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# Sensitivity of Vertebrate Embryos to Heavy Metals as a Criterion of Water Quality, Phase II: Bioassay Procedures Using Developmental Stages as Test Organisms

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# SENSITIVITY OF VERTEBRATE EMBRYOS TO HEAVY METALS AS A CRITERION OF WATER QUALITY

## PHASE II

## Bioassay Procedures Using Developmental Stages as Test Organisms

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ABSTRACT

Chick, amphibian and fish embryos were evaluated as bioassay and bioindicator organisms. Test procedures were developed by which embryonic stages may be used 1) in bioassay systems to evaluate the toxicity of particular metallic or metal-containing trace contaminants, and 2) as bioindicators to monitor the quality of natural water resources.

A bioassay technique was devised in which metallic toxicants were administered to chick embryos by "needle tract" injection into the yolk sac. This provided more uniform distribution of test metals throughout the yolk mass than can be obtained by conventional yolk sac injection methods, and gave more sensitivity and uniformity of test results. Metals such as arsenic, cadmium, mercury, lead and zinc are easily detectable at a level of 1 ppb.

An in vitro culture technique was developed by which embryos of aquatic vertebrates may be "maintained" for bioassay and bioindicator purposes. Five test species were identified, suitable synthetic culture water was formulated, and culture monitoring procedures were determined. Most toxic metals (e.g., mercury) may be detected at 1 ppb or less with the use of more sensitive embryonic species (e.g., trout). Early cleavage stages of the leopard frog (Rana pipiens) proved more sensitive to cadmium than older embryos, similar to results obtained in Phase I with mercury treatment of trout and frog embryos. Early developmental stages, therefore, have proven especially important for use in bioassay and bioindicator systems.

Discussions are also given to a number of environmental and experimental variables which are important in performing and interpreting test results of bioassays.

Descriptors: Bioassay, Bioindicator, Heavy Metals, Water Quality

Identifier: Vertebrate Embryos

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# CHAPTER 1

## INTRODUCTION

Operating upon the premise that water quality standards for metallic pollutants should be established at levels which are safe for the most susceptible stages in the life cycles of living organisms, consistent with the recommendations of the National Technical Advisory Committee on Water Quality Criteria (1) and other investigators (2-5), we proceeded under Phase I of this investigation to determine the aqueous concentrations of certain toxic metals which prove lethal and/or teratogenic to embryos of avian, amphibian and piscine species.

Embryos were treated continuously until hatching with sodium arsenite and chloride salts of cadmium, lead, mercury, methyl mercury and zinc (12). Compared to survival in control populations, all metals produced significant degrees of lethality and/or gross anatomical anomalies in one or more species when used at 10 ppb or less. Early frog embryos were found to be at least 1,000 times more sensitive to inorganic mercury than adult stages, with 100% of cleavage and blastula stages suffering lethality when exposed to a concentration of 10 ppb. Treatment with methyl mercury at 10 ppb produced 66%, 12% and 2% mortality rates in populations of rainbow trout, channel catfish and goldfish embryos, respectively. In addition, 20% of the surviving trout embryos bore gross morphological deformities. Lethality or severe developmental impairment occurred in 53% and 33% of trout embryos treated with 5 ppb and 2 ppb methyl mercury. Rainbow trout and chick embryos suffered 10-22% lethality when exposed to 1 ppb of either inorganic or methyl mercury. Zinc, lead and cadmium at 1 ppb produced 17%, 26% and 34% lethality in chick embryos, respectively.

Arsenic was found to be highly toxic to chick embryos, producing 35% mortality at a concentration of 1 ppb. However, arsenic-induced lethality was significantly less in the trout embryo, with a $TL_{50}$ value of approximately 0.5-0.75 ppm. It appears that eggs and/or embryos of homeothermal species (e.g., chick) are much more susceptible to arsenic than those of poikilotherms. This may be due in part to the higher rate of oxidative metabolism in the former, as arsenic oxides are known to uncouple oxidative

phosphorylation (57). Survival values reported above were based upon all embryos which lived to complete the hatching process, including those bearing gross teratologies. The latter often comprised 1/4 or more of surviving populations.

Based on a comparison of survival values approaching threshold (90-95% hatchability), embryos of the trout and catfish, in respective order, were approximately 50 and 10 times more sensitive to mercury than goldfish embryos. This differential sensitivity to mercury correlates with differences in both egg size and hatching time of these species, indicating that fish with larger eggs and/or longer periods of embryological development are more susceptible targets of mercurial poisoning. In the face of increasing levels of pollution, such data raise concern regarding 1) proper ecological balance in vertebrate communities, and 2) fate of commercially important vertebrate species which are subject to both economic and environmental stress.

A number of other investigations also show that fish eggs and fry are extremely sensitive to metallic poisoning. Most current studies in this area concern the toxic effects of zinc. Affleck (6) indicated a high sensitivity of eggs and alevins (fry) of brown and rainbow trout to zinc poisoning, where concentrations as low as 0.01 ppm were found to be lethal or toxic. Similarly, Leitritz (7) has reported that zinc at a concentration of 0.04 ppm is lethal for young rainbow trout of two to four weeks of age. Pickering and Vigor (8) have shown an acute toxicity of zinc to eggs and fry of the fathead minnow. They found median tolerance limits ($TL_m$) of 3.92-3.98 milligrams/liter for 1-day old eggs. The $TL_m$ values dropped to 2.47-2.63 after two days of development and were 1.57-1.69 by completion of the 12th day. The newly hatched fry were still more sensitive to zinc sulfate ($TL_m$ of 0.95 mg/l for 2 days exposure). Corresponding $TL_m$ values for adults, under the conditions imposed in the study, were not given. However, other reports indicate lethal concentrations of zinc sulfate for adult minnows ranging from 10 mg/l for 48 hours in freshwater (9), to values as high as 400 mg/l for 200 minutes in distilled water (10). Skidmore (2, 11) has shown that zebrafish eggs also increase in sensitivity to zinc sulfate during the course of embryonic development. The later embryonic

stages (2-4 days) and newly hatched fry (4-13 days old) were highly susceptible to the toxic effects of zinc. Young fish up to 40-days of age were still found to be more sensitive to zinc than adults, as illustrated by their threshold concentration of 1.3 ppm (zinc sulfate) compared to a value of 10 ppm for adults.

The fact that zinc appears to be more toxic to late embryonic stages and newly hatched fry, at least in certain species, is interesting in view of our findings (12, 13) that mercury is distinctly more harmful to early embryonic stages. Though toxic at certain concentrations, zinc has been demonstrated to be an essential trace element for normal embryonic development, and zinc deficiency may lead to serious embryonic abnormalities (14-17). Thus, the pattern of zinc toxicity to embryonic systems may vary significantly from that of non-essential elements like mercury. Similar to the effects of zinc, recent studies indicate that eggs of the king salmon (18) and brook trout (19) are somewhat less sensitive to copper than newly hatched alevins.

It is becoming increasingly apparent that, concerning the toxic effects of most metallic trace contaminants, embryonic, larval, and early post-hatched stages constitute the critical "sensitive links" in the life cycles of many piscine, amphibian and avian species (2, 8, 11, 13, 18-20). It appears plausible that reproductive potential of natural vertebrate populations may be severely restricted or abolished by trace levels of metals which are harmless or sublethal to most adult organisms. Accordingly, protective environmental standards and pollution abatement policies, which have been based to a considerable extent on tolerances of adult animals, may not provide adequate protection for embryonic development and continued reproduction of many vertebrates. Considering the great susceptibility of vertebrate embryos and early posthatched stages to many trace metals, and the alternative pathways through which they may receive embryopathic exposure (13), it is important to formulate more adequate guidelines with which to 1) evaluate the impact of metallic pollution upon reproductive potential of vertebrate species, and 2) determine whether recently promulgated environmental standards are adequate to protect sensitive developmental stages.

