorine and ammonia. Heavy metals, PCB's, PAH's, and radionuclides are not detected at high levels in routine water sampling, however, chlorine and ammonia are routinely present at the uppermost sites, and are occasionally detected below the outfall of the Oak Ridge Sewage Treatment Plant (Stewart and Kszos, personal communication, 1992).

It is important to note, however, that when chlorine was detected in water samples from sites used in this study, it was found only in samples that had been collected for chlorine analysis. These samples were collected in dark bottles, brought back to the laboratory and analyzed immediately. When water samples were analyzed following routine treatment where no attempt was made to preserve chlorine (i.e. collection in clear bottles, returned to the laboratory and sterile filtered under air vacuum), no chlorine contamination was found. Therefore, it is unlikely that the cause of the acute toxicity is due to chlorine. Also, while ammonia was present and detectable in the water samples collected for use in the assays, this was only at specific upper sites and would not account for the high toxicity at the lower sites. Further studies done by Stewart and Kszos (personal communication, 1992) identified a small pipe within the facility that was contributing high concentrations of ammonia to the headwaters of EFPC.

An observed phenomenon may provide some information on the reason for the disparity between embryo and fry sensitivity. In these exposures, the embryonic chorion apparently decayed during incubation in the water samples from EFPC. It was not apparent whether the flaking and bulging seen in the chorion was a direct effect of the exposure, or instead, an indirect effect resulting from problems within the embryo. However, this phenomenon was present in all embryos exposed to water from EFPC. In the few cases where exposure did not result in 100% lethality, the embryos that survived emerged from the chorion within 48 - 72 h PF. Development was then completed outside the chorion. The

resulting pre-larval stage maintained a large yolk sac and remained inactive until the time at which they would normally hatch (8 - 10 d PF). Also, in many cases, the chorion appeared ruptured and the embryo had begun to emerge, unsuccessfully. In these cases, the yolk sac would partially herniate through the rupture, leaving a constricted area in the yolk sac at the point of the rupture in the chorion. Pooling blood in the herniated region would then be followed by death of the embryo. In many other cases, when the daily observations were made at 48 - 72 h PF), dead embryos were observed outside and separated from their chorions. This information may indicate that the toxic component of the EFPC water samples could be specifically attacking the structure of the chorion, or affecting the hatching enzymes within the embryo. Premature hatching of medaka embryos has been documented during exposure to the water-soluble fraction of crude oil (Leung and Bulkley, 1979). The toxic component of EFPC may also cause premature hatching--so early that survival is not possible. Such a mode-of-action would not be as hazardous to the already-emerged fry.

The difference in sensitivity between embryo and fry life-stages is neither unique to this stream effluent nor to the medaka fish. Differences in life-stage sensitivity are recognized in most fish species (McKim, 1985; APHA, 1985), and may be due to metabolic differences or to differences in available exposure routes. In this case, it may be the removal of the site-of-action (i.e. chorion) in the fry that offers it protection from the toxic component.

5.2 Comparison of Different Incubation Methods

While few differences were seen in the comparison of group incubations and single-embryo incubations with control solutions and single-compound laboratory-prepared solutions (Parts II and III of this document), data obtained in the EFPC-exposures indicates that group incubations are not feasible for field-collected water samples (Table 5.7). The most obvious example of this is site 11, a reference site. While single embryo incubations were successful, and generated adequate control data during each use, neither type of group incubation produced comparable results. In fact, both types of group incubations resulted in 100% mortality for both reference and EFPC sites. While the data for single embryo and group incubations did not differ for the EFPC sites (100% mortality) the time to death for the group incubations was faster. There were problems with white, cloudy accumulations of fungi and rotifers in all cases where field collected water samples were used in group incubations.

Since all water samples were sterile filtered (0.1 μm nalgene filters), it is improbable that the fungi was present in the water samples. Contamination most likely results from indigenous bacteria, fungi and rotifers on the embryos, originating in water in which the adults live. The environmental water samples merely provide more nutrients for survival of fungi and rotifers than does sterile water or sterile-filtered ERM. Group incubations would not be recommended for experiments using environmental water samples, since most would contain higher nutrient levels than sterile water.

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CONCLUSIONS

The three phases of this project involved: (1) the development of a protocol for the incubation of individual embryos, and comparison of data from control solutions to insure that it was feasible to utilize individual embryos, (2) testing these protocols on single-compound, laboratory-generated solutions, and (3) using the protocols to evaluate the toxicity of both soil extracts and environmental water samples.

Data collection, analysis, and observations provided the opportunity to draw important conclusions from each phase of the project. During the first phase, protocol development (Part II), it was determined that while medaka embryos will develop and hatch in a wide range of water salinities, all laboratory water sources are not optimal for use as controls. Tests with those sources available in this laboratory demonstrated that sterile water (water filtered through a Millipore filtration system) worked best. This water can either be used as it is, or with the addition of a mixture of rearing salts (i.e. ERM). Experiments also demonstrated the benefits of 24 or 48 h media-exchange regimes with individually incubated embryos, as well as the benefits of routine aeration. Of course, during toxicological studies, aeration would not always be recommended, because of the loss of volatile compounds.

The incubation of single embryos was compared to two different types of group incubations during each phase of the project. Differences were seen between embryos exposed to control solutions, and those exposed to either BaP or contaminated soil extracts. The data was inconsistent within these groups.

