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TOXICITY TESTS FOR FRESHWATER ORGANISMS

Edited by
E. Scherer

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Edited by

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PREFACE

In Canada as elsewhere, hardly a decade has passed since ecologists saw their young, if not obscure science suddenly pushed to center stage. Pollution was the cue, DDT and mercury emerged as villains, dramatically shaping and changing public consciousness. Identifying and describing the environmental actions of some of the 50,000 or so most common man-made substances (an estimated 1,000 or more being added every year) has been an ongoing effort ever since.

Of course, the ultimate goal is not description and documentation of existing problems, but prevention or reduction of future environmental deterioration. To this end, new legal tools were devised in most industrialized countries. In Canada, the Environmental Contaminants Act of 1976 is the general framework designed to restrict, and prevent from entering the environment, chemical substances of a kind and quantity that "cause biological change". Since 1977, amendments to Canada's Fisheries Act have been aimed specifically at protecting aquatic habitats.

Enforcement of these well-intentioned laws, however, requires the capacity to evaluate and predict the kinds and extent of harmful changes a chemical might cause upon entering air, soil or water at various concentrations and under various biological, chemical and physical background conditions. Unfortunately, this capacity is still very limited.

Already the early alerting experiences with chlorinated hydrocarbons and mercury had taught the lesson that conventional acute-

lethal "bioassays" could not be relied upon to foresee and avert environmental danger. Sudden, easily recognizable kill-offs did not constitute the challenge, but inconspicuous, slow, gradual derangements of functions at all levels of biological organization, from biochemistry to behaviour of a species, and from bacterium to vertebrate predator. In addition to basic information on physical and chemical properties of existing and new compounds (e.g. solubility, adsorption/desorption, persistency), a new, much more comprehensive kind of toxicity testing was needed - and still is.

In 1971, Dr. R.D. Hamilton of the Freshwater Institute chaired a federal task force that investigated the state of toxicity testing vis à vis the new environmental laws being planned at that time. Their conclusion, valid then and now: enforcement would be limited due to lack of sound testing procedures.

While no single country has surged significantly ahead in eco-toxicological methodology, some international organizations such as the International Organization for Standardization (ISO), the Food and Agriculture Organization of the United Nations (FAO), and the Organization for Economic Co-operation and Development (OECD), have begun to work jointly with member nations towards incorporating individual tests into standardized, tiered (step-sequence) testing procedures ("protocols"). There are strong political and economical reasons for standardized tests, and for cost-saving tiered testing. The problem is - and may well remain so for a number of years - to put the right kind and number of tests into the right kind of tiered system to predict hazard at an acceptable level of certainty, at least for a given class

of chemicals in a given environmental "compartment".

In 1972, the Freshwater Institute, housing a large group of aquatic biologists with a wide range of expertise, added toxicity test development to its research activities. This publication summarizes what has been achieved so far.

Considering the state of the art, we chose not to present our 20 tests in some form of tiered system. Rather, they are described as discrete elements, to be used independently or in any desired combination. The sections called "Examples of Application" and "Discussion" with literature references will give the reader an indication of the test's state of development; "Costs and Staff Requirements" contain estimates for specifically needed items, in 1979 Canadian dollars, with basic laboratory supplies and equipment including toxicant delivery systems usually assumed to be available.

As the reference sections will show, some of the tests described are based on previously published material, reviewed and rewritten to fit a user-oriented common format. Others are proposed for the first time. All should be considered guidelines or proposals rather than cut-and-dry standard recipes. The authors are only too aware of the gap between test development at the research stage and the testing of tests in routine use by regulatory agencies. In fact, trying to bridge this gap has been a major motive for this publication. Accordingly, readers and potential users are invited to contact the editor and individual authors for changes and improvements, expanding on procedural details, etc. This publication can only be a start. Any future editions would benefit, I believe, not only from critical outside

comments, but also from external authorship under an enlarged editorial panel, thus pooling and focusing the somewhat scattered Canadian testing expertise.

Tests dealing with invertebrates frequently refer to a "Manual for the Culture of Selected Freshwater Invertebrates" edited by S.G. Lawrence, planned to be published shortly in the same "Special Publication" series as this volume. Further, while compiling and editing material for these toxicity tests, a comprehensive bibliography on testing methods was assembled; this will also be available (probably in this series) within the next few months.

E. Scherer
Winnipeg, November 1979

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First and foremost, this publication was only possible through the non-abating cooperation of 24 authors, going diligently through the various stages, from collating and reviewing material over adopting a common format to several revisions and final drafts. Drs. R.D. Hamilton and J.F. Klaverkamp, former heads of the F.W.I. Toxicology Section, provided much encouragement and helpful discussions, from preceding research to write-up. Miss M.P. McLean, later joined by Mr. R.E. McNicol, assisted most commendably in many editorial aspects including proofreading and reference and symbol standardization, with technical advice by Mrs. M. Layton and Mr. K.E. Marshall. Reviews by Drs. G.B. Ayles, W.G. Franzin, J.A. Mathias and Mr. D.G. Wright led to many substantial improvements. Drawings, except those previously published, were done by Mr. and Mrs. L.D. Taite. Certainly not least: Mrs. G.M. Decterow transcribed some of the early, at times not very legible, handwritten outlines, notes and comments; Miss M.D. Ziprick typed in a most reliable and efficient manner all the seemingly never-ending drafts without, miraculously, ever losing her patience or friendliness.

ABSTRACT

Scherer, E. (ed.) 1979. Toxicity tests for freshwater organisms. Can. Spec. Publ. Fish. Aquat. Sci. 44: 194 p.

Twenty tests are proposed for determining toxic effects of environmental chemicals. Test species are representatives of seven major groups of freshwater organisms: algae, higher plants, protozoans, crustaceans, insects, snails, fish. Most of the tests described can be adapted for alternate species or other groups of aquatic organisms. The emphasis is on sublethal and chronic effects, using biochemical, histological, physiological (including growth and reproduction) and behavioural criteria.

Key words: algae; phytotoxicity; freshwater invertebrates; fish; pollutants; environmental toxicants; hazard assessment.

RESUME

Scherer, E. (ed.) 1979. Toxicity tests for freshwater organisms. Can. Spec. Publ. Fish. Aquat. Sci. 44: 194 p.

On propose vingt essais pour la détermination des effets toxiques des produits chimiques dans l'environnement. Les espèces pour ces essais sont représentatives des sept groupes principaux d'organismes d'eau douce: algues, plantes d'ordre supérieur, protozoaires, crustacés, insectes, gastéropodes et poissons. La plupart des essais décrits peuvent être adaptés pour d'autres espèces ou pour d'autres groupes d'organismes aquatiques. L'emphase est placée sur les effets sublétaux et chroniques, utilisant des critères biochimiques, histologiques, physiologiques (y inclus croissance et reproduction) et éthologiques.

Mots-clés: algues; phytotoxicité; invertébres d'eau douce; poissons; polluants; toxiques de'l'environnement; évaluation d'hazard.

REPRODUCTION AND GROWTH IMPAIRMENT TESTS WITH THE AMPHIPOD *Hyalella azteca*

by B.G.E. de March

ABSTRACT

A technique for detecting small changes in growth and reproduction parameters of a multigeneration population of *H. azteca* is proposed. Measurable response parameters in the parent generation are: tendency to commence or terminate the reproductive resting stage, size and numbers of eggs produced, frequency of pairing, and hatching success. Measurable features in the young produced are: survival, maturation time, and size at maturity. The technique is designed so that different morphs and life stages of the animals are tested during a lab-simulated "year".

RATIONALE

Hyalella azteca (Saussure) is a freshwater amphipod common in temperate North America. Its manageable size and ease of identification and culture have made it a popular organism for scientific studies for more than a century. (For a complete literature review, see de March, in prep.). Many aspects of *H. azteca*'s growth and reproduction in relation to environmental influences have been described (Table 1), providing a good basis for testing the effects of toxicants.

METHOD

(i) Holding and Culturing

Culture conditions at which animals continue to reproduce are: daylength of 16 h or more, high light intensity (≏6000 lx measured under fluorescent light in my lab), oxygen levels near saturation, and a water replacement rate of 25%/h (less may do). I use aquaria 40 x 20 x 15 cm, water depth about 10 cm, supplied with a layer of fine sand and a fish spawning mat for cover. The animals are fed ad libitum three times a week. Tetra-Min B®, a 40% protein fish food, is rapidly and cleanly assimilated (plant material requries some decomposing before it can be ingested, causing dirty tanks; cf. Hargrave 1970).

Reproduction is slow at temperatures under 18°C, and relatively rapid at temperatures between 18-28°C. All activity, including reproduction, normally ceases at a temperature slightly colder than 10°C. At 10°C reproduction can be induced with longer daylengths; in this case, induction may take 2 months, copulation 3 weeks, and embryonic development more than 3 months while at 26°C these processes take 3 d, 1 d, and 6 d, respectively (de March 1977, 1978).

Normal reproductive resting conditions in H. azteca are: daylengths of 12 h or less, light intensity less than 2000 lx and temperatures less than 20°C. Food may still be required.

Under natural conditions, seasonal changes in adult animal sizes and their reproductive condition are caused by the timing of seasonal photoperiod and temperature changes. Actively reproducing animals usually occur in water ≥ 20°C, and are small, short lived, and not very

hardy. Overwintering specimens are large, in a reproductive resting stage, and hardier. This natural seasonality is incorporated into the proposed test procedure, so that all morphs and life stages of animals are tested.

(ii) Testing

The proposed test starts with animals in normal overwintering conditions (condition 1, Table 2). Animals should be acclimated to the test water chemistry, pH, light regime, and food type in test tanks for at least one month.

Animals used are of uniform age and size if they are collected from the field in winter. If this is not possible, then animals at the desired stage can be produced for the experiment by simulating a normal "fall" (conditions 8 and on, Table 2). The use of mixed size and age classes is not recommended since random differences among the cultures could cause growth and reproductive differences greater than those caused by the toxicant.

Animals must be transferred between tanks at various stages of the experiment. Either 4 tanks or a tank with 4 compartments is required at each test and control concentration. These chambers should be set up so that photoperiod and temperature changes can be manipulated. Photoperiods, temperatures, and particularly light intensities should be as similar as possible between tanks.

The test may be done in a flow-through system or in static tanks with replacement of at least 10% of the water each week.

It may be necessary to thin cultures at various stages of the test.

TABLE 1. Hyalella azteca - Measurable life cycle responses to environmental factors.