Under Phase II of our investigation, we will consider the use of vertebrate embryos for bioassay/bioindicator purposes. Noting the great reliance that EPA and other agencies have placed on the use of bioassay and bioindicator techniques (1, 21-23) in evaluating the biological effects of trace contaminants and in establishing protective environmental standards, we contend that sensitive developmental stages should be included among the test organisms used for such purposes. We note further that one recommendation accruing from the 2nd Annual NSF-RANN Conference on Trace Contaminants was that more attention should be directed to development and growth, since such processes appear more sensitive to trace levels of environmental contaminants (24).

In the chapters which follow, we will 1) treat background considerations essential to the proper selection, design and application of bioassay systems, and 2) describe procedures developed in this study for the use of chick, amphibian and fish embryos as bioassay and bioindicator test organisms.

## CHAPTER 2

## BIOASSAY PROCEDURES - TERMINOLOGY AND GENERAL CONSIDERATIONS

Laboratory bioassays are extremely useful tools with which to assess the toxicity of pollutants to living organisms. There are several types of pollutant bioassays which routinely are conducted on vertebrate animals. In terms of exposure time, bioassays generally are classified as either acute or chronic. An acute test is one in which the organisms' response to a toxicant is measured over a relatively short period of time (8, 25, 26), with results most commonly expressed as median tolerance values for 4-7 day periods (e.g., 96-hr $TL_{50}$). In comparison, a chronic test usually signifies a longer response time (treatment period), often one-tenth or more of the life span of the test organism (26). In most instances, test results are given as survival frequencies within the treated animal populations. In addition to acute and chronic toxicity measurements, bioassay systems may be based on observable behavioral responses, such as avoidance (27), increased activity, or alterations in respiration rate (28). Though tests on behavioral responses usually are more sensitive than acute or chronic

bioassay procedures, results are not necessarily quantifiable in terms of animal survival. The greatest value of such monitoring procedures rests with the early detection of changing environmental conditions which produce increased animal stress, thereby giving "early warning" in advance of more acute levels of pollution.

The majority of bioassays involve aquatic organisms and usually are conducted under either static or continuous flow-through conditions. Static tests consist of adding toxicants to standing, nonflow aquatic cultures. In flow-through systems, the culture water is continuously perfused through the test chambers during the treatment period. The principal disadvantages of the former derive largely from 1) progressive deterioration of culture conditions (increasing $NH_3$, $CO_2$; decreasing pH), and 2) inability to maintain a constant level of toxicant throughout the test period. Flow systems, given adequate monitoring of toxicant level and other variables, provide the best means of maintaining stable, constant test conditions, and enable more reassuring extrapolations of test results to actual environmental situations.

Most bioassays involve exposure to a single toxicant. However, since often a number of chemical poisons are present simultaneously in the aquatic environment, bioassay tests also may be used to study the combined toxic effects of certain mixtures of toxic agents. Such studies enable detection of synergistic, antagonistic, or additive effects produced by particular combinations of pollutants. Synergistic interaction between two or more poisons is revealed if the toxicity of a mixture is greater than the sum total of their individual effects. For example, synergistic actions have been shown in toxicity experiments with rainbow trout for mixtures of copper and zinc, as well as for alkyl benzene sulfonate and zinc (29, 30). Likewise, a mixture of selenium and mercury produces a marked synergistic effect on carp and catfish embryos (31, 32).

Antagonism may be described as an interaction between two pollutants which leads to a net reduction in toxicity (33). For example, an antagonistic relationship has been found in which long-term exposure to $H_2S$ provides bluegills with increased resistance to acutely toxic copper concentrations (34). Although this particular interaction involves two toxic substances

($H_2S$; copper), a non-toxic element such as calcium may interact antagonistically to reduce the toxicity of metallic poisons such as zinc (9). Further explanation of such antagonistic interactions is given below in a discussion on water hardness as a bioassay variable.

Certain combinations of toxicants show simple additive effects. In 48-hr acute tests on rainbow trout, the toxicities of mixtures of copper and phenol; copper, zinc and phenol; and copper, zinc and nickel have been found to equal the sums of the fractional toxicities of the particular poisons present in the test solutions (35). Considering the various possible interactions of different toxic agents, it becomes increasingly important to evaluate the combined effects of those pollutants which may occur simultaneously within a given ecosystem. As noted by Cairns et al. (28), "It is difficult, if not impossible, to predict the biological effects of a complex, continuously changing industrial waste from chemical analyses alone." As such evaluations cannot be derived simply from physiochemical monitoring data, bioassay systems provide a most useful tool for estimating the biological impact produced by particular combinations of pollutants.

## CHAPTER 3

## PHYSIOCHEMICAL VARIABLES IMPORTANT IN BIOASSAY DETERMINATIONS

In laboratory bioassays, concern must be given to the extent to which certain physiochemical parameters may alter the form and/or toxicity of various classes of pollutants. Furthermore, physical and chemical characteristics of aqueous habitats may differentially stress test organisms, affecting their responses to particular toxicants. While it is not possible to control all water quality variables in bioassays, one should at least measure the important parameters, and apply them to the collection and interpretation of toxicity data. Several major variables will be discussed in light of their significance to aquatic bioassay systems.

Temperature. Control of temperature is essential in most bioassay experiments as it 1) affects the metabolic rate of poikilothermal organisms and the physiological action(s) of toxicants, 2) governs the rate of embryonic

development and hatching time of aquatic species, and 3) alters solubility and reaction kinetics of toxicants, as well as certain chemical agents which may interact with toxic substances. It is well known that variations in temperature differentially affect the toxicity of certain poisons to fish. For example, zinc toxicity and temperature have been demonstrated to be positively correlated (36), while the toxicity of phenols increases as temperature decreases (37).

It is most desirable to conduct experiments within temperature ranges which are optimal for the test organism. During the use of bioassay procedures, it is recommended that temperature be checked daily and that the average temperature be calculated to represent the test. Ideally, the day-to-day variation should be no greater than $\pm$ 0.5° C (26).

Dissolved oxygen. Another physical parameter very closely related to temperature is dissolved oxygen. As temperature increases, the solubility of oxygen in water decreases. Additionally, temperature affects oxygen requirements of fish and other bioassay organisms. The standard oxygen uptake by goldfish increases by over 250% when water temperature is raised from 10° to 20° C (38). The minimum oxygen requirement optimal for most fish is 5 mg/l. Few fish are found in streams in which the oxygen level falls below 4 mg/l (39). For most fish bioassays, the dissolved oxygen level should be no more than 2 mg/l below saturation (26). It also has been established that the dissolved oxygen level can influence the toxicity of various compounds. Alkyl aryl sulfonate detergents, cyanides, and cresols exert greater toxicity to fish as dissolved oxygen decreases (40). Oxygen not only affects the physiological aspects of a bioassay, but also the redox potential of aquatic test systems (41).

Hydrogen ion concentration. As different forms of a particular compound may vary as to their toxic effects, it is desirable to conduct bioassays so that results may be interpreted in terms of the specific species of a given toxic agent. Hydrogen ion concentration affects the solubility and ionization of many toxic compounds, and many different inorganic metal complexes may be formed under conditions of varying pH. For example, in a rather simple equilibrium system involving copper salts ($10^{-6}$ M copper, $10^{-2}$ M total carbonate), a number of different copper species predominate at various levels

of pH. Below a pH of 5, the copper ion ($Cu^{+2}$) is present; at pH 5-9, soluble copper carbonate forms; and between pH 8-12, an insoluble copper species, tenorite (CuO) may be present. Above pH 12, the anion $CuOH_4^{-2}$ exists. Six other copper compounds or ions of less significance also may form, including $Cu_2(OH)_2CO_3$; $Cu_3(OH)_2(CO_3)_2$; $CuOH^+$; $Cu_2(OH)_2^{+2}$; $Cu(OH)_3^-$; and $Cu(CO_3)_2^{-2}$. The cupric ion and copper carbonate are thought to be the most toxic species of this metal (44). Sprague (26) suggests that a pH variation of $\pm$ 0.3 is reasonable in most toxicity determinations, and Roberts and Allen (45) have dealt extensively with techniques for controlling pH, alkalinity, and total carbonate levels in bioassay systems.

