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TOXICOLOGICAL ASSAYS WITH FISH

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Fish, among other aquatic vertebrates, occurred naturally in streams, lakes, and the oceans prior to the era of modern man. People have harvested this "taken for granted" resource for food and income for many years, and some populations of people are dependent upon fish for survival. The abundance of desirable fish fluctuates, however, according to such factors as food availability, changes in water quality and temperature due to pollution, the quantities of algae and other aquatic weeds supported by nutrifying runoff, siltation, and eutrophication or the accelerated aging of natural waters. People are beginning to realize the importance of fish in sport and commercial fisheries, and the present scientific community is emphasizing the control and manipulation of fish populations.

The Fish Control Laboratories at La Crosse, Wisconsin, and Warm Springs, Georgia, are assigned to discover and develop tools which are useful in the culture and management of fish. These tools may include general and selective toxicants, anesthetics, collecting aids, surfacing agents, attractants, repellents, chemosterilants, therapeutic agents, growth stimulators and any other method or device for enhancement of the fish industry. The route to success for any of the tools just mentioned begins in the research laboratory. The basic ingredients for this research are healthy fish, test water of known physical and chemical characteristics, proper-sized test containers, and educated people to observe and record the responses of the fish to various chemical stresses.

Why are toxicological assays with fish necessary? What is their significance in developing fish control chemicals? These control chemicals for fish or diseases of fish must be cleared and registered by regulatory agencies just as medical drugs and veterinary preparations must be cleared for human or animal use. In particular, fish that are destined for human consumption must not contain residues of drugs or contaminants which exceed the tolerances defined by regulatory agencies. The general procedures followed for drug clearance were presented in The Progressive Fish-Culturist in 1967 by Dr. Robert E. Lennon. He reviewed the laws set forth by the U.S. Department of Agriculture, the Public Health Service, and the Food and Drug Administration to insure that chemicals are safe and efficacious prior to their use by the public. Our basic responsibilities with chemical clearance at the Fish Control Laboratory include the toxicity, efficacy, and in certain cases the residues of candidate chemicals.

Toxicological assays are necessary in defining lethal concentrations of chemi cals against fish. These data are not only important for developing candidate fishery tools, but also for evaluating the effects of industrial pollutants or pesticides in our aquatic environment. In fact, fish are presently among the most important indicator organisms, and only resistant species can survive in many of our deteriorated aquatic habitats. Clean water is essential for healthy and productive fisheries as well as for human health and survival.

Fish bioassays at the Fish Control Laboratories are conducted according to standard procedures with some modifications being necessary for our facilities and specialized objectives. Our testing procedures are outlined in the Bureau of Sport Fisheries and Wildlife Circular

185 by Dr. Robert E. Lennon and Charles R. Walker. The test fish are obtained from private, state and federal fish hatcheries located primarily in the Midwest. The fish are fasted prior to and during the bioassay to reduce the metabolic wastes in the solution. The preliminary bioassays include the following representatives of warmwater and coldwater species: rainbow trout, goldfish, carp, white suckers, black bullheads, green sunfish, bluegills, and yellow perch. These eight species represent fishes which are extremely resistant, moderately resistant, and susceptible to physical and chemical stress. In the more definitive tests, a single chemical may be bioassayed against 50 or more species of fish and other aquatic organisms.

A minimum of ten fish are exposed to each chemical concentration in reconstituted water which is prepared by adding sodium bicarbonate, calcium sulfate, magnesium sulfate, and potassium chloride to deionized water to yield a consistent and known quality of water. The routine test water has pH, 7.2-7.6; total hardness, 40-48 p.p.m.; and total alkalinity, 30-35 p.p.m. Water quality is perhaps the single most important factor affecting the efficacy of chemicals against fish. In order to measure the effects of different water qualities on the activity of chemicals against fish, we artificially prepare several different solutions of reconstituted water. The quantities of reconstituting salts and corresponding water qualities are listed in table 1. The four classifications indicate a pH range of 6.4 to 8.4, a total hardness range of 10 to 320 p.p.m. and a total alkalinity range of 10 to 245 p.p.m.

Generally, chemicals are more toxic in acid waters, but some are enhanced in alkaline waters, and others are toxic in neutral waters only. In fact, our bioassay experience has shown that lethal concentrations of certain chemicals may be rendered nontoxic with pH changes of only one unit. We have found that efficacy is highly dependent on the pH of the test solutions and the pKa's of the test chemicals.

The normal range of pH tested is from 6 to 9, and buffers are used to attain and hold selected levels. Most natural waters in the United States are within this range. Waters that exceed pH 9 often cause rapid chemical degradation.

The literature presents a number of good buffer systems which maintain pH over a desired period of time, but chemical concentrations of the buffers themselves are sufficient to kill the more susceptible fish, and occasionally the resistant species as well. We have chosen weaker buffers and the quantities of each have been reduced to a minimum. The chemical buffers added to standard reconstituted water and corresponding pH values are listed in table 2. The bioassay solutions are checked daily for changes in pH and adjusted when necessary. The data from these tests assist us in selecting candidate chemicals by predicting the degradation or enhancement of chemicals in natural waters. Thus, the candidate compounds which offer little or no potential in the fisheries field are eliminated.