Response	Relation to Environment	Reference
a) Resting stage termination	1) is rapid (<10 d at 20°C, 20-30 d at 16°C) when proper long daylengths imposed.	de March 1977
	2) 100% termination in a population occurs within 10 d at 20°C, and over a longer period of time at lower temperatures.	de March 1977
b) Number of eggs produced	is directly proportional to the size of the female. Between 4 and 30 eggs have been reported in females of head length between 0.34 and 0.80 mm.	Strong 1972 Cooper 1965
c) Egg size	is probably related to developmental temperatures.	Steele and Steele 1975a, 1975b de March 1978
d) Gestation time	is inversely related to temperature: Time (in days) = 1/(0.00864°C - 0.0759).	de March 1978 Bovée 1950
e) Mating frequency	is same as gestation time unless overwintering conditions are introduced.	
f) Maturation time	is inversely related to temperature: Time (in days) = 1/(0.00188°C - 0.0130).	de March 1978 Bovée 1950
g) Size at maturity	is inversely related to embryonic development temperatures. Head lengths of adults vary from 0.34 mm to 1.00 mm.	de March 1978
h) Induction of resting stage	1) occurs when daylengths reduced to less than 12 h light.	de March 1977
	2) may take several weeks, especially if temperatures are low.	de March 1977

TABLE 2. Outline of a simulated year in the proposed test.

Condition and (or) Change made	Duration	Parameters measured	Transfers or other Changes
1. Animals acclimated to 2 control tanks and any number of test tanks. 10°C 8 h light/d (8L:16D)	1 month	none	
2. Introduce toxicant.		none	
3. 10°C 8L:16D	2 weeks	survival	Animals can be transferred to another test tank during survival count.
4. Transition	4 d		
5. 15°C 16L:8D	1 month	survival	same as above
6. Transition	3-5 d		
7. 20°to 25°C 16L:8D	≥2 months	a) time to induce reproduction; synchrony of first pairing; % of population pairing.	Count animals and transfer to another tank as they are observed pairing.
		b) number of eggs/female; size of eggs.	Preserve animals to measure these.
		c) gestation time (or frequency of mating).	As animals pair for the second time, following release of the first brood, count and transfer to another tank. The first brood released remains in first tank.
		d) maturation time of juveniles.	Transfer animals into another tank as they are observed pairing.
		e) survival of the parents; survival of the young; reproductive success of young (same as a).	
		a, b, c, d can be measured for any number of generations.	Maintain a breeding culture of any but the first generation of juveniles.
8. Transition	5 d		
9. 15°C 16L:8D	1 month	a) reduction in pairing frequency (i.e. pairing/d).	Isolate animals pairing in the last 2 weeks, of this month.
		b) egg sizes, no. of eggs/females.	Preserve animals.
10. Transition	4 d	survival of adults	
11. 15°C 8L:16D	2 months	growth rate of juveniles	Since these do not mature under these conditions, they must be measured at regular intervals.

I recommend using no more than 20 mating pairs to start a new stage of the experiment. Densities should be no more than 1 adult/cm^2 of substrate during the test. Thinning is possible because the growth rate of the population is not measured, only some components of it.

In the proposed test, the effects of a toxicant are monitored during a simulated "year". All responses in Table 1 are monitored at appropriate times as shown in Table 2.

Acclimation rates to various temperatures and photoperiods during transitions should be no greater than 2°C/d and 2 h light/d.

The frequent and conspicuous pairing of animals must be observed. Pairing tells us that 1) animals have come out of the resting stage, 2) they have reached maturity, or 3) they are releasing a previous brood. Paired animals must often be moved to another tank in preparation for the next part of the test (a 1 cm diameter pipette may be used). A sample of paired animals must be preserved to measure size at maturity or egg numbers and size.

EXAMPLES OF APPLICATION

Known data on responses of H. azteca to toxicants and other stresses are summarized in Table 3. From this it is apparent that growth and reproduction are impaired by lack of food and by low oxygen (Wilder 1940; see also Strong 1972). In a series of tests, the author (de March 1979) determined the effects of four different pH conditions (ranging from 4.8 to 7.9) on the survival of eight healthy laboratory populations of H. azteca. The results demonstrated significant differences in sensitivity, related to different life stages and morphs. Since the procedure

TABLE 3. Responses of H. azteca in stress and toxicity tests.

Author	Response Parameter	Result
Wilder 1940	survival in standing water, crowded conditions and various food types and levels	- size of animals and population density decreases with stress.
Bovée 1949	survival at various temperatures	- ranges defined
Sprague 1963	24-h LD50 on low oxygen and high temperature	- ranges defined
Nimmo 1968	osmotic balance tests	- H. azteca regulates to different conditions easily. Is an osmoconformer.
Maciorowski 1975	96-h LC50 on various effluents	- control mortality problems - high survival in organic effluents (sugar beet and meat processing wastes).
de March (unpubl.)	survival of gravid females in low concentrations of Cadmium (Cd^{++})	- 1 µg/L Cd^{++} causes moulting difficulties, hence young not released.
de March 1979	MST (time to 50% mortality) at pH values (4.8 to 7.9) with 8 different populations of H. azteca	- survival and MST values depend on age, culture condition and other factors. - useful culture condition recommended. - resorbtion and/or abortion and/or high juvenile mortality is a common response to stress.

here proposed allows to work with all morphs and stages, the most sensitive ones in regard to particular toxicants can be identified.

DISCUSSION

H. azteca offers potential as a standard test organism. It occurs from the tropics to the northern tree-line and on coastal islands (Bousfield 1953). Breeding stocks are easily obtained and cultured.

High control mortality has been an undesirable characteristic of H. azteca in many short-term tests. Reasons may include the use of senile animals, changes in ionic concentrations, light shock, and changes in food type. The procedure described here is designed to overcome these difficulties. The population is held at conditions at which it has a high probability of surviving, apart from the toxicant introduced, thus allowing the measurement of additive and cumulative toxic effects over several generations.

COSTS AND STAFF REQUIREMENTS

Dissecting microscope with micrometer	$2500
Twelve aquaria, 10 L ea.	$ 150
Light and temperature control	$1500
Air pump(s), tubing, air stones	$ 100
Pipettes, beakers	$ 100

A culture system producing several hundred H. azteca per day can be maintained by one individual (well-trained in procedures as described) with one day's work per week. Overwintering animals can be maintained

with one hour of work per week. The multi-generation test as proposed requires one or two hours per day for several months.

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A HISTOCHEMICAL TEST FOR OLFACTORY FUNCTION IN FISH

by R.E. Evans and T.J. Hara

ABSTRACT

Phospholipids are highly localized in the olfactory receptor neurons of several species, present in the membranes and in the cytoplasm. This localization is in accordance with a role in olfactory function. The Baker acid hematein staining method shows clear morphological features of the olfactory neurons (cell body, dendrite, and axon) beyond those obtainable by routine histological methods. The technique allows the determination of phospholipid alterations and/or morphological changes of olfactory neurons caused by toxicants. Results are in agreement with electrophysiological tests on olfactory function.

RATIONALE

The initial sensory process of olfactory reception is considered to take place at olfactory receptor membranes. The protein and lipid molecules in these membranes seem to be the receptors for chemical stimuli. Kurihara et al. (1972) have shown that 60% of the total content of plasma membranes of bovine olfactory epithelia were lipids, and that more than 76% of the total lipids were phospholipids. Dodd (1971) presented evidence of odorant binding to a phospholipid region of the olfactory sensory membranes but not to receptor proteins. Therefore, if there is some alteration of the phospholipids we might expect a change in the olfactory response the neurons transmit to the brain. This test demonstrates phospholipid alterations associated with decreased

olfactory responses measured electrophysiologically.

METHOD

The olfactory rosettes, comprised of the olfactory lamellae in the olfactory capsules of fish are removed intact from anaesthetized fish. They are then immediately fixed in formol-calcium fixative for 6 h and processed according to the Baker acid hematein method (Baker 1946) from Pearse 1968.

(i) Preparation of Solutions

a) Formol-Calcium Fixative

40% formalin	10 mL
10% $CaCl_2$ (anhydrous) aqueous solution	10 mL
Distilled water	80 mL

To this is added a piece of chalk to maintain neutral pH.

b) Dichromate

Potassium dichromate	5 g
$CaCl_2$ (anhydrous)	1 g
Distilled water	100 mL

c) Acid Hematein. Place 0.05 g hematoxylin in a flask, add 48 mL distilled water and exactly 1 mL of 1 per cent $NaIO_4$. Heat until the water begins to boil, cool and add 1 mL of glacial acetic acid. (The author recommended B.D.H. reagent hematoxylin). This solution should be used on the day of preparation.

d) Borax-ferricyanide Differentiator

Potassium ferricyanide	0.25 g
Sodium tetraborate, 10 H_2O	0.25 g
Distilled water	100 mL

This solution should be kept in the dark.

(ii) Fixing, Staining and Mounting

1) Fix small blocks of tissue in formol-calcium, 6-18 h.
2) Transfer to dichromate-calcium for 18 h at 22°C.
3) Transfer to dichromate-calcium for 24 h at 60°C.
4) Wash well in distilled water.
5) Cut frozen sections at 10 µm(embed in gelatin if necessary).
6) Mordant in dichromate-calcium for 1 h at 60°C.
7) Wash in distilled water.
8) Transfer to acid hematein solution for 5 h at 37°C.
9) Rinse in distilled water.
10) Transfer to borax-ferricyanide for 18 h at 37°C.
11) Wash in water.
12) Mount in glycerin jelly.

The tissues thus processed are embedded in gelatin to prevent the delicate olfactory lamellae from breaking up after sectioning and cut in the cryostat at -25°C (sections of 10 µm in thickness). To avoid prolonged exposure at 37°C, the 2 h method of gelatin embedding for enzyme histochemistry is used; Baker's pyridine extraction test is used as a control: both methods are described below following Pearse 1968.

(iii) Gelatin Embedding for Enzyme Histochemistry

This process is usually carried out when it is necessary to support delicate tissues so that sections can be cut on the freezing microtome, and to minimize damage to enzymes.

1) Fix thin slices or portions of tissue in 15% cold neutral formalin (4°C) for 10-16 h.
2) Wash in running water for 30 min.
3) Embed in gelatin* for 1 h at 37°C.
4) Cool and harden in formalin (40% formaldehyde) for 1 h at 15-22°C. Wash.
5) Store at 4°C or below until frozen sections are required.

* Gelatin pulv. . . . . 15 g
Glycerin . . . . . 15 mL
Distilled water . . . . . 70 mL
Thymol . . . . . a small crystal

(iv) Baker's Pyridine Extraction Test

1) Fix in a weak solution of Bouin's fixative (sat. aqueous picric acid 50 mL, commercial formalin 10 mL, glacial acetic acid 5 mL, distilled water 35 mL) 20 h.
2) Wash in alcohol to remove picric acid.
3) Immerse in pyridine at 17-22°C, 30 min.
4) Immerse in pyridine at 60°C, 24 h.
5) Wash in running water, 2 h.
6) Transfer to the dichromate-calcium mordant of the acid hematein method (q.v.).