Ionic strength and water hardness. The concentration of salts in bioassay media may substantially influence toxicity measurements. The toxic actions of zinc and ammonia to fish diminish considerably as salinity is increased to the isotonic point (46). On the other hand, phenol becomes more toxic under similar conditions (37). Calcium, the major contributing factor to water hardness, greatly alters the effects of many toxic agents, especially heavy metals. Copper concentrations as low as 0.015 mg/l have been found toxic in soft water to certain species of fish, whereas in hard water concentrations as high as 3.0 mg/l may be required to produce toxic effects (25). The decreased toxicity of heavy metals due to water hardness may result from calcium-induced antagonism (36) and/or to complexation and precipitation of metal ions as carbonates or hydroxides (47). At a hardness of 30 mg/l $CaCO_3$, copper has been found to be approximately five times more toxic to fathead minnows than at a hardness of 200 mg/l (48). Similarly, Lloyd (36) has observed a decrease in zinc toxicity to rainbow trout paralleling increased water hardness, and Mount (49) has found that with an increase in hardness from 50 to 200 mg/l $CaCO_3$, zinc toxicity to minnows decreases approximately 50-70%. Also, 3-trifluoro-methyl-4-nitrophenol (TFM), a non-metal used as a lampricide in the Great Lakes, is more effective in soft than in hard water (41).

Careful attention must be given to water hardness during both the use of bioassay procedures and in the extrapolation of test results to actual environmental conditions. Also, fish and other aquatic test organisms should be acclimatized to particular levels of hardness and tonicity (50) selected

for bioassays. Acclimatization is a salient feature of bioassay experimentation, since toxicity determinations will not have precise meaning unless test organisms are first conditioned to the bioassay test medium.

Organic complexing agents. In natural systems, metallic toxicants may be adsorbed onto sediments or chelated by various organic compounds. Several major chelating and sequestering agents in the aquatic environment include fractions of suspended plant and animal detritus, microbial metabolites, domestic sewage effluent, certain clay minerals and various humic compounds associated with bottom sediments. Many studies indicate a reduction in toxicity of metals when organo-metal complexes are formed. Grande (52) in his investigation of several Norwegian lakes demonstrated that humic colloids reduce copper toxicity to salmonid fishes, and Nielson (53) has found that organic substances such as polypeptides reduce copper toxicity to algae. Also, Fitzgerald (51) has shown that citric acid chelation reduces the toxicity of copper to minnows. Other organics such as phenols, petroleum, and carbon disulfide can reduce the amount of free copper present in natural ecosystems, thus altering copper-induced toxic effects on living organisms (54). In addition, marine copepods have been found to survive copper treatment longer in seawater containing sediment extracts thought to be humic in nature (55). Some other naturally occurring organic ligands which complex copper also have been found to reduce copper toxicity to fish. The amino acid glycine reportedly is more effective than humic acid in reducing acute copper toxicity (56).

From the above discussion, it seems clear that metal toxicity may be altered significantly by the presence of chelating and sequestering agents, and that such factors must be considered in establishing the parameters for bioassay procedures, evaluating the significance of test results, and in applying test data to natural environmental problems.

## CHAPTER 4

## THE CHICK EMBRYO AS A BIOASSAY TEST ORGANISM FOR METALLIC TRACE CONTAMINANTS

Sensitivity to metals. As noted in previous studies by Birge and Just and Birge et al. (12, 13), the chick embryo is extremely sensitive to metallic toxicants. Selenium, arsenic and cadmium produce quite similar responses, giving survival rates (hatchability) at 1 ppb of 64-66% and approximate $TL_{50}$ values of 0.01, 0.01 and 0.05 ppm, respectively. These extremely embryopathic metals also produce substantial percentages of gross embryological anomalies among survivors (e.g., hydrocephaly and other brain deficiencies,absent eyes, various skeletal defects), with frequencies of 11% and 29% for selenium and arsenic at 0.5 ppm, and 33% for cadmium at 1.0 ppm. Lead, methyl mercury, mercury and zinc also are highly toxic to chick embryos, giving survival rates of 74-83% when used at 1 ppb (Table 1). Combining the frequencies of lethality and gross anomalies, $TL_{50}$ values for lead, methyl mercury and mercury are 0.1, 0.1 and 0.5 ppm, respectively. Occurrences of teratologies among survivors range from 7-24% at 0.05-5.0 ppm lead, and 7-22% at 0.1-5.0 ppm methyl mercury. Teratogenic development for all metals diminishes at reduced concentrations, resulting in frequencies under 3% at 0.01 ppm.

As noted in Table 2, there are three principal patterns of vertebrate reproduction which differ significantly concerning the avenues through which embryos receive exposure to environmental trace contaminants. Assessing the effects of metallic toxicants on avian reproduction depends largely upon 1) levels of contamination in avian food sources, and 2) the rates at which metals consumed by laying females are incorporated into eggs during oogenesis.

A number of investigations reveal that mercury administered to adult female birds may accumulate in eggs at concentrations which reduce hatchability (58-70). Backstrom has studied the accumulation of mercury in eggs and embryos of the quail (59). Depending upon dosage level, as much as 50% of the methyl mercury given to adult hens becomes concentrated in eggs. Backstrom suggests that the accumulation of mercury in eggs of female

quail may constitute a "safety valve" pathway for excretion. It would appear that such a physiological mechanism, though affording substantial protection for egg-laying adults, may constitute a formidable hazard for reproduction of the species. Tejning (70) has shown that domestic hens fed mercury-treated grains produce eggs which contain high levels of mercury, and that mercury accumulates within the tissues of the developing embryos during incubation, frequently producing embryonic mortality. Kuwahara (69) has administered subcutaneous injections of methyl mercuric chloride to adult hens, recovering up to 80-90% of the mercury in developing eggs. This apparent high level of accumulation of mercurial compounds and other metals (13) in avian eggs is of obvious importance in assessing the validity of pollution control standards. Egg accumulation of metallic toxicants, together with the high sensitivity of chick embryos to metallic poisoning (12, 13), emphasizes the need to ascertain egg incorporation rates for a number of trace metals and to determine limits on food contamination which are adequate to protect avian reproduction.

Bioassay applications. The chick embryo possesses a number of advantages as a bioassay organism with which to assess the toxic effects of actual or potential metallic pollutants important in the management of water resources. These advantages include 1) high sensitivity to trace levels of metallic and metal-containing contaminants, 2) wide availability and low cost of fertile eggs, and 3) assessibility and ease of handling for test purposes. The chick embryo may be used to investigate three principal categories of bioassay responses--including toxicant-induced lethality, teratogenesis, and certain observable behavioral or functional impairments (e.g., defective locomotion).

Essential equipment required for most chick embryo bioassays includes a forced-draft incubator (500 egg capacity minimum), egg candler, autoclave or other sterilizer, stereoscopic microscope, and general laboratory facilities for handling reagents (e.g., pH meter, analytical balance). In addition, a forced-draft sterile hood may be used to minimize contamination when toxicants are administered to test eggs. Should it prove desirable to extend test procedures to include histopathological analyses

of tissues and organs, a research grade, compound microscope and general facilities for histological technique also are necessary. As equipment required for test purposes is minimal, chick embryo bioassays may be performed in most laboratories and in many field stations. Accurate and reproducible bioassay results can be obtained, providing the following procedures are observed.