Water temperatures also affect the efficacy of many chemicals against fish. In general, chemicals are more toxic to fish at warmer temperatures; however,

TABLE 1. *Quantities of salts and characteristics of reconsituted waters.*

Water type	Salts added in mg. per l.				pH range	Total as p.p.m. $CaCO_3$	
	$NaHCO_3$	$CaSO_4$	$MgSO_4$	KCl		hardness	alkalinity
Very soft	12	7.5	7.5	0.5	6.4 - 6.8	10-13	10-13
Soft[1]	48	30.0	30.0	2.0	7.2 - 7.6	40-48	30-35
Hard	192	120.0	120.0	8.0	7.6 - 8.0	160-180	110-120
Very hard	384	240.0	240.0	16.0	8.0 - 8.4	280-320	225-245

[1] Routine bioassay water

TABLE 2. *Buffer chemicals for maintaining the pH*

pH	Ml. of solutions for 15 l. of water 1N NaOH	1M KH_2PO_4
6.0	1.3	80.0
7.0	10.0	30.0
8.0	18.8	20.0
8.5	12.0	11.5
9.0	11.4	10.0
9.5	14.0	10.0
10.0	15.5	10.0

the reverse has also been demonstrated. Test temperatures of 7, 12, 17, and 22° C. are maintained in water baths in our bioassay program.

The bioassays are observed and the mortalities of fish are recorded at least four times on the first day and daily thereafter for the duration of the 96-hour static test. The observations include general behavior of the fish; their swimming activity; pigment discoloration; coagulation of mucous; respiration rates; alimentary responses; and sensitivity to movement, sound and touch. The response data usually indicate whether a compound should be tested further and categorized as a candidate, such as a toxicant, an anesthetic, an irritant, a surfacing agent, etc.

Preliminary bioassays, in which the fish are exposed to three chemical concentrations, 0.1, 1.0 and 10 p.p.m., yield estimates of the toxic effects. Delineative tests, in which greater numbers of fish are exposed to additional concentrations, enable us to define tolerance limits in terms of lethal or effective concentrations. For example, a 96-hour LC50 of a toxic substance is that concentration which kills 50 percent of the fish in 96 hours. The EC50 of an anesthetic is that concentration which effectively anesthetizes 50 percent of the fish exposed to it.

Disease control chemicals for fish or other organisms are tested according to the rigid bioassay standards. In fact, the first step in developing a therapeutic agent is to determine concentrations that are lethal to the fish and also, the concentrations that are absolutely harmless to the fish. Fish diseases are usually treated somewhat differently than diseases of other animals although identical chemicals are sometimes used. The administration of drugs to fish is usually accomplished by incorporating the drug in the diet or by immersing the fish in a solution containing the appropriate amount of drug. Injections are used occasionally to combat diseases in fish, but this technique is usually impractical and very cumbersome. Can you imagine injecting several million fingerling lake trout to prevent or cure a bacterial or fungal infection?

Diseases in fish are recognized by experienced culturists through unusual behavior of the fish, a failure to feed, formation of lesions or sores, and unusual rates of mortality. The disease should be diagnosed by an experienced fish culturist or pathologist, and only recommended medications should be used. A few of the common fish diseases and possible treatments suggested by Dr. Fred P. Meyer, BSF&W Fish Farming Experiment Station, Stuttgart, Arkansas are listed in table 3.

TABLE 3. *Some fish diseases and treatments*

Disease	Possible control
External protozoans (i.e. Chilodonella, Trichodina, Trichophrya)	Potassium permanganate, copper sulfate, formalin
Ichthyopthirius multifilis ("Ich")	Formalin, malachite green, copper sulfate
Monogenetic flukes (i.e. Gyrodactylus)	Dylox, formalin, potassium permanganate
Parasitic copepods (i.e. Argulus, Lernia)	Dylox, benzene hexachloride
Intestinal worms (i.e. tape worms, flukes)	di-N-butyl tin oxide in diet
Bacterial infections	Terramycin, nitrofurazone, chloramphenicol

Wayne A. Willford, a Chemist, at the Fish Control Laboratory, La Crosse, Wisconsin reported the toxicity of 22 therapeutic compounds to fish. His toxicological assays differentiated the toxic and nontoxic chemicals in 48-hour static tests. These data indicate, for example, that LC50's of malachite green range from 0.1 to 0.4 p.p.m. Formalin, nickel sulfate, and quinacrine hydrochloride, on the other hand, are relatively nontoxic and LC50's are usually above 100 p.p.m. Saturated solutions of sulfa drugs did not kill any fish within 48 hours.

Fish are susceptible to nearly every type of disease which afflicts higher animals. They are vulnerable to viruses, rickettsia and bacteria. Tuberculosis has been diagnosed in fish, and tumorous growths are common. It is extremely important, however, to propose only treatments in food fish which are recognized and approved by regulatory authorities.

As previously mentioned, the toxicity of chemicals to fish is influenced by the dissolved ingredients in the water. Copper sulfate, for instance, is harmless to fish in hard water at 1 or 2 p.p.m., but in soft water one hundredth this amount or .02 p.p.m. may kill the fish. Zinc contamination in malachite green has been known to kill fish at supposedly sublethal concentrations.

A simple procedure to prevent fish kills in the case of disease treatment, or to achieve a complete kill in the case of a fish toxicant, is to bioassay the control chemical on-site with the particular water and fish in the management area. A toxicological assay in such situations is good assurance for efficacy at the lowest possible cost.

I have discussed toxicological assays as they are performed at our Fish Control Laboratory. The static assays are very effective in evaluating candidate fish control compounds and establishing toxic concentrations of a wide variety of chemicals to fish under laboratory conditions. These short-term tests measure the effects of temperature, pH and water hardness on the chemical. These parameters usually influence the biological activity of chemicals to fish.

My presentation has included the mechanics of short-term, static, toxicological assays. Unfortunately we are not equipped to do continuous-flow assays which are becoming popular as the result of unique pollution problems. Long-term tests are desirable to define chronic or no-effect concentrations. The flowing assays offer certain advantages over the static ones, but do not replace them.