All lipids should be removed by the pyridine extraction procedure which can be followed by Sudan black B or other colorant methods as well as by the acid hematein method. A positive result for phospholipids with the Baker acid hematein technique should be accompanied by a negative reaction after pyridine extraction. In the absence of such a negative extraction test, the dark blue or blue-black staining material is not phospholipid. In a positive result, nucleoprotein and phospholipids stain dark blue or blue-black; mucin dark blue, fibrinogen pale blue; cytoplasm pale yellow.

EXAMPLES OF APPLICATION

The test has been successfully applied so far to six freshwater fish species: rainbow trout (Salmo gairdneri), whitefish (Coregonus clupeaformis), arctic char (Salvelinus alpinus), brook trout (Salvelinus fontinalis), lake trout (Salvelinus namaycush), and black bullhead (Ictalurus melas) (Evans and Hara 1977). Studies with whitefish and rainbow trout have shown that the method can detect decreased phospholipid in the neurons after exposing fish to metals such as copper, cadmium or mercury (Fig. 1-4). Concomitant with decreased phospholipids, electrophysiological data have demonstrated an impairment

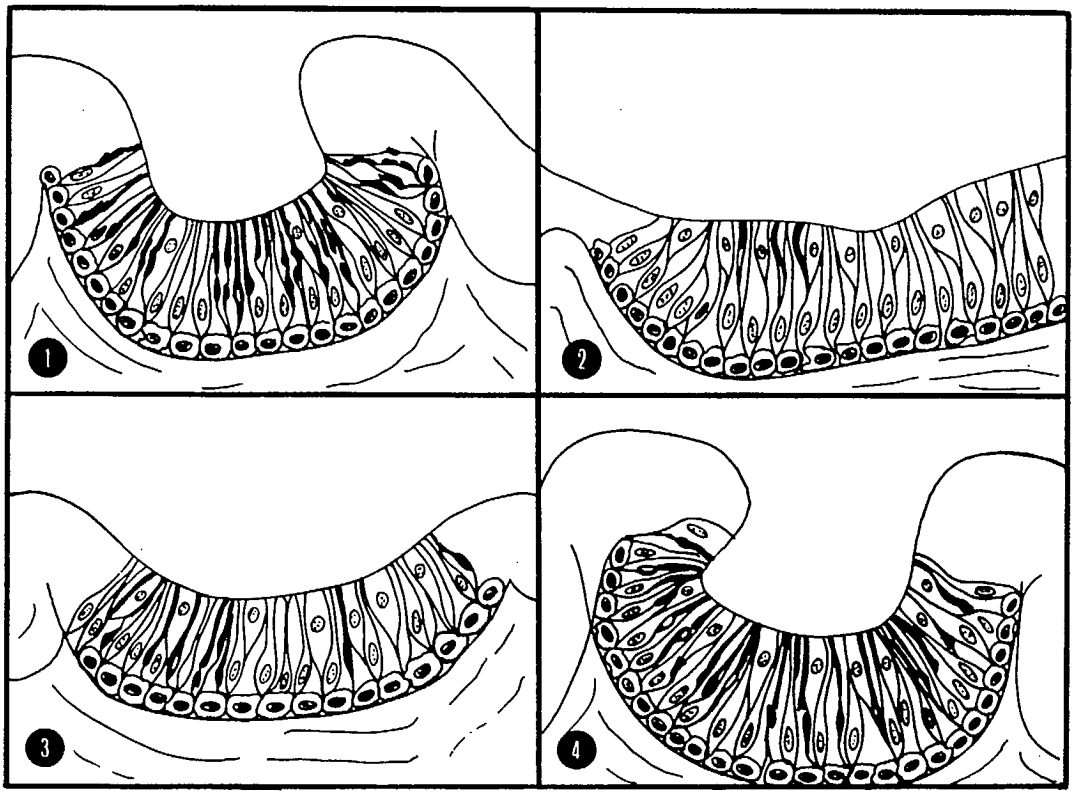

Figure 1. Olfactory rosette sensory epithelium from control whitefish, olfactory neurons stained black; Baker acid hematein method.

Figure 2. Olfactory rosette sensory epithelium from whitefish exposed to 0.15 mg Cu/L for 2 weeks showing the lack of phospholipid-staining in neurons.

Figure 3. Whitefish olfactory sensory epithelium after 4 weeks recovery from 0.15 mg Cu/L showing some phospholipid recovery.

Figure 4. Whitefish olfactory sensory epithelium after 6 weeks recovery from 0.15 mg Cu/L showing completed phospholipid recovery.

of olfactory response to standard test substances after metal exposure (Thompson, Evans and Hara, unpubl. data). Our data have shown that in rainbow trout exposed to high levels of copper (50-150 µg/L for 2 weeks) most olfactory neurons, normally high in phospholipid content, showed no reaction to the acid hematein stain. Electrophysiological observations showed a definite diminished response. An exposure to 0.5 mg/L of copper, cadmium and mercury was tested for up to 3 weeks. Fish were sampled at various time intervals. Each metal showed its own specific olfactory response inhibition rate and diminution of phospholipid levels. Phospholipid levels gradually returned to normal at about six weeks after the toxicant was removed (Fig. 3 and 4).

DISCUSSION

This technique is quite simple and results may be obtained in one week. Because of the precise timing required for each step, only a few samples can be done at one time by one person. The results presently are not quantifiable, but effects can be recognized when comparing with controls. The results back up quantifiable electrophysiological data. Further testing will have to be carried out on substances other than metals to determine the general applicability of the method.

The techniques largely follow standard methods given above. However, it has yet to be determined how long samples can safely be retained in fixative prior to completion of the method. If samples can be stored in fixative over several weeks it would be feasible to process more samples in a specific study.

Beyond providing additional evidence to physiological tests, the

method is useful as an indicator of the olfactory ability of fish where sophisticated electrophysiological equipment is not available or practical to use.

COSTS AND STAFF REQUIREMENTS

Chemical supplies for this method cost about $125. Equipment required for the technique includes a cryostat (min. $3500), a small oven (min. $500), and an electric hot plate with magnetic stirrer (min. $100).

A technician could be trained within two weeks to perform this method. Processing five samples requires 10-14 man-hours over a one-week period.

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THE USE OF THE SNAIL Helisoma trivolvis IN A MULTIGENERATION MORTALITY, GROWTH AND FECUNDITY TEST

by J.F. Flannagan and D.G. Cobb

ABSTRACT

A multigeneration toxicity test using the freshwater snail Helisoma trivolvis (Say) is presented. Methods for collecting, culturing and testing of this species are given. Mortality, growth and fecundity are the parameters used to determine toxic effects. Results of tests using trisodium nitrilotriacetic acid are discussed in light of the phenology of the snail.

RATIONALE

The test described here provides information on the effects of toxicants on the mortality, growth and fecundity of successive generations of the Ram's horn snail, Helisoma trivolvis (Say). This information can be used to determine detrimental effects of environmental toxicants as well as the potential of the animal to adapt or acclimate to such compounds. We selected the Ram's horn snail because it is available locally, is widely spread throughout central and Eastern Canada and the U.S.A. (Clarke 1973), is readily adaptable to laboratory culture, has a short generation time and is amenable to handling and short-term exposure to air.

METHOD

(i) Collection

H. trivolvis is easily collected from lakes or streams using a

simple dip net or by collecting egg masses from stones, aquatic plants, etc. Transportation back to the laboratory can be successfully achieved either by wrapping the snails or egg-bearing stones in damp moss or by placing them in plastic bags half filled with water.

(ii) Culture Conditions

Factors important in culturing this snail, and freshwater snails in general, have been described in the literature. Krull (1937) recommended alkaline water and had little or no success in acid water. However, Flannagan (1971) found that a pH of 9.1 or above induced significant mortality in H. trivolvis. A pH between 7 and 9 is therefore recommended.

Water depth also is an important consideration. Cheatum (1937) states that H. trivolvis thrives better in aquaria with 5-8 cm of water than 25-30 cm of water. Water temperature and changes in water temperature have been shown to profoundly affect both growth and fecundity of snails. Van der Schalie and Berry (1973) concluded that optimum growth and survival of H. trivolvis occurred at 28°C and optimal egg-laying at 30°C. Growth and fecundity restrictions have also been associated with overcrowding (Levy et al. 1973) and inadequate diets (Krull 1937). In nature, H. trivolvis has a life span of approximately 2 years (Boerger 1975). However in the laboratory Flannagan (1974) showed that the generation time (egg to egg laying) was about 70 d at 23°C, indicating that approximately five generations can be produced in a year. A daily diet ($\simeq$ 10% of body weight of snail) of Tetramin® staple fish food meets the food requirements of these snails.

(iii) Test Procedure

Using a stock concentration and dechlorinated water, nominal concentrations of a toxicant are delivered by a proportional dilutor (Mount and Brungs 1967) to six mixing chambers one of which is used as a control. The flows are then directed to experimental tanks each of which is divided into three compartments by glass partitions. The whole system is maintained at a constant temperature with a 12 h light: 12 h dark photoperiod provided by fluorescent lighting. In each tank, temperature is measured daily and dissolved oxygen, pH and toxicant concentrations are analyzed weekly.

Sixty juvenile H. trivolvis (mean weight about 10 mg) are selected from stock cultures, divided into 6 groups of 10 with approximately the same mean weight. The groups are placed in the first compartment of the experimental tanks. The snails are fed 10% of their body weight per day of Tetramin® staple fish food. Once a week the animals are removed from their tanks, allowed to dry for 5 min at room temperature (about 22°C), weighed individually, and returned to their tank. The resultant weights are used to calculate their food ration for the following week and these weights can be plotted as mean weight/concentration to measure growth rate.

Ten snails selected from the first offspring of each exposure group are weighed and transferred to the second compartment in each tank to form the F_1 generation; 10 from the first offspring of the F_1 to the third compartment to form the F_2 generation; and 10 from the F_2 to the first compartment (P_1 eliminated by this time) to form the F_3 generation. At the end of each month the remaining offspring are removed

from the compartment and counted.

The laboratory growth curve for H. trivolvis (Fig. 1) is essentially sigmoid with the time from hatching to the start (i.e. day 0 in Fig. 1) of the given growth curve ($\simeq$ 30 mg) being approximately 25 d. Thus snails in the 0-30 mg range although very similar in weight could well be very different ages (in days). Experimental groups of snails starting within this range could give rise to growth curves appearing to be different. To avoid this complication the comparative growth curves (Fig. 2) should show only the straight line (exponential) part of the curve (Fig. 1).