Incubation and care of eggs. Fertile chicken eggs with normal hatchability of 85% or more should be used. A minimum fertility level of at least 75% should be maintained, as we have found eggs of lower viability to produce variable results when used for bioassays. Best results are obtained when fresh eggs are used for test purposes. However, eggs may be stored up to 1 week at a reduced temperature of 8-10° C. Fertility generally will drop rapidly with longer storage (72, 73). The physiological zero for chick development is approximately 27° C (72). A regular source of high fertility eggs is essential to maintain an extensive, critical testing program. We have used White Leghorn, White Plymouth Rock and White Rock strains with equal success.

Twenty-four hours prior to treatment, eggs should be oriented with their axes in a horizontal plane. In most instances, the egg yolk will rotate so that the embryonic blastodisc will be positioned upper most. This positioning enables toxicants to be administered by yolk sac injection without damaging the blastodisc, from which the chick embryo develops. The blastodisc represents a very restricted superficial area of the egg cell, with the remainder comprised largely by yolk. Any incidental mechanical damage to the blastodisc may seriously alter test results. It should be noted that chick development involves a megalecithal egg, meroblastic (discoidal) cleavage, and gastrulation with primitive streak formation. Technicians who have not been trained in avian embryology should refer to standard references for further background information (71-76).

After prior orientation of the eggs, they may be treated with the selected toxicant or test material by the procedure described below and placed under incubation. Eggs should be maintained in a forced-draft incubator at a temperature of 38° C (100-101° F) and with a relative humidity of 60-65%, as previously described by Birge (77, 78) and others

(72,73). Temperatures exceeding 41° C and/or excessive variations in humidity will generally preclude development or greatly reduce hatchability (72).

Incubating eggs should be rotated frequently to prevent adhesions of extraembryonic membranes (72, 73). Egg development may be examined at 1-3 day intervals by candling, though eggs should not be allowed to cool appreciably during such observations. Chick embryos may be staged according to developmental time using the normal series by Hamburger and Hamilton (98). In the course of normal chick development, there are two periods at which mortality rates generally are highest. These occur on days 4 and 19. A third mortality period may occur around day 14, presumably induced by certain dietary deficiencies (72). We have observed that the periods of mortality which normally accompany chick development frequently represent the stages at which toxicant-induced lethality is greatest. Thus, these periods deserve careful scrutiny during bioassay tests. It also should be noted that for any given strain of domestic fowl, yolk volume, yolk composition and egg fertility may vary seasonally, as well as with the age of the laying stock. These are all factors which may influence the accuracy and dependability of chick embryo bioassays, and should be given careful consideration in formulating test procedures.

Administration of toxicant. Three methods have been examined by which metallic toxicants may be administered to avian embryos:

1) Injection of metals directly into the air space of the egg, using amounts calculated to dilute egg volume to specified test concentrations, after the method of Franke et al. (79).

2) Administration of metals directly onto the surface of unincubated blastoderms and primitive streak stages, in order to study responses during early development (up to 3 days).

3) Injection of test metals into the yolk sac (prior to incubation) in sufficient amounts to dilute the yolk to desired test concentrations.

Air and yolk sac injections were made directly with a hypodermic syringe, after cleaning the appropriate area of the shell surface with an alcohol swab. Immediately after each injection, the point of entry through the egg shell was sealed with paraffin. Direct application to the blastodisc was made through a small opening (1/4 inch diameter) in the egg shell, after which it was sealed with a glass window (78). The

embryos were inspected at regular intervals to determine their extent of development. Control eggs received identical treatment as given the experimentals, except the metallic toxicants were withheld (12).

During the course of this study (Phases I, II), each of the above procedures was used to administer metallic toxicants to a minimum of 1500 eggs, and the results may be summarized as follows. Air cell injection was found to be the least sensitive and most variable form of administration. Replicate treatments involving identical metal concentrations often yielded results varying by as much as 25-30%, measured as embryonic mortality. Also, concentrations of 1-5 ppb mercury and cadmium, which produced substantial lethality when administered by yolk sac injection, frequently yielded no toxic effects when applied by means of the air cell injection procedure. Accordingly, we have discounted air cell injections as a suitable method for bioassays.

The open window technique, allowing direct application of toxicants to the embryonic blastodisc, also gave variable results. Replicate measurements generally varied by 15-20%, though sensitivity approached that obtained with yolk sac injections. Other disadvantages of this procedure included greater opportunity for experimental error, and significantly reduced survival of control embryos. The latter were treated with avian Ringer's (73) instead of toxicant, and survival generally ranged from 55-75% for egg sources which normally averaged 85-90% hatchability when "control" treatment was omitted. In addition, the open window technique was found considerably more expensive and time consuming compared to the yolk sac injection procedure. Though the open window method of treatment is not recommended for routine bioassays, it may prove of limited value for screening various classes of compounds for lethal and/or teratogenic effects on early developmental stages (1-3 days). The method of window preparation used was essentially as described earlier (73, 77).

Of the three methods used to administer metallic toxicants to chick embryos, yolk sac injection was found to be the procedure of choice. Initially, each test metal was dissolved in a 0.3 ml aliquot of sterile avian saline (0.9% NaCl) and injected into the egg yolk center in an amount sufficient to dilute total yolk volume to the desired test concentration. Control eggs were treated with avian saline, withholding the

metallic toxicant. Two difficulties arose from the use of this procedure. First, the 0.3 ml aliquot of avian saline proved toxic to control animals, killing from 10-20% of treated populations. Secondly, this injection procedure failed to provide uniform distribution of toxicant throughout the yolks of treated eggs. The latter was confirmed by injecting $^{203}Hg$ and subsequently photographing the treated eggs after 5 days of exposure, using Anger scintillation photography. The results showed poor distribution of the mercury (Figures 1-4).

These two problems were obviated by administering the test metal in a 0.1 ml aliquot of sterile, distilled water, deposited in a "needle tract" extending across the diameter of the egg yolk. Similarly, control eggs were treated with 0.1 ml aliquots of distilled water, withholding only the metallic toxicant. With this procedure we were able to maintain control survival at 92-100% of that obtained with uninjected eggs, and much more uniform metal distribution was obtained with experimental eggs. This technique was used to collect the data reported in Table 1. Chick embryos proved much more sensitive to metallic toxicants administered by this method, compared to the traditional yolk injection procedure used by Ridgeway and Karnofsky (80).

Our recommended yolk injection technique may be summarized as follows:

1) The blunt end of the egg should be cleansed with an alcohol swab (area approximately 3/8 inch diameter).

2) A small hole (approximately 1/64 inch diameter) is drilled through the center of the blunt end of the egg, opening into the air cell. A hand drill, cut to 1/8 inch in length, may be fashioned from a dissecting needle.

3) A 1 ml hypodermic syringe, equipped with a 1 1/4 to 1 1/2 inch 27 gauge needle, is used for the injection. The needle is inserted into the air cell and through the diameter of the egg yolk, to within approximately 1/8 inch of the opposite wall of the vitelline membrane. The needle is then withdrawn slowly, injecting a 0.1 ml aliquot of test solution in a "needle tract" extending across the diameter of the egg yolk. The injection should be completed just prior to removing the needle from the

yolk, and the point of entry through the egg shell should be sealed with melted paraffin. Treated eggs are then ready for incubation.

During the injection care should be taken to avoid rupturing the vitelline membrane. A cork stop may be positioned at the base of the syringe needle to regulate penetration depth. The latter may be determined by measurements taken on sagittal sections of boiled eggs, or by observing the injection procedure through an open window cut through the egg shell. Initially, injections may be made using a dye solution, to permit visual observations on the extent and position of the needle tract. This method is reproducible with high accuracy, given reasonable care and experience.