Mortality was often high in one Petri dish or 20 ml scintillation vial, while lower in another containing the same solution. The most obvious reason for these inconsistencies appeared to be fungal, bacterial, and rotifer contamination in the vessels where there was higher mortality. Such contamination was visible to the naked eye, and under the dissection microscope. The most dramatic differences between the data appeared during the EFPC exposures. While high mortality was seen in embryos exposed to EFPC water in both the individual incubations and the two group incubations, it was also evident in all group incubation vessels containing water samples from the reference site. Embryos incubated individually in water from the reference site experienced mortality levels comparable to controls. All of the vessels (Petri dishes or 20 ml scintillation vials) containing water collected from the environment (EFPC and reference site) produced very high, rapid mortality. Fungal, bacterial, and rotifer contamination was very evident in all of these cases, whereas it was not evident in individual incubations.

As noted in Part V, the source of the contamination is probably the embryos themselves. Since the water samples were all sterile filtered ($0.1\ \mu\text{m}$ Nalgene filters), there is little reason to assume that the water samples were the source of contamination. The embryos, however, were exposed to the same water that the adult medaka live, eat, and defecate into, for up to one hour prior to collection. Once on the outside of the embryos, the microbes would be transferred to the incubation media. The water samples may then provide more nutrients than the ERM for the microbial growth. Single embryo incubations with

48 h exchange regimes prevented both uncontrolled growth of the microbes, and spreading of the microbes from one embryo to another. Incubation of groups would provide ample opportunity for both microbe growth and embryo-to-embryo contamination.

The later phases of the project, with tests on a single-compound, laboratory generated solution (BaP), contaminated soil extracts, and water samples from a stream receiving industrial discharges, demonstrated the need for a variety of endpoints. As was evident in the embryos exposed to the lower concentrations of BaP and to the soil extract (TP-04-65) from the diesel-fuel-contaminated soil, mortality is not necessarily the most sensitive indicator available. In both cases, the occurrence of developmental abnormalities, visible in the growing embryos under dissection microscope, proved to be more indicative of exposure than did the level of mortalities. Altered time-to-hatch was observed in BaP exposures, and altered developmental timing was observed in the soil extract exposures. Thus, it is important to monitor various endpoints for different exposures. Those found useful in this project include:

- (1) percentage mortality,
- (2) percentage abnormalities,
- (3) altered time-to-hatch, and
- (4) altered developmental timing.

The flexibility of this particular medaka embryo assay was also demonstrated by the later phases of the project. While the incubation of individual embryos worked well in the traditional use of the assay (single-compound, laboratory generated solution [BaP]), also provided a better method for the applied approaches (soil extracts and stream effluents). In the experiments where embryos were exposed to extracts of contaminated soils, the individual vials provided for better observation of developmental abnormalities. In this case, the occurrence of developmental abnormalities and developmental delays was different between the two soil extracts, whereas the percentage of abnormalities was not so clearly different in group incubations. Observation of developmental abnormalities or developmental delays was not as easily accomplished in the group incubations. In the experiments comparing water samples from a stream receiving industrial discharges (EFPC) and samples from a reference stream, group incubations were not possible due to the high mortality in all group incubations. When individual embryos were incubated in the water samples, differences in the percentage mortality between the reference and experimental streams were very evident.

Areas of future research still exist within each phase of this project. In the development of controls, factors such as light and movement need to be investigated. It is important to determine the role of each of these in the hatching process. Of course, each phase could be expanded to include other chemicals, for direct comparison with previously documented studies (e.g. PCB's, heavy metals

and insecticides). Such studies were not done in this project due to the breadth of the goals and the Environmental Health and Safety regulations within our facility. Likewise, experimentation with various types of soil extracts, extraction procedures, and types of contamination would prove useful toward documentation of this protocol.

While other stream systems within the East Tennessee region have been tested with this protocol (Niemela, et al., 1992), further experimentation is necessary. This should include studies designed to determine the temporal and seasonal changes in effluent toxicity and water chemistry.

Most importantly, direct, side-by-side toxicity tests are needed to make good sensitivity comparisons for medaka, fathead minnow, and daphnia. These tests would evaluate the sensitivity of the medaka in relation to these other species that are frequently used in toxicity testing. Limited information is available on some compounds and species; however, the different incubation methods may affect sensitivity.

Side-by-side comparisons are most important on the Oak Ridge Reservation for other reasons. As was mentioned in Part V, the fathead larvae that are routinely used to test the toxicity of the streams on the reservation do not show the extreme sensitivity seen in the medaka embryos. It is important to determine whether this difference is due to actual variation in sensitivity, or to a specific effect seen in medaka embryos, or to a sensitivity in fish embryos, in general, that is not seen in larvae. The possibility still exists that this difference

may be specific to the medaka embryos, possibly as a result of the general water chemistry interactions (i.e. pH, alkalinity, hardness), although much effort was made to rule out this possibility. Since the Biological Monitoring and Abatement Program is done to insure compliance with NPDES regulations, there will be legal implications to the source of the differences, no matter the cause.

In conclusion, this medaka embryos assay has proven to be flexible enough to be used on a wide variety of media. These include laboratory-generated, single-compound solutions, soil extracts and stream effluents. As expected, one endpoint of the numerous ones monitored, did not prove ideal for all types of exposures. It would, therefore, be necessary to monitor all endpoints available when working with complex environmental contaminants.

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