If mortality occurs during the egg-laying period, the fecundity results can be adjusted, using the formula given below, to permit direct comparison of these results:

$$\text{adjusted fecundity} = \text{number offspring produced} \times \frac{\text{standard number of egg-laying days}}{\text{actual number of egg-laying days}}$$

To ascertain whether any change in fecundity is due to reduced egg-laying or is associated with mortality of eggs or embryos, counts of egg masses should be carried out for a 5-d period on the F_1 generation and compared to the total production of juveniles in that generation.

EXAMPLES OF APPLICATION

The above test was used to test effects of Na_3NTA (trisodium nitrilotriacetic acid, a phosphate replacement in detergents) which had been found to be acutely toxic to H. trivolvis at concentrations

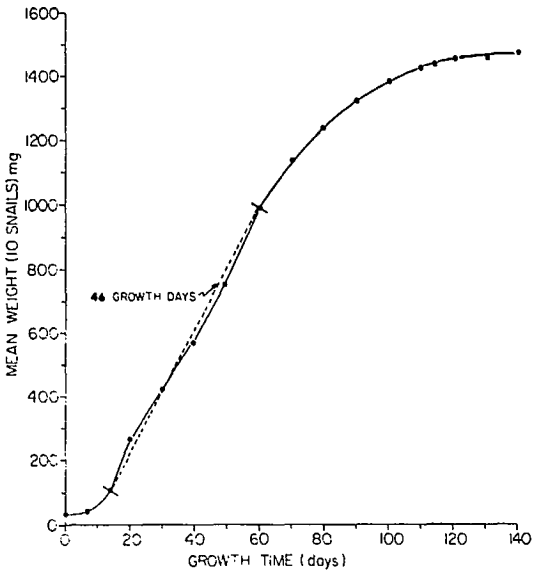

Figure 1. Typical growth curve of laboratory population of Helisoma trivolvis (means for 10 specimens) indicating part of the curve used in comparison of growth rates. From Flannagan (1974).

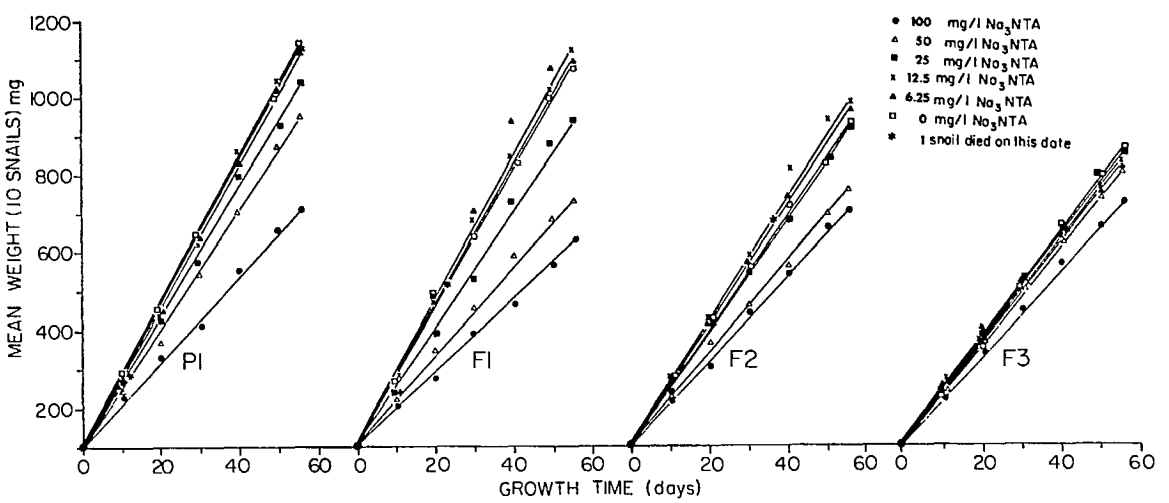

Figure 2. Mean growth rates of four generations of H. trivolvis (10 per test) in various concentrations of Na_3NTA during the 46 days represented by linear part of curve in Fig. 1. From Flannagan (1974).

> 250 mg/L (Flannagan 1971). Using a stock concentration of 50 g/L and activated charcoal dechlorinated Winnipeg tap water (TDS around 117 mg/L), nominal concentrations of 0, 6.25, 12.5, 25, 50 and 100 mg/L Na_3NTA were delivered to experimental tanks, and the procedures described above were carried out.

The results from the above experiment indicated that Na_3NTA at the three higher concentrations produced a significant reduction in growth rate (Fig. 2) and fecundity (cf. Fig. 3 in Flannagan 1974). However, these functions could be interrelated if size and fecundity are interdependent. If this is the case (but see Flannagan 1974) then the effects recorded could be the result of the toxicant acting on only one of the measured physiological parameters.

DISCUSSION

The testing of NTA showed measurable effects on mortality, growth and fecundity. Also, this species adapted to environmental stress in succeeding generations. This multigeneration toxicity test is probably applicable to most toxicants, and to most, if not all gastropod species that can be successfully reared under laboratory conditions.

COSTS AND STAFF REQUIREMENTS

Mount-Brungs diluter	$200
pH meter and probe	$600
O_2 meter and probe	$600
Misc. glassware, tubing etc.	$100

The construction of the Mount-Brungs (1967) dilutor can be inexpensively carried out by one technician in one week. Although long-term in nature, this multigeneration procedure is not labour-intensive. Six days a week one technician requires one hour to feed the snails, check for mortalities and egg masses, to record parameters (pH, temperature, O_2, etc.) and make minor adjustments to the system if required. Once a week two hours are required to weigh the snails, weigh out the following week's food supply and make up new stock solutions.

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USE OF EGGS OF THE BURROWING MAYFLY Hexagenia rigida IN TOXICITY TESTING

by M.K. Friesen

ABSTRACT

Hatch parameters (start of hatch, hatch rate, final percent hatch) and embryonic development of eggs of Hexagenia rigida are used to determine lethal and sublethal effects of toxicants. Methods for the collection, dissection, incubation, and counting of eggs are described. Use of the test for the insecticide methoxychlor and other applications are briefly discussed.

RATIONALE

Burrowing mayflies of the genus Hexagenia are distributed throughout the U.S.A. and south and central Canada, and are among the largest mayflies found in North America. Large numbers of nymphs commonly inhabit lakes, rivers and streams having silt substrates, and constitute an important food item for fish (Neave 1932; Hoopes 1960). Further information on the role of these mayflies in the ecosystem is given by Edmunds et al. (1976).

The life cycle of H. rigida McDunnough, as that of other Hexagenia species, is relatively long compared with most aquatic insects. In Lake Winnipeg (Manitoba, Canada) the life span was found to be 14 to 24 months (Flannagan 1979), consisting mainly of the larval (nymphal) stage. The larvae may reach a length of over 3 cm before emergence. Adults, which are terrestrial, emerge during the summer and live for several days at most,

during which time mating and egg laying occur. Most egg hatch seems to occur within several weeks, although overwintering may occur in the egg stage. The egg stage was chosen for toxicity testing because eggs of this organism are easy to handle, have uniform hatching characteristics, involve the use of simple equipment, and can be held in cold storage for up to ten months of the year and be transferred to higher temperatures without considerably affecting hatch parameters (Friesen et al. 1979).

METHOD

In brief, the method involves the incubation of eggs in various concentrations of toxicant and monitoring of embryological development and egg hatch.

(i) Equipment and Material

1) plastic bags: used to transport fertilized females from the field and for holding prior to dissection
2) forceps and scissors: dissection
3) beaker (200-1000 mL): into which eggs are dissected
4) Pasteur pipette: transfer of eggs to Petri dishes
5) Petri dishes: incubation of eggs
6) water for incubation
7) appropriate concentrations of toxicant
8) dissecting microscope (10x and 25x power) and counter

(ii) Collection

Large numbers of viable eggs can be obtained from female imagines collected during emergence and mating periods (June to July in the Red

River, Winnipeg, Canada). Collections are made after dusk under lights located near bodies of water inhabited by mayfly nymphs. The females may be held at 6-10°C for several days if they are not to be dissected immediately, and are moved to the higher temperatures at least three hours prior to dissection.

(iii) Storage

Eggs collected during peak emergence and mating periods can be stored at 12°C and 8°C for prolonged periods of time. Eggs allowed to develop to about mid-way of their embryological development first can be stored at 8°C for up to ten months and returned to 20°C without considerably affecting hatch parameters. Eggs stored for ten weeks have been used in toxicity testing (Friesen 1979).

(iv) Dissection

A live female is dissected by grasping her by the abdomen and cutting off the thorax and head portion of the body. The egg packets are gently squeezed out of this large opening, are placed directly into a beaker containing water, and are teased from the ovarian tissue. Eggs of at least several females should be pooled since some may not have been fertilized. Eggs are mixed thoroughly and aliquots are transferred to Petri dishes containing the appropriate concentrations of toxicant. Number of eggs transferred may vary but density (number per dish) does not seem to affect hatch unless eggs are clumped. A recommended number for a standard size Petri dish (9 cm diameter) is 300-500 eggs, but more or less may be used.

(v) Incubation

Eggs are incubated in Petri dishes in a depth of about 10 mm of water. Water may be natural, i.e. river water, or reconstituted (distilled water and appropriate salts). Water changes are possible since eggs normally adhere to the Petri dish surfaces. Temperatures at, or slightly above, 20°C were selected for the studies described below but other temperatures may also be used (Friesen et al. 1979).

(vi) Embryonic Development

Eggs of H. rigida are ovoid in form with dimensions of approximately 0.2 mm x 0.3 mm; they have been described in detail by Neave (1932). The gross embryology may be observed using a dissecting scope at 25x magnification without interfering with normal development. In early stages of egg development only the yolk is visible. About midway through the developmental period the embryonic form becomes distinct, but eyespots and appendages are not yet clearly discernible. Monitoring the embryological development will indicate whether development is affected; sensitive phases in the embryo's development may become apparent.

(vii) Hatch Monitoring, Computing

An adequate monitoring schedule consists of daily checks for first hatch to occur, followed by daily counts of hatched nymphs for the next 4-5 d, and another check at least one week later to determine final percent hatch. Only nymphs which have freed themselves completely from the egg cases are considered hatched. Dead eggs can be easily identified because the contents will disintegrate or become brown with time.

Counts are made using a dissecting scope at 10-25x power. Percent hatch can be determined by counting number of hatched eggs per total number of eggs in each dish. If a very large number of eggs has been used, and time is limited, the dish may be subsampled, either by counting hatch in a known area of the dish (provided all eggs have adhered to the dish bottom) or by counting a constant number of eggs in random areas.