As noted above the toxicant under study should be prepared in distilled water with the concentration adjusted so that a 0.1 ml aliquot will dilute total egg yolk volume to the desired test level. If necessary, 0.15-0.20 ml aliquots may be used. However, larger quantities will induce measurable lethality among control animals. It should be noted that the distilled water does not directly contact living embryonic tissue, thus there are no problems with osmotic balance. Also, compared to other carrier fluids, the 0.1 ml of distilled water produces the least change in the chemical composition of the egg yolk. Water used for test purposes should be distilled and/or deionized, with a conductivity approaching 0.1-0.2 micromho/centimeter, and it should be monitored routinely for possible contaminants. Yolk volume will vary with chicken strain, age of hens, and laying season (71, 76). As final test concentrations depend on yolk volume, mean values for the latter should be determined routinely at weekly or monthly intervals during testing periods.

A minimum sample size for bioassay determinations should be set at 100 eggs for both control and experimental populations, and each test should be performed using 2-4 replicates, as experience dictates. Control eggs should be treated in a manner identical to experimentals, except the toxicant is withheld from the 0.1 ml aliquot of distilled water.

It is also advisable to incubate equal samples of untreated eggs, along with injected control and experimental populations. This will provide a measurement of the optimum fertility level for the particular source of

test eggs, and will give a baseline with which the hatchability of control populations may be compared. For best results, untreated eggs should average at least 80% hatchability, and controls injected with water blanks should give a hatching frequency at least 90% of that obtained for untreated eggs. Greater disparity generally denotes faulty injection technique. Treatment (exposure) time should run the full course of chick development (20-21 days), and observations should be continued for 7-14 days posthatching.

Test results are best expressed as the frequency of embryonic survival (experimental populations/control samples), with survival defined as the ability to complete the hatching process. In addition, threshold and $TL_{50}$ values should be adequately documented at hatching for each toxicant. Where possible, each toxicant should be tested at sufficient concentrations to produce a complete survival curve (e.g., 0-100% survival). In studies of teratogenesis, the types of gross anomalies should be classified and the frequency of occurrence should be determined for each category. In addition, data for all types of serious anomalies should be combined, giving the percentage of surviving animals rendered defective by each concentration of toxicant studied (e.g., Table 1).

It is important to note that toxicants dispersed in egg yolk reach the embryo primarily via the yolk sac circulation. Development of the yolk sac, the primary organ of nutrition for avian embryos, continues throughout much of the incubation period, steadily increasing in complexity and efficiency until the 19th day (72). Though it becomes well vascularized by the third day of development, it covers only a fraction of the yolk surface. Not until 4-6 days of incubation does the vascularized yolk sac cover a substantial portion of the yolk mass (74). The embryonic assimilation of toxicants present in egg yolk obviously must parallel yolk sac development and the progressive utilization of the yolk mass. Much of the yolk is withdrawn into the gut just prior to hatching, to support the early chick until feeding habits are developed. Thus, the initial injected dose of toxicant is never fully delivered to the embryo. Early developmental stages (1-4 days) likely receive quite reduced exposure, and the maximum assimilated dosage presumably is delivered to later embryonic stages and

early posthatched chicks. Thus, toxicity experiments which are terminated prematurely may give inconclusive results. Though a single, initial injection of test metal is administered to the egg yolk just prior to the start of incubation, exposure is progressive with development. Accordingly, it is essential to extend the test period through the hatching process (20-21 days). Furthermore, observations should be maintained for at least one week posthatching, commensurate with the final utilization of the last remaining egg yolk constituents. In the event that postembryonic lethality or behavioral impairments result, the frequency of such occurrences may be expressed separately for the posthatching period observed (e.g., 7 days), supplemental to embryonic survival data.

## CHAPTER 5

## USE OF FISH AND AMPHIBIAN EMBRYOS AS BIOASSAY AND BIOINDICATOR ORGANISMS

Assessment of the magnitude of direct exposure of aquatic embryos to waterborne trace metals is dependent upon a number of factors, among which are 1) release rates of metallic pollutants into natural waters, 2) various physical characteristics such as oxygen level, pH, water hardness, and flow rate (9, 81-87), 3) the capacity of eggs and embryonic tissues to bind or accumulate available metals, in competition with organic detritus or other metal acceptors (41, 88), and 4) the extent to which metals are remobilized from bottom sediments to reach the biomass of aquatic ecosystems. The latter may be particularly important since such metals as arsenic, cadmium, lead, mercury and zinc are known to accumulate in bottom sediments at concentrations of 10-100 ppm or more (89-94). Bothner and Carpenter (94) recently have reported a half-life of 1.3 years for mercury when present at 2-10 ppm in a generally oxidizing sediment, and Jernelov (95) has shown that fish concentrate methyl mercury to levels of 3 ppm within 15 days, when exposed to organically-rich sediment spiked

to 100 ppm with inorganic mercury. A concentration of 3 ppm mercury is 3,000 and 600 times the levels which produce measurable lethality in trout and channel catfish embryos, respectively (13). The desorption of metals from bottom sediment (94-96) could result in substantial exposure for fish and amphibian eggs, particularly those spawned directly on bottom substrates.

Selection of test species. Considering the great sensitivity of embryonic and early posthatched stages of certain fish and amphibian species to metallic trace contaminants (12, 13), and the ease with which such developmental stages may be cultured in vitro, they constitute excellent test organisms for use in either bioassay or bioindicator systems. In developing the techniques described below, we made use of embryonic and posthatched stages of five species of aquatic vertebrates, including the rainbow trout (Salmo gairdneri), channel catfish (Ictalurus punctatus), goldfish (Carassius auratus), largemouth bass (Micropterus salmoides), and the leopard frog (Rana pipiens). Under Phase I of this investigation, it was established that trout and catfish embryos generally are more sensitive to metallic pollutants than developmental stages of the goldfish (12, 13) or bass, and that sensitivity for certain toxicants (e.g., mercury) correlates with the length of developmental (hatching) time.

All of the above species have proven useful for bioassay purposes, and it is recommended that the evaluation of actual or potential waterborne toxicants be based on the use of at least 2 to 3 test species. Selecting test species which differ significantly in sensitivity (hatching time) will provide a better indication of the range of toxic effects to be expected in natural ecosystems. The bioassay procedure given below is suitable for culturing most freshwater fish embryos. Thus, in considering pollution problems which are regional in nature, test species generally may be chosen which are endemic to the area of interest.

Preparation of culture water. Culture medium for aquatic embryos should be prepared from distilled, charcoal-filtered, deionized water (conductivity of 0.1-0.2 micromho) which is routinely monitored for background contamination. Calcium, magnesium, potassium and sodium salts

are added to give either modified Holtfreter's (73) solution (sodium bicarbonate reduced by 50%) or to approximate Mount's formula for spring water (49). As needed for particular test purposes, these solutions may be adjusted to provide various water hardness levels (50, 100 and 200 ppm, measured as calcium carbonate), and the final concentration of sodium bicarbonate may be adjusted to aid in stabilizing pH within selected ranges.

Holtfreter's solution has been used extensively and found to possess a number of advantages for embryonic culture. As compared to a number of other water media we have tested, the use of Holtfreter's provides 1) maximum survival of control animals, presumably minimizing culture stress and mechanical trauma introduced with the handling of embryos, 2) significantly lower incidence of fungal and bacterial contamination of cultures, and 3) good retention properties for low concentrations of metallic toxicants.

We have varied the NaCl content of Holtfreter's solution over wide ranges and have observed no major effects on mercurial toxicity to embryos. Also, where comparisons have been made, the toxicity of metallic pollutants tested in Holtfreter's solution closely approximates that found in natural, hard waters (180-240 ppm). In the event the high salt content is not desired, the concentration of NaCl may be reduced to 0.1 gm/l. This provides a very simple, definable culture medium which gives both optimum survival for control populations of embryos and few constituents which are likely to affect the degree of toxicity of test agents. The principal advantage of Mount's formula is that it more closely approaches the mineral content of natural waters, and may permit more reassuring extrapolations to natural conditions. Natural water is not recommended for bioassay tests due to substantial geographical and seasonal variations in mineral content, hardness and background contamination.