Start of hatch, hatch rate (cumulative percent hatch per treatment) and final percent hatch are the hatch parameters monitored. Final percent hatch in treatments is adjusted to final percent hatch in the control to account for non-treatment related hatch reduction, e.g. caused by non-fertilized eggs (Abbott 1925), and the adjusted final percent mortality is then plotted versus concentration to estimate an incipient LC50 (as in Sprague 1969).

For example: if final percent hatch of total number of eggs in the control is 92.0%, and in samples exposed to 3 treatments at concentrations of 20, 40 and 60 mg/L are 69.9%, 36.8% and 19.3%, respectively, then using the formula -

$$\text{percent mortality} = \frac{X - Y}{X} \times 100\%$$

where: X = % hatch in the control

Y = % hatch in the treatment

percent mortality in the treatments would be 24.0%, 60.0% and 79.0%, respectively, with an estimated incipient LC50 of approximately 33 mg/L as derived from a log/probit plot.

When replicates are run, variance and standard deviations are calculated on arcsin transformed data because percentages are not normally distributed (see Snedecor and Cochran 1967).

EXAMPLES OF APPLICATION

Eggs have been used in toxicity tests with the insecticide methoxychlor (Friesen 1979), saline groundwater and the heavy metal cadmium (Friesen, unpubl. data). One group of eggs, just released from females, was exposed to methoxychlor for the full developmental period, another for approximately the last half of the developmental period. The latter part of the test was repeated 10 weeks later using stored eggs. Effects ranged from partial suppression of hatch at the lowest concentration to a delay in hatch or total suppression at the higher concentrations, with the incipient LC50 estimated to be < 0.06 mg/L. Hatch rates were not appreciably affected. The estimated LC50's for "fresh" eggs exposed during the latter period of development and for stored eggs were 0.07 mg/L and 0.10 mg/L, respectively.

In those eggs which failed to hatch, embryonic development ceased during the latter stages of development, whether eggs were exposed for their whole, or just the last half, of the development period. This indicates that the embryo is probably most sensitive to methoxychlor in the advanced stages of development.

When testing saline groundwater and cadmium, embryos were more sensitive to these toxicants in their early stages of development for all lethal and sublethal parameters monitored.

DISCUSSION

The determination of an incipient LC50 for this stage of the animal's life cycle and the ability to expose eggs during readily defined periods of development should be useful in comparing results from tests performed with different toxicants.

This method should be applicable to other Hexagenia species and other mayflies whose eggs develop under static conditions. A drawback in working with this life stage lies in obtaining eggs, since "wild" viable eggs are available only in the summer when emergence and mating of adults occur. However, eggs collected in the summer may be held in cold storage and used at a later time (Friesen et al. 1979). Viable eggs of H. rigida may also be obtained from laboratory cultured animals, although in inconsistent and unpredictable quantities, either through artificial insemination (Friesen, in prep.) or parthenogenetically (Friesen and Flannagan 1976). Toxicity testing of the egg stage may or may not be indicative of effects on the nymph, since some substances which are toxic to the nymph may not penetrate the egg case. McCart et al. (1977) studied the effects of methanol on duration of development and hatching of eggs and nymphal mortality of Ephemerella infrequens, and found both life stages were sensitive to methanol.

COSTS AND STAFF REQUIREMENTS

Based on testing at 20°C (first hatch about day 16) with a control, 5 concentrations with 3 replicates per concentration and about 500 eggs per replicate, approximate costs are:

Petri dishes	$100

Dissection tools, Pasteur pipettes etc.	$ 200
Dissecting microscope	$2000

Professional supervision is desirable. Testing can be done by one non-professional person performing the following duties (with estimates of workdays required):

a) Preparation (washing Petri dishes, preparing test solution, etc.)	5 d
b) Day 0 - collection, dissection and transfer of eggs to toxicant	1 d
c) Days 1-15 inclusive* - periodic checks for hatch (30 min/day)	1 d
d) Days 16-20 inclusive - checks and counts	5 d
e) Day 34 (approx.) - final check	1 d
f) Total egg counts - may be made anytime	1 d
Total workload:	14 d over a space of ≃40 d

* more time is necessary for observations of embryos.

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THE RESIDUAL OXYGEN TEST: A RAPID METHOD FOR ESTIMATING THE ACUTE-LETHAL TOXICITY OF AQUATIC CONTAMINANTS

by M.A. Giles and D. Klaprat

ABSTRACT

The residual oxygen concentration is the concentration of oxygen dissolved in water in a closed container containing an aerobic organism at the time of death of the organism. At toxicant concentrations below a threshold value the oxygen residual for a particular set of test conditions is approximately equal to that of control vessels. As toxicant concentrations are increased beyond this threshold, the amount of oxygen the test organism can extract from the water before death declines, and the residual oxygen progressively increases. Under appropriate test conditions this threshold often occurs at toxicant concentrations approximately equal to the median lethal toxicant concentrations (96-h LC50) as derived from more time-consuming conventional acute-lethal bioassays. To date, this technique has been applied only to fish although it should be possible to employ other aerobic aquatic organisms.

RATIONALE

Reduced dissolved oxygen concentration increases the toxicity of a variety of contaminants to fish (Lloyd 1961; Alderdice 1963; Pickering 1968; Hicks and DeWitt 1971). Carter (1962) observed that the dissolved oxygen residual measured at the time of death of brown trout (Salmo trutta) held in glass bottles containing serial dilutions of cyanide or copper increased with increasing toxicant concentrations, and suggested that a residual oxygen bioassay would permit the rapid detection

of solutions containing low concentrations of toxicants. More recent studies have demonstrated that for such diverse toxicants as dehydroabietic acid, zinc, phenol, lindane, bleached kraft mill effluent, mercuric chloride, and sodium pentachlorophenate, a threshold toxicant concentration exists above which the residual oxygen concentration increases (Ballard and Oliff 1969; McLeay 1976; Gordon and McLeay 1977; Vigers and Maynard 1977). Furthermore, in the majority of these studies this threshold toxicant concentration ranged from 0.5 to 1.5 of the 96-h LC50 or the incipient lethal threshold as determined from conventional bioassays. Since the residual oxygen test can be performed within 6 to 10 hours and requires a minimum of specialized equipment and space, it offers a number of advantages over conventional bioassays in assessing the acute-lethal toxicity of aquatic contaminants.

METHOD

(i) Experimental

Logarithmic serial dilutions of toxicant in water to which the fish are acclimated are prepared and air-saturated with oxygen. The thermally equilibrated solutions are delivered into glass jars such as BOD bottles or reagent bottles which can be sealed without entrapping air bubbles. Although a single vessel per test concentration has been used in some studies, our results suggest that a minimum of three replicates per test concentration, including controls, are superior. If the toxicant or test water has a high BOD a second set of vessels may be prepared to measure extraneous oxygen consumption. Fish are put into each test vessel with a minimum of disturbance and the vessels are

sealed. One or more fish may be put in each vessel if a consistent loading density is maintained. The vessels are placed in a water bath at the required acclimation temperature. The time to death and the residual oxygen concentration at death are recorded. Dissolved oxygen is most readily measured with a low current oxygen electrode, such as a Radiometer PHM-72 acid base analyzer system, but can also be measured chemically by the Winkler method. Water samples may be taken for toxicant analysis for comparison with initial concentrations.

The loading density (weight of fish/volume of test vessel) will influence the time to death and the sensitivity of the test, especially in heavy metal bioassays. Loading densities of 1 to 4 g/L solution have commonly been employed (McLeay 1976; Vigers and Maynard 1977; Giles and Klaprat, in prep.). In general the loading density should be such that fish die in control vessels within 6 to 10 hours from the initiation of the test. Fish should be acclimated to the test temperature for at least two weeks and not fed for two days prior to the bioassay. Acclimation temperatures may be anywhere within the zone of tolerance of the species. Species which have been tested using this method include juvenile rainbow trout (Salmo gairdneri), coho salmon (Oncorhynchus kisutch), brown trout (Salmo trutta) and bluegill sunfish (Lepomis macrochirus), as well as adult zebrafish (Brachydanio rerio).

(ii) Data Analysis

Residual oxygen concentrations as percent of air saturation or as mg O_2/L are plotted against the logarithm of toxicant concentration and the relationship examined for a well defined inflection point (Fig. 1). An estimate of the inflection point can be derived from a simultaneous

solution of the least squares regression lines fit to the data above and below the inflection. If sufficiently high toxicant concentrations are tested, little or no oxygen will be extracted prior to the death of the fish as illustrated in the upper dashed lines of cyanide and copper bioassays (Fig. 1). Since these data would artificially lower the estimated inflection point they should not be included in the regression analyses. Residual oxygen expressed as percent saturation should be subjected to an arc-sine transformation prior to the regression analyses in order to statistically normalize the data. If computer facilities are available the inflection point can also be estimated using an iterative program to fit the relationship and define the point of maximum change in slope.

EXAMPLES OF APPLICATION

Significant residual oxygen responses at toxicant concentrations of 0.5-1.0 of the 96-h LC50 have been reported using dehydroabietic acid, zinc, phenol and lindane with juvenile rainbow trout (McLeay 1976). Coho salmon showed a significant response to bleached kraft mill effluent, whereas juvenile rainbow trout did not (McLeay 1976). Vigers and Maynard (1977) however, found no significant difference between the 96-h LC50 derived from a conventional static bioassay and the residual oxygen response when testing three different effluents from a pilot plant kraft pulp mill, a chloralkali effluent and sodium pentachlorophenate using juvenile rainbow trout. Giles (unpubl. data) observed a significant increase in residual oxygen at cyanide concentrations in the range of 0.8-1.0 of the 96-h LC50 for rainbow trout,

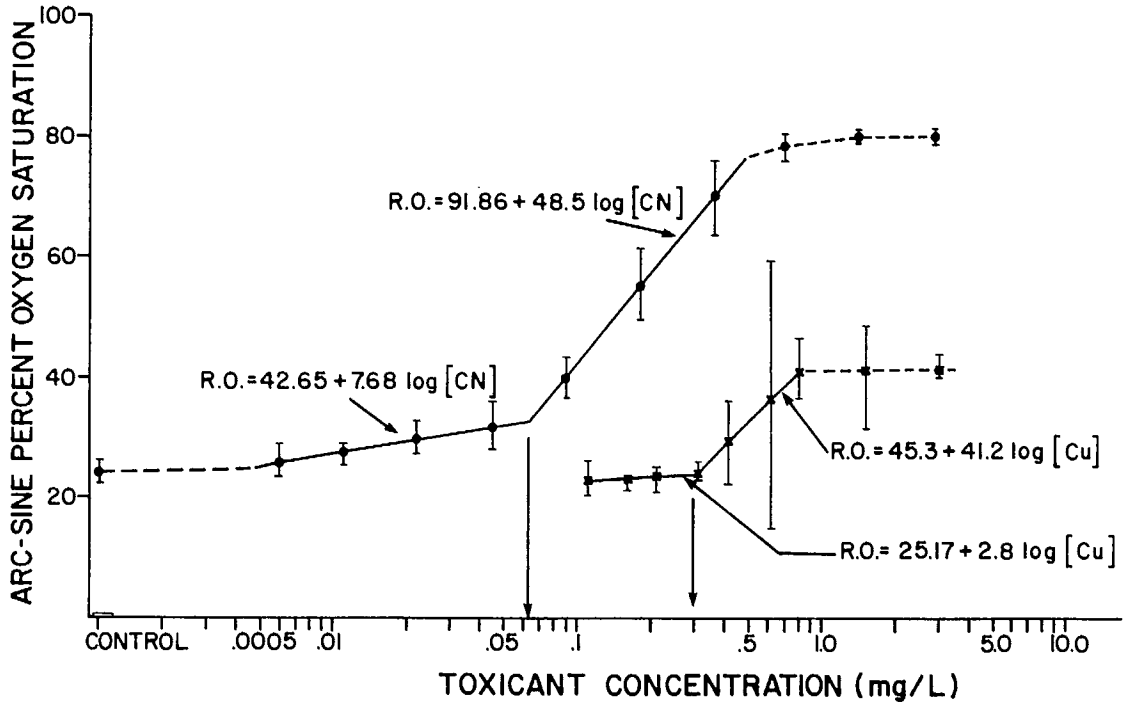

Figure 1. Residual oxygen bioassays of cyanide and of copper with rainbow trout, Salmo gairdneri. The NaCN bioassay (•) was performed at 10°C using 2 to 5 g trout in 300 mL BOD bottles with five replicates per toxicant concentration. The $CUSO_4$ bioassay (x) was performed at 15°C using 2 to 5 g trout in 1100 mL glass bottles with three replicates per toxicant concentration. The residual oxygen (R.O.) results are presented as the arc-sine transformation of the percent oxygen saturation with the horizontal bars indicating the 95% confidence interval of the means. The vertical arrows denote the inflection point of the residual oxygen: toxicant concentration relationship calculated from the least squares fit of the lines above and below the inflection point. The incipient lethal concentrations of cyanide and of copper are 0.05 mg/L (McKee and Wolf 1963) and 0.19 mg/L, respectively (Giles and Klaprat, in prep.).

zebrafish and pumpkinseed(Lepomis gibbosus). The residual oxygen response of rainbow trout to copper approximated the 96-h LC50 only under certain test conditions while no response to cadmium was observed under any test conditions (Giles and Klaprat, unpubl. data). In toxicity studies with the marine fish Ambassis safgha residual oxygen responses closely approximated the median lethal concentrations for mercuric chloride and phenol (Ballard and Oliff 1969).

DISCUSSION

The residual oxygen bioassay has been studied only sparingly, although several recent papers and toxicity workshop discussions indicate an increasing interest in this technique. Several facts would tend to support continued development and application of this test procedure for aquatic toxicology. First, after optimum test conditions for vessel size, fish size, etc. have been determined, the bioassay can be completed within one day using a minimum of space and equipment. This not only circumvents the inherent difficulties in maintaining longer term conventional bioassays,but also permits easier replication of toxicity experiments. Secondly, the test can be performed under field conditions with a minimum of portable, battery-operated equipment. Thirdly, potentially useful data on the effects of toxicants on metabolic rate may be derived by relating oxygen consumption to survival time. Finally, the results do approximate the 96-h LC50 for some specific contaminants and whole effluents.

Several unresolved problems still exist, however. Temperature, bottle size, loading density and differing sensitivities of various fish species

have been demonstrated to modify the sensitivity of the test for various toxicants (Ballard and Oliff 1969; McLeay 1976; Gordon and McLeay 1977). The residual oxygen response would appear to be less sensitive to heavy metals than to organic toxicants. With copper, for instance, test conditions can be applied which do result in a reasonable approximation of the 96-h LC50 using rainbow trout, but analysis of the data suggests that major interactions exist among several of the factors such as test temperature, bottle size, fish size, loading density and genetic strain of fish (Giles and Klaprat, in prep.). It would appear, therefore, that the residual oxygen bioassay is a useful tool for specific applications, but widespread use must await further definition of the factors influencing the sensitivity of the response to specific toxicants and classes of contaminants.

COSTS AND STAFF REQUIREMENTS

Basic equipment required for this test are 50 glass jars ($300), an oxygen meter ($800-$1000), and a large constant temperature bath (a fish-holding tank can be employed).

One person can perform the entire bioassay and analyse the data within 1-2 workdays. Training in preparation of toxicant dilutions, operation and maintenance of the oxygen meter, and basic statistical analysis is necessary.

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AN ELECTROPHYSIOLOGICAL TEST FOR NEUROTOXICITY IN FISH

by T.J. Hara

ABSTRACT

Acute-sublethal effects of toxic chemicals on the olfactory response of fish are tested by perfusing water containing the toxicants through the olfactory organ while recording the olfactory bulbar electrical responses to standard chemical stimuli, e.g. food extract and amino acids. Response parameters (magnitude, frequency, and latency of electrical response) during treatment are compared to controls before treatment. Chemical threshold concentrations for toxic effects are estimated from dose-response relationships. In chronic tests, olfactory responses of control and treated groups to standard stimuli are compared. The method is rapid, specific, and sensitive, and can be applied to most chemicals, various physical conditions of water, and fish species.

RATIONALE

The nervous system is one of the most susceptible and vulnerable parts of the animal body. Many poisons including most insecticides owe their toxicity to their ability to attack the nervous system (Eto 1974).

The olfactory system of fish can be used as a model to test aspects of neural interactions with the environment. Olfaction plays an important role in the survival of fish by mediating such diverse phenomena as feeding, recognition of prey and predator, sexual and social behavior,

and orientation and migration (Hara 1970, 1971, 1975). The initial sensory process of olfactory reception takes place at olfactory receptor membranes. Because they are not protected by external barriers, man-made alterations of water quality interfere with their functioning, resulting in impairment of communication among fish and between fish and environment (Bardach et al. 1965; Atema et al. 1973; Sutterlin 1974).

METHOD

(i) Preparation of Fish

The fish is initially anesthetized by immersion for a few minutes in an aqueous solution of tricaine methanesulfonate (MS 222®). Although the response to the anesthetic varies somewhat in different species of fish, the dosage of 1:10,000 to 20,000 is normally used for salmonid and cyprinid fishes. The fish is then immobilized by intramuscular injection of a muscle relaxant; d-tubocurarine chloride and gallamine triethiodide (Flaxedil®) are widely used at the dosages of 2-4 mg/kg body weight. These dosages are sufficient to keep the fish immobilized for a period of several hours. The fish is wrapped in a wet tissue and secured on a holding apparatus placed in a plastic trough. During the experiment the gills are perfused with water through a tubing inserted into the mouth; this water, after passing over the gills, is drained into the trough. Since the oxygen requirement varies for different sizes and species of fish, a suitable flow rate should be established after several trials. A flow rate of 1 L/min for rainbow trout (Salmo gairdneri) of 15-20 cm in body length is satisfactory for an experiment lasting over

several hours. The brain is exposed by removing the top of the skull with a dissecting knife. The loose fatty tissues surrounding the brain are removed by aspiration and by sponging with tissues. Since even a small injury of the brain could be a cause of an immediate decay of the electrical activity, special care should be taken not to make any direct contact to the brain with knives, forceps or aspirating devices during the operation. The anterior part of the brain cavity thus formed is filled with mineral oil after the recording electrodes are implanted (Hara 1973).

(ii) Recording of the Bulbar Electrical Responses

Electrical activities are led through a pair of stainless steel bipolar electrodes (e.g. David Kopf Inst. Type NEX 200) placed on the surface of the posterior part of the olfactory bulb(s) and recorded on a polygraph (e.g. Grass Model 79). Spontaneous and stimulant induced waves are amplified and integrated by an a.c. preamplifier and integrator. This integrator is in turn coupled to a d.c. integrator in order to measure the accumulated activity during the stimulation period (Hara 1977).

When electrodes are placed on the surface of the olfactory bulb, spontaneous electrical activities, the typical electroencephalogram (EEG), can be recorded. Infusion of odorous stimulants such as food extract, hand rinse, and amino acid solutions into the nostrils induces large rhythmic oscillation; the spontaneous activity is immediately interrupted by oscillatory waves that are terminated on cessation of the stimulus (Fig. 1).

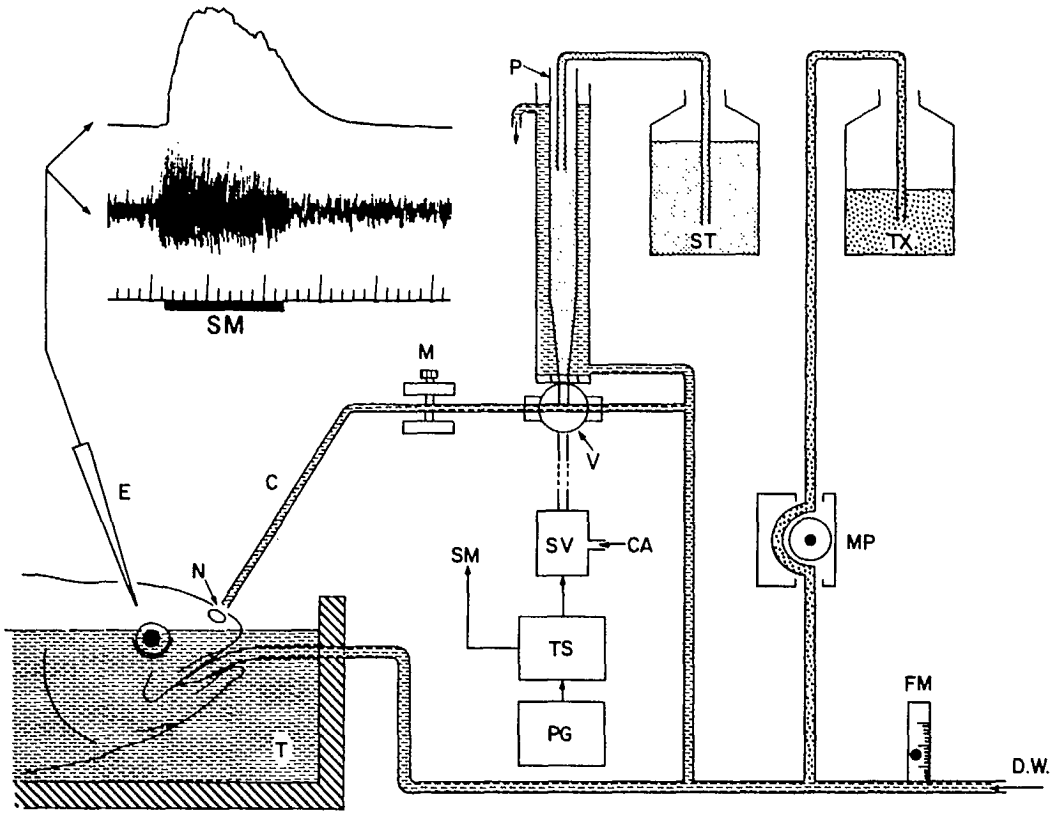

Figure 1. Experimental setup for recording olfactory responses and for toxicant injection system. The standard stimulant solution (ST) is led via plastic tubing into a glass pipette (P). By switching the three-way valve (V), the stimulant is poured into the nares (N) through a pair of glass capillaries (C) adjusted by a micromanipulator (M). CA, compressed air; DW, dechlorinated water; E, recording electrodes; FM, flow-meter; MP, metering pump; PG, programmer; SM, signal marker; SV, solenoid valve; T, trough; TS, time switch; TX, toxicant. From Hara et al. (1976).