Administration of toxicants. Agents to be examined for toxic effects should be added to the culture water used for the experimental test eggs, and continuous exposure should be maintained throughout the full hatching period of the test species. Control eggs should be maintained simultaneously

using the same prepared culture medium, except the toxic agent is omitted. Initially, the toxicant should be administered at high concentrations, in an attempt to produce complete lethality in test populations of embryos. Once the lethal dosage has been established, successively lower concentrations should be used until a "safe" level is determined for the most sensitive embryonic stage. It is important to initiate treatment at or immediately following fertilization of test eggs. During and immediately following the fertilization process, egg permeability is altered and there is substantial water imbibition. This may represent the stage when certain water soluble contaminants are most prone to enter fish and amphibian eggs. Elimination of this period from bioassay tests may lead to spurious test results. Also, it is important to maintain exposure continuously through development and for 4-7 days posthatching. Certain water contaminants (e.g., zinc, copper) may prove more toxic to early posthatched stages (e.g., trout alevins, catfish fry), while other agents, such as mercury, are more deleterious to early embryonic stages (12). As the developmental process involves rapid morphological and physiological changes, continuous treatment from fertilization through early posthatching is the only sure means by which to adequately quantify the sensitivity of developmental stages to environmental contaminants.

Bioassay culture techniques. The culture system developed to maintain fish and amphibian embryos under bioassay treatment is described in Figure 5. Fertilized eggs are cultured in vitro, using Pyrex chambers with a minimum capacity of 300-500 ml. Deep Petri dishes are adaptable for this purpose, each accommodating 150 or 300 eggs for trout or goldfish, respectively. The culture dishes should be provided with an outlet positioned approximately 5 mm from the bottom. The outlet of the culture dish is coupled to both culture water and waste water reservoirs, using a three-way selector valve (Figure 5). The selector valve is used alternately to drain used culture water into the waste reservoir and to refill the egg culture chamber from the culture water reservoir. The latter may serve six or more egg chambers, allowing simultaneous testing for embryos of several different species.

Continuous oxygenation should be provided to each egg chamber, using

filtered air. In this "maintained" culture procedure, water is changed at intervals (usually 6-12 hr) sufficient to meet the half-life requirements of the toxicant used, and to prevent deterioration of the culture medium. Metals administered by this system may provide somewhat lower toxic responses than those obtained in a flow system. However, this method of maintaining cultures has been used extensively for tissues and embryos, and affords certain advantages. Particularly, this system is simpler and less expensive to maintain, making it available for bioassay purposes to laboratories which are not equipped with elaborate facilities to maintain and monitor flow systems. It provides a simple, rapid and reliable means of screening many elements and compounds for embryopathic properties, providing cultures are adequately maintained.

Culture water should be monitored at regular intervals for temperature, oxygen level, ammonia content, water hardness, and pH. For these purposes, we have used a YSI tele-thermometer with thermocouple, YSI oxygen meter (model 51A), Orion ammonia and water hardness electrodes, and a Corning digital pH meter (model 110, with expanded millivolt scale). Temperature should be regulated within the optimal range for each given test species (e.g., 54-56° F for trout). Fluctuations should be kept minimal, as the rate of embryonic development in fish and amphibians varies directly with temperature. Oxygen level should be held at saturation and the content of ammonia should be kept under 1 ppm. Higher concentrations of $NH_3$ generally indicate overcrowding in cultures, or excessive time between changes of culture water. For routine bioassays, the pH of culture water should be adjusted initially to 7.5-8.0 and should not drift below 7.0 within the time interval allowed between the changes of culture medium. The level of water hardness may be selected to represent the particular bioassay application. However, as toxicities of certain contaminants (e.g., zinc, cadmium) vary greatly with hardness, it is desirable to duplicate bioassay tests at two or more ranges (e.g., 50-100 and 200-250 ppm, measured as calcium carbonate). In addition to monitoring for culture conditions, periodic measurements should be made to verify the test concentrations of the administered toxicants. Eggs and embryos should

be examined daily to gauge extent and frequency of development, and to remove dead specimens.

Particular attention should be given to trout embryos, especially during the "green stage". For best results they should be maintained in a constant temperature environmental room at 55° F, with a pH of 7.5-8.0 (7), and embryos should be protected from harmful exposure to artificial light. In the event of fungus or soft egg disease, periodic treatments may be given with formaldehyde (1/1000 for 10 minutes) or acriflavine (1/2000 for 20 minutes).

Expression of data. Control eggs and embryos should be cultured simultaneously with experimentals and under identical conditions, except for omission of test agents. A minimum sample size of 300 eggs (3-5 replicates) should be observed for determinations on both experimental and control survival rates. In bioassays using fish and amphibian embryos, responses normally are grouped under three principal categories: lethal, teratogenic or normal. Certain behavioral impairments also may be categorized, providing adequate screening procedures are used. Lethality and teratogenesis are best expressed as frequencies within experimental populations/frequencies in control samples. The resulting frequency of each type of response should be expressed for each embryonic species and for each toxicant evaluated. Concentration-mortality or survival curves may be plotted for continuous exposure from fertilization through hatching, and the median tolerance limit (TLm) should be calculated for 1) the entire developmental period, and 2) the first 4-7 days posthatching (e.g., 96 hr TLm).

Embryos as bioindicators. Fish and amphibian embryos also may be used as bioindicators with which to monitor the quality of selected water resources. For this purpose, natural water samples are used in the egg chambers for culturing test embryos. Survival is compared against that of control embryos reared in "uncontaminated" natural water or in prepared culture medium. With this exception, cultures are maintained as described above. Sensitive early embryonic stages are particularly suitable as bioindicators, since trace levels of metallic pollutants may produce detectable effects after 1-3 days of exposure (12).

The culture procedure given above has been used to evaluate the toxic effects of cadmium, mercury and several other metals on fish and

amphibian embryos (Phase I; ref. 12), in which more sensitive species were affected by concentrations ranging down to 1-10 ppb. Early embryonic stages were most sensitive to mercury. Trout embryos treated at 5 ppb, the $TL_{50}$ concentration for both inorganic and methyl mercury, suffered highest lethality during the first 3 days of the 24-day exposure period. In experiments with the leopard frog, 10 ppb of inorganic mercury killed 100% of the cleavage and blastula stages, while 80-93% of gastrula, neurula and tail bud stages survived this concentration.

To further examine the differential sensitivity of early and late developmental stages, embryos of the leopard frog (Rana pipiens) have been exposed to cadmium (Table 3). Initiating treatment during the cleavage stage, complete lethality was observed following 6 days of exposure at 2.5 ppm cadmium. At 0.5 and 0.1 ppm cadmium, 30% and 53% of treated cleavage stages survived 15 days, compared to 68% survival in the control population. Therefore, the $TL_{50}$ value for cleavage stages is in the range of 0.1-0.5 ppm. When treatment was delayed until neurula and tail bud stages, 37% and 50% of treated embryos survived 0.5 ppm cadmium for 15 days. At 0.1 ppm, 70% of neurula stages survived, compared to 83% for control animals. As originally found for mercury (12), frog cleavage stages are more susceptible to cadmium than are older embryos, particularly tail bud stages. Consequently, it is most important to include the early developmental stages when fish and amphibian embryos are used as bioassay test organisms.

## EXPLANATION OF FIGURES

Figure 1. Photographs taken of a White Plymouth Rock chicken egg, using an Anger scintillation camera. It shows the localization of $^{203}Hg$ five days after a 1 ppm dose (16.2 mCi/mg; New England Nuclear) was injected into the center of the egg yolk. Three images of the same egg were taken at different exposure levels. The images, in order of increasing exposure levels appear at upper left, lower left and right center. The latter is labeled, showing the position of the embryonic blastodisc (E) and air cell (AC). The blunt end of the egg (air cell) is oriented to the right. The principal yolk mass is situated just under the embryonic disc. The three dot-like structures which appear around it are "labeled" reference markers. At the lowest exposure level (upper left), only the very center of the yolk is discernible. Approximately two-thirds of the yolk mass is evident at the intermediate exposure level (lower left) and the entire yolk appears labeled at the optimum exposure (right center). In the latter, however, considerable fall-off is evident at the periphery of the yolk, adjacent to the margin of the yolk sac wall. This sequence of exposures clearly indicates that the yolk center contains the highest concentration of the injected $^{203}Hg$, with considerably less appearing in outer layers of the yolk mass. This pattern of uneven distribution obviously would result in decreased assimilation of mercury by developing embryos, and increased exposure to posthatched stages which utilize the final yolk reserve. All photographs in Figures 1-4 were taken at 1/2X, and all eggs were treated by identical procedures.

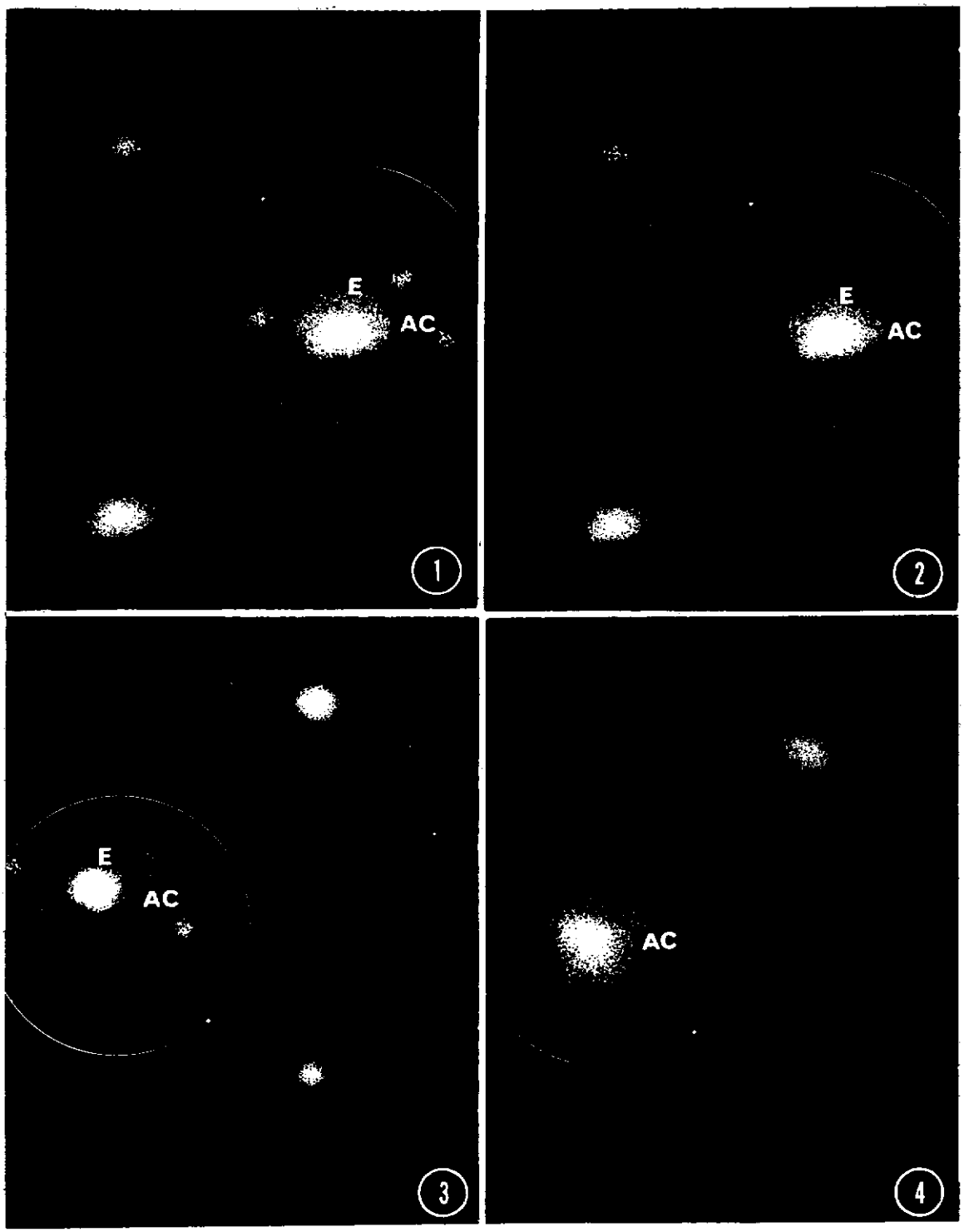
Figure 2. Sequence of scintillation photographs similar to that given in Figure 1. The $^{203}Hg$ distribution in this egg correlates closely with the pattern described above.

Figure 3. Scintillation photographs of an injected egg showing little or no $^{203}Hg$ in the peripheral yolk layers. The labeled mercury remains largely in the central one-half of the yolk mass, where

it was originally injected five days prior to taking the photograph. This egg was treated with the same dose of $^{203}Hg$ as those shown in the above figures. Exposure levels for the three images were increased progressively, as shown at lower right, upper right and left center. Comparisons of Figure 1 with other figures indicate that injections deposited in the central yolk mass may produce variable patterns of mercury distribution.

Figure 4. Sequence of scintillation photographs similar to those shown in preceding figures, except taken with the air cell (AC) oriented upper most.