L-serine solution at 10^{-5} mol/L has been satisfactorily employed as a standard stimulus. The stimulant solution is delivered to the paired nares at the rate of 0.1 mL/naris per second for 10 s, at intervals of 2 min. The use of an automatic stimulatory apparatus is recommended (Fig. 1; Hara et al. 1973). All measurements are taken after a stable maximum response to the standard stimulus is reached by repeated applications.

(iii) Treatment of the Olfactory Organ

The olfactory organ is treated with toxicants by injecting the stock solutions into the main stream of water infusing the nares and gills of the fish (Fig. 1). This is done by the use of a peristaltic infusion pump. Either the nares alone or both nares and gills can be infused with toxicants by minor alterations of the apparatus.

(iv) Definition and Interpretation of the Data

The integrated bulbar response is a function of the amplitude and frequency of the induced waves, and varies in magnitude depending upon either the intensity of the stimulus or the sensitivity of the olfactory system. Thus, the toxicity of chemicals to be measured can be determined in terms of the depression of the bulbar response to a standard stimulus. Fig. 2 illustrates typical responses recorded from the olfactory bulb of the same fish when the nares are stimulated with 10^{-5} mol/L L-serine before, immediately after exposure to 0.25 mg/L $HgCl_2$, and after rinsing. The patterns of response and depression of the response caused by $HgCl_2$ are qualitatively similar regardless of types of stimulants, such as other kinds of amino acids, food extracts, and hand

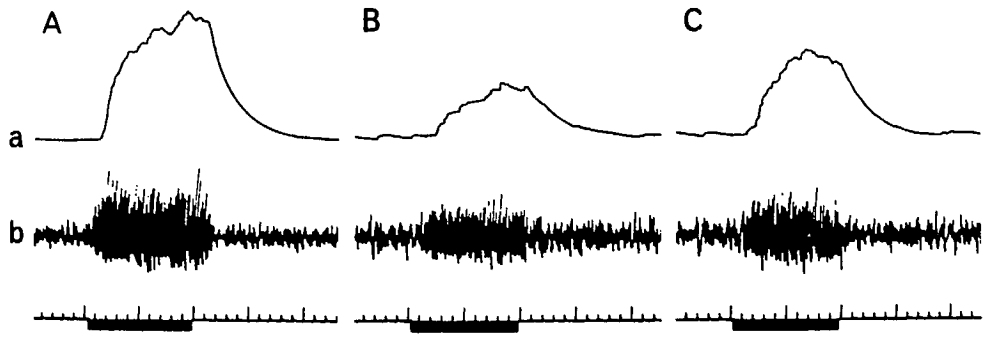

Figure 2. Electrical responses recorded from the olfactory bulb of the same fish when the nares were stimulated with 10^{-5} mol/L L-serine before (A), after exposure to 0.25 mg/L $HgCl_2$ (B), and 1 h after rinsing (C). The upper tracing (a) of each pair is the integrated responses of the lower (b). Duration of stimulation is indicated by heavy lines below each record. From Hara et al. (1976).

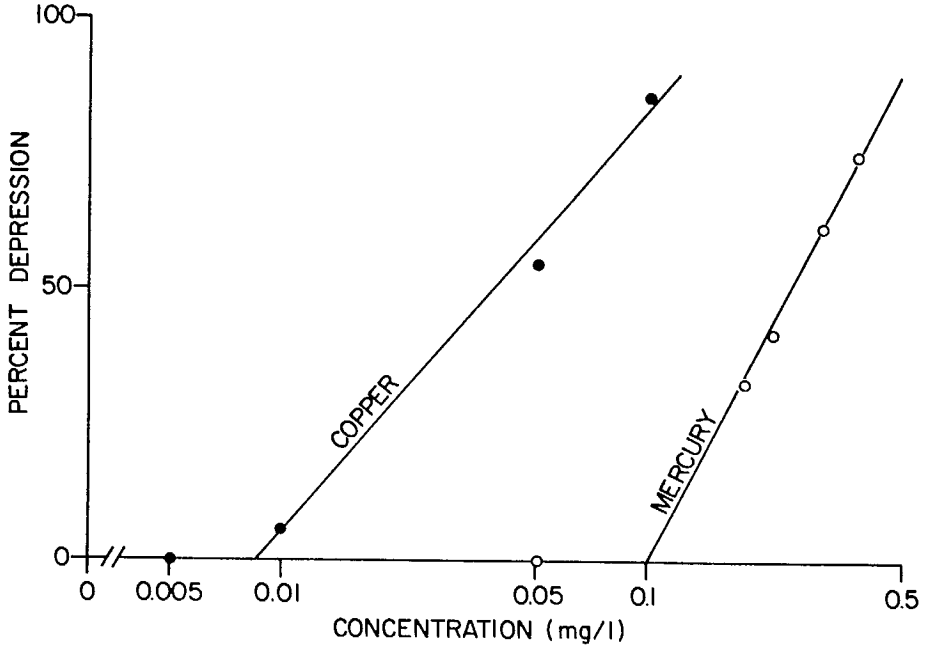

Figure 3. Relationships between percent depression and concentrations of $HgCl_2$ and $CuSO_4$. From Hara et al. (1976).

rinse. The threshold concentration of toxicant required to cause minimal depression of the bulbar response is determined from a concentration-percent depression relationship (Fig. 3).

EXAMPLES OF APPLICATION

Effects of $HgCl_2$ and $CuSO_4$ on the olfactory response were determined in rainbow trout (Salmo gairdneri). The lowest concentration of mercury and copper needed to cause minimal effects within 2 h were estimated at 0.10 and 0.008 mg/L, respectively (Hara et al. 1976).

The toxicity of natural lake waters contaminated with heavy metals from mining effluents was studied in Arctic char (Salvelinus alpinus). Chemical analysis of lake waters and single component experiments showed that Cu and Cd are probably most responsible for the depressive effect. The depressive effect of natural lake water was less than that of an artificial lake water containing heavy metals at natural lake concentrations (Thompson and Hara 1977).

An anionic detergent, sodium lauryl sulphate (SLS), at sublethal concentrations depressed the olfactory bulbar responses to odorants in whitefish (Coregonus clupeaformis). The lowest concentration of SLS to cause minimum effects within 15 min was 0.1 mg/L. Whitefish were attracted to this substance at the same concentration range, when tested in a avoidance/preference trough (Scherer and Nowak 1973; Scherer 1977; Hara and Thompson 1978; Scherer, this volume).

DISCUSSION

There are several ways in which toxicants might interact with

olfactory receptors, thus resulting in reduction of sensitivity, i.e. elevation of the threshold for detecting odorous chemicals in water. Most pertinent is probably cell impairment due to membrane dysfunction caused by binding or coupling between the toxicants and the receptors. Persistent binding of a pollutant to receptor sites might cause irreversible damage resulting in death of functional chemoreceptor cells. A second possible effect of aquatic toxicants is the masking of biologically important odors. If toxicants compete with naturally occurring molecules for receptor sites on chemoreceptor membranes, this might also result in reduced sensitivity of the animal to important biological information either by elevating the threshold or altering the quality of natural odors. In such a case, however, the sensitivity usually returns to the original level due to sensory adaptation. For better interpretation of the data obtained from the electrophysiological test described, combination with histological, biochemical, and behavioral tests is recommended.

The lowest concentrations of mercury and copper to cause minimal effects within 2 h were estimated at 0.10 and 0.08 mg/L, respectively, for rainbow trout. These are approximately 3 and 5 times lower than their respective lethal levels.

The electrophysiological method described here is a sensitive, fast test on the function of one specific organ, but can be applied to various toxicants and fish organs.

Species tested in my laboratory so far are Salmo gairdneri, Coregonus clupeaformis and Salvelinus alpinus.

COSTS AND STAFF REQUIREMENTS

Oscilloscope	$3000
Polygraph	$4000
Stereo microscope	$2000
Micromanipulator	$ 200
Misc. glassware, tubing etc.	$ 500

Tests can be carried out by technical staff after 2-4 weeks of basic training in electrophysiological techniques; professional supervision desirable. One test requires about 4 man-hours, i.e. testing of a chemical at five concentrations with one replicate each takes about 5 man-days.

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THE USE OF GROWTH RATE FOR TESTING TOXICANT EFFECTS ON ALGAE

by F.P. Healey

ABSTRACT

The measurement of growth rate in batch cultures at low cell concentrations offers many advantages over other methods for the routine testing of the toxicity of potential pollutants. Because of its simplicity, the use of batch cultures allows greatest replication with least investment of time and expense. Since growth rate is a summation of cellular metabolism it will reflect effects on a wide variety of cellular processes. Changes in growth rate in turn have profound effects on species composition and primary productivity in natural situations. Since measurement of growth rate extends over a period of many days, it has the potential of revealing acclimation. Measurement of short-term responses (such as photosynthetic and nutrient uptake rates, motility and flotation) and the use of continuous cultures are helpful in the study of some special aspects of toxicity.

RATIONALE

Obtaining some estimate of effects on primary producers is an important part of testing the potential influence of toxicants on aquatic systems, since changes in rates and species composition at this level can have profound effects throughout the aquatic food chain. A number of approaches could be used to test toxicant effects on microscopic algae, which are a major component of aquatic primary production. These include measurement of effects on rates of growth, photosynthesis,

and nutrient uptake, on motility and flotation, and on competition between species. Of these possibilities, measurement of growth rate at low cell concentrations in batch cultures has many features attractive for routine toxicity testing. Growth rate represents a summation of all metabolic reactions. An effect on any essential reaction should be reflected in the growth rate, unless the organisms can compensate by increasing the concentration of an affected enzyme to offset a decrease in its specific activity. Use of growth rate as an indicator of toxicity offers both a sensitive general indicator of a toxic effect and a measurement of effects on an important determinant of species competition. The measurement is simple, inexpensive, rapidly performed (allowing greater replication) and, if properly done, should not be subject to artifacts.

METHOD

(i) Species.

A wide variety of microscopic algae are available in culture or can be readily isolated from natural waters. Some thought should be given to the choice of strains before work begins. If results are to be of general application, strains should be selected to include representatives from the major algal classes (green and blue-green algae, diatoms, etc.) or from diverse ecological origins (marine, freshwater, eutrophic and oligotrophic waters). A wide variety of algal cultures are available at nominal cost from the Culture Collection of Algae at the University of Texas (Starr 1978). Where application of results to a particular area is to be emphasized, isolates from the waters in question may be

preferred, but where waters are polluted, the possibility of acclimation should be kept in mind (Stokes et al. 1973; Jensen and Rystad 1974).

Detailed descriptions of the procedures involved in the isolation, culturing, and measurement of growth rates of microscopic algae can be found in manuals (Stein 1973). The following procedural considerations will be confined to aspects of special importance in toxicity testing.

Essentially, the test alga is inoculated into a number of containers of test medium including a series of concentrations of the toxicant. The preparation of the inoculum should be standardized in terms of conditions of growth and cell number reached. The experimental cultures can be as small as 5 mL in test tubes but would more usually be larger volumes in flasks. The rate of increase in cell biomass or number is followed with time and variations in this rate with increasing concentration of toxicant are used as a measure of toxicity. The choice of temperature and light source and intensity used during growth of the algal cultures will probably be determined by what is available. A temperature of 20°C (commonly found in summer epilimnion of temperate lakes) is a reasonable choice. Certain commercially available fluorescent lights offer spectral compositions resembling that of sunlight. An intensity of about 100 $\mu E/m^2/s$ (approximately 1.2 mW/cm^2) can be expected about 10 cm from a bank of four fluorescent lights (20 or 40 W each), and should result in near maximum growth rates of most algae.

(ii) Medium

Since medium composition can affect the responses of algal growth

rates to environmental factors (Maddux and Jones 1964), the composition of the medium should be as similar to that of the natural water of interest as feasible. This will involve the use of the minimum nutrient concentrations required to avoid nutrient limitation.

The containers, including the medium, and often the toxicant, should be autoclaved and allowed to stand overnight before inoculation. If there is a possibility that the toxicant may be heat labile, it can be sterilized by filtration or by treatment with ethylene oxide (see Chapter 12 in Stein 1973) before addition to the autoclaved medium. Because the growth rate measurement usually need not last more than a week, the toxicant could be added to the autoclaved medium without sterilization if necessary, but any dilutions before addition should be made with sterilized water and glassware. Chemical measurement of the total concentration of toxicant remaining in solution or suspension at the end of the growth period is needed to determine whether adsorption to container walls or breakdown of an organic toxicant has lessened the total concentration of toxicant presented to the alga.

Studies on copper toxicity strongly support free dissolved ions as the toxic species (Sunda and Guillard 1976; Anderson and Morel 1978; Sunda and Lewis 1978). Thus the relationship between total metal concentration and toxicity will depend on the extent to which the metal is complexed. As a result, rather than attempting to use media with minimal chelation and relating toxicity to total metal concentration, it is more realistic to add an appropriate chelator and relate toxicity to calculated dissolved species (Morel et al. 1978; Anderson and Morel 1978). This has the advantage of holding free metals constant at a known

concentration, in a manner analogous to use of a buffer to hold pH constant, and of offering greater insight into the mechanisms of metal toxicity. At present, this approach suffers from the disadvantage of giving results in terms of free ion concentration while what is generally measureable in natural waters are total metal concentrations. However, methods either of measuring free metals in solution or a labile fraction approximating this (Gachter et al. 1973; Ramamoorthy and Kushner 1975), or of calculating free metal concentrations from natural water composition (R. Wagemann, pers. comm.) offer hope that this difficulty can be overcome. Where results are to be applied only to a local water, that water or a medium of similar complexing ability can be used. A final alternative would be to use a medium of minimal complexing capacity (an unpolluted soft water or dilute medium without added chelators or buffers) on the assumption that metal toxicity would probably be lessened in harder and more polluted waters; that is, the results obtained would be the maximum expected toxicity for a given metal concentration.

(iii) Inoculum Size and Cell Concentration

The use of batch cultures can be criticized because of variations which occur with time as the culture grows. These include changes in medium composition, in cell physiology, and in the ratio of cell number to toxicant, all of which could potentially affect the outcome. These changes can be minimized or effectively eliminated by using low cell concentrations so that (following the lag period) exponential growth occurs throughout the experiment and the composition of the

medium is little affected. A lowering of apparent toxicity by production of chelators (Fogg and Westlake 1955) or by dilution of toxicant among a large number of cells, thus lowering the effect on individual cells (Steemann Nielsen and Wium-Andersen 1971), would be minimized by use of the lowest practicable cell concentrations. That is, at low cell concentration, the distribution of toxicant is primarily determined by the physical (type of container, surface to volume ratio, temperature) and chemical (medium composition and pH) conditions imposed by the researcher and minimally affected by the presence of the alga. This approach has been successfully used to study growth rates of algae at low nutrient concentrations, where an effect of the growing alga on its medium must be minimized (Tilman and Kilham 1976; Steemann Nielsen 1978). The use of low cell concentrations also allows the use of low nutrient concentrations recommended above.

(iv) Measurement of Growth Rate

Following inoculation, each culture should be sampled several times (usually at daily intervals) through the growth of the cultures to determine the length of any lag period, the rate of exponential growth, and the final yield (see Chapter 19 of Stein 1973). Measurement of growth rate is preferred to a single measurement of yield because various combinations of lag period and growth rate can result in the same yield. The use of low cell concentration precludes insensitive methods of measuring changes in biomass (such as optical density and dry weight). The use of extracted chlorophyll as an index of biomass is not recommended because of possible effects of toxicants on the chlorophyll/

biomass ratio. Cell counts, either under a microscope or by a particle counter (such as a Coulter counter) would be most generally useful but should be accompanied by observations on variation in cell size. Use of a particle counter is faster than counting under a microscope but requires a larger sample and generally a higher cell concentration. Counting may be done either directly on the sample or following a concentration step involving either simple centrifugation or a more complex procedure (Steemann Nielsen 1978). Some large cells and colonies can be counted directly under a dissecting microscope (Klaveness and Guillard 1975; Tilman and Kilham 1976), allowing growth rates to be measured at cell concentrations found in natural waters.

EXAMPLES OF APPLICATION

The use of growth rate measurements of algae in culture to determine the effects of potential toxicants is illustrated in several papers. Studies on metals include Steemann Nielsen and Wium-Andersen (1971), Sunda and Guillard (1976), Hart and Scaife (1977) and Morel *et al.* (1978), and on pesticides, Krishnakumari (1977) and Harding and Phillips (1978). In none of these studies does any attempt seem to have been made to minimize cell concentrations. Changes in cell number or optical density have been most commonly used to monitor growth rates; Rivkin (1979) provides a comparison of several methods of measuring growth rate in a study of the effects of lead on an alga.

DISCUSSION

Besides measurements of effects on growth rate in batch culture,

a number of other methods have been used to determine the effects of toxicants on algae. Each has a number of limitations for routine testing of toxicants when compared with the approach described above, but each also has a role in more detailed studies of toxicants.

(i) Continuous Cultures

Unlike batch cultures, continuous cultures, once in steady-state, provide a constant environment, eliminating the variations with time found in batch cultures and providing a relationship between a constant environment and growth rate. But this can be done only at increased expense and decreased opportunity for replication for a given amount of effort. Two types of continuous culture are used (Kubitschek 1970). In the turbidostat, medium input occurs to maintain the cell concentration constant and the alga grows at the maximum growth rate allowed by the light and temperature provided. At similar low cell concentrations, results should be the same as found in batch cultures, since both are the effect of a toxicant on the alga growing at its maximum growth rate in complete medium. In the chemostat, a constant dilution rate sets the growth rate at some fraction of the maximum growth rate, with the alga being limited by a specific nutrient deficiency. Use of a chemostat may underestimate the effect of a toxicant because an effect may not show up until enough of the toxicant is present to depress the growth rate below that set by the dilution rate, although lower concentrations may depress at higher dilution rates. Thus, selection of the dilution rate could potentially affect the outcome of the experiment. On the other hand, an alga may be more sensitive to toxicants in a

chemostat than when growing in complete medium because it is subjected to two stresses: nutrient deficiency and toxicant. Thus, results from a chemostat may be more ecologically meaningful since algae in nature are probably more often nutrient limited than not. Whether either of these effects is significant should be evaluated if a chemostat is to be used.

(ii) Photosynthetic Rate

Short-term responses to a toxicant can be easily and quickly measured as the effect on photosynthetic rate. However, the results may be misleading. The alga may adapt to the toxicant, in which case the effect may lessen with time (Steemann Nielsen and Wium-Andersen 1971; Stockner and Antia 1976). Alternatively, the toxicant may take some time to reach equilibrium with the medium used, giving too great an initial effect, followed by results which mimic adaptation (Anderson and Morel 1978). Finally, the toxicant may affect some process not required in short-term photosynthesis, in which case, the effect would increase with time beyond a short-term photosynthetic measurement (Erickson 1972).

Providing time to detect acclimation is one of the major advantages of growth tests over short-term measurements such as rates of photosynthesis. However, even where acclimation occurs, and long-term growth results differ from short-term responses, this does not mean that the short-term results are without ecological significance. Any inhibitory effect on certain species could have major effects on competition between species before acclimation could occur, and this in

turn could influence food chains.

(iii) Other Short-term Responses of Ecological Interest

The growth rate of an axenic culture in complete medium may be unaffected by a particular addition while the same addition to the same alga in nature might markedly affect its competitive position. Effects on nutrient uptake at low concentration, or on motility or flotation, could have profound effects on species competition in nature which could be overlooked in growth studies on individual cultures. Short-term studies of such specific responses would supplement studies on growth rates, as would studies on the effect of toxicants on competition between algae, either in simple systems (such as those used in studies of nutrient competition, Tilman and Kilham 1976) or in more