Figure 5. Culture system for bioassay determinations on developmental stages of fish and amphibians. Prepared culture water containing the toxicant to be tested is stored in the reservoir shown at upper right. Drawing from the culture water reservoir, the culture chamber (500 ml capacity) is emptied and refilled at regular intervals, using the three-way selector valve. Used culture water is exhausted into the waste reservoir. Arrows indicate the direction of flow. Culture water should be oxygenated by supplying filtered air directly to the culture chamber. Culture water in the egg chamber should be changed at 8 to 12 hr intervals to prevent deterioration of culture conditions. Sets of 6 or more culture chambers may be served by each culture water reservoir, allowing simultaneous testing of several bioassay species.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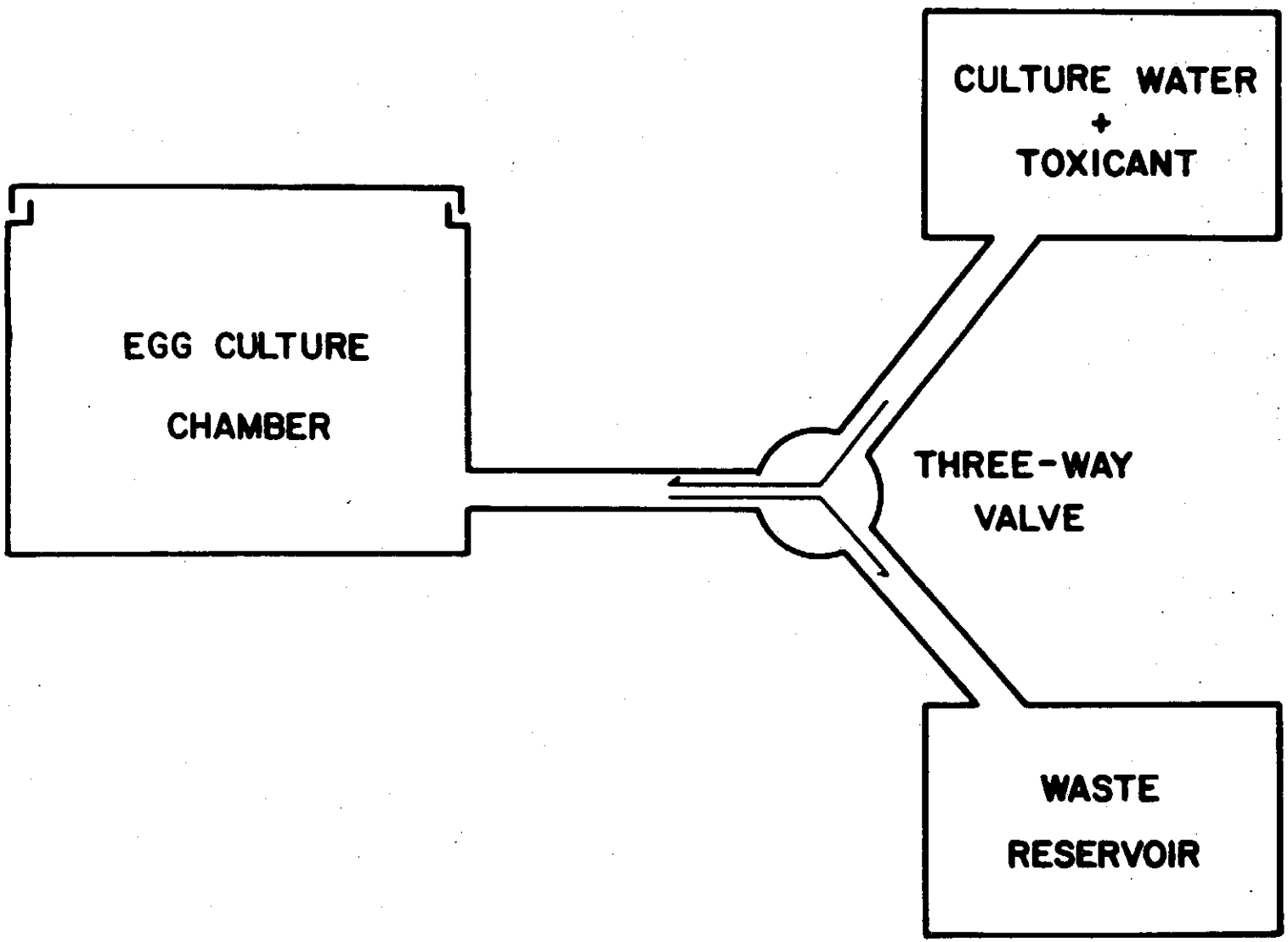


FIGURE 5

CULTURE SYSTEM FOR BIOASSAY DETERMINATIONS ON DEVELOPMENTAL STAGES OF FISH AND AMPHIBIANS

TABLE 1

TOXICITY OF METALS TO CHICK EMBRYOS

| CONCENTRATION[1] (ppm) | PERCENT SURVIVAL[2] | | | | | | |
|---|---|---|---|---|---|---|---|
| | $Se^{+6}$ | $As^{+++}$ | $Cd^{++}$ | $Pb^{++}$ | $CH_3Hg^{+}$ | $Hg^{++}$ | $Zn^{++}$ |
| 0.001 | 64 | 65 | 66 | 74 | 78 | 78 | 83 |
| 0.010 | 43 | 54( 2) | 55 | 73( 1) | 71 | 73 | 75 |
| 0.050 | 38( 7) | 47( 6) | 48 | 74( 7) | 65( 4) | 64 | 69 |
| 0.100 | 30( 9) | 43(17) | 41( 6) | 63(10) | 54( 7) | 61( 2) | 64( 2) |
| 0.500 | 24(11) | 18(29) | 25(15) | 57(16) | 45(11) | 56( 3) | 58( 9) |
| 1.000 | 0 | 0 | 8(33) | 52(14) | 26(15) | 51( 8) | 49( 8) |
| 5.000 | 0 | 0 | 0 | 23(24) | 23(22) | 37(12) | 35(17) |
| 10.000 | 0 | 0 | 0 | 0 | 10(25) | 8(33) | 9(29) |
| 50.000 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

1 Metals were administered by yolk sac injection in amounts calculated to dilute egg yolk to specified concentrations.

2 Each percentage represents frequency of survival for 200 experimental embryos/200 controls. Percentages of surviving embryos possessing gross teratologies are given parenthetically.

TABLE 2

AVENUES OF EMBRYONIC EXPOSURE TO METALS FOR THREE PRINCIPAL PATTERNS OF VERTEBRATE REPRODUCTION

1. PLACENTAL MAMMALS:
   UPTAKE BY MATERNAL ORGANISM AND PLACENTAL TRANSMISSION

2. TERRESTRIAL EGG LAYERS (e.g., BIRDS, REPTILES):
   A. UPTAKE BY MATERNAL ORGANISM AND INCORPORATION OF TRACE METALS INTO EGGS DURING OOGENESIS (SECONDARY EXPOSURE)
   B. DIRECT EXPOSURE TO ATMOSPHERIC CONTAMINANTS WITH EXCHANGE ACROSS EGG MEMBRANES

3. AQUATIC EGG LAYERS (e.g., FISH, AMPHIBIANS):
   A. DIRECT CONTACT WITH AQUATIC CONTAMINANTS (PRIMARY EXPOSURE)
   B. UPTAKE BY MATERNAL ORGANISM WITH INCORPORATION INTO DEVELOPING EGG, GIVING SECONDARY EXPOSURE

TABLE 3

PERCENT SURVIVAL OF FROG EMBRYOS (RANA PIPIENS) WITH CADMIUM TREATMENT INITIATED AT CLEAVAGE (CL), NEURULA (N) AND TAIL BUD (TB) STAGES [1,2]

| DAYS | 10 ppm | 7.5 ppm | 5 ppm | 2.5 ppm | 1 ppm | 0.5 ppm | | | 0.1 ppm | | 0 ppm | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CL | CL | CL | CL | CL | CL | N | TB | CL | N | CL | N | TB |
| 1 | 7 | 11 | 67 | 73 | 96 | 77 | 53 | 90 | 93 | 74 | 97 | 85 | 100 |
| 2 | 0 | 0 | 51 | 48 | 90 | 70 | 53 | 90 | 85 | 74 | 94 | 85 | 100 |
| 3 | | | 36 | 36 | 86 | 50 | 51 | 90 | 78 | 74 | 85 | 85 | 100 |
| 4 | | | 11 | 8 | 82 | 33 | 51 | 90 | 68 | 74 | 84 | 85 | 100 |
| 5 | | | 2 | 3 | 82 | 30 | 51 | 90 | 63 | 72 | 74 | 85 | 100 |
| 6 | | | 2 | 0 | 82 | 30 | 51 | 90 | 63 | 70 | 70 | 85 | 100 |
| 7 | | | 0 | | 80 | 30 | 51 | 90 | 63 | 70 | 70 | 85 | 100 |
| 8 | | | | | 80 | 30 | 51 | 85 | 63 | 70 | 69 | 83 | 96 |
| 9 | | | | | 80 | 30 | 49 | 85 | 63 | 70 | 68 | 83 | 96 |
| 10 | | | | | 80 | 30 | 41 | 70 | 63 | 70 | 68 | 83 | 96 |
| 11 | | | | | 76 | 30 | 41 | 70 | 63 | 70 | 68 | 83 | 96 |
| 12 | | | | | 76 | 30 | 41 | 50 | 63 | 70 | 68 | 83 | 90 |
| 13 | | | | | 76 | 30 | 41 | 50 | 53 | 70 | 68 | 83 | 90 |
| 14 | | | | | 76 | 30 | 41 | 50 | 53 | 70 | 68 | 83 | 90 |
| 15 | | | | | 41 | 30 | 37 | 50 | 53 | 70 | 68 | 83 | 90 |

[1]Sample size was set at 100 embryos, and cultures were inspected daily

[2]Tests were conducted at room temperature (70-72° F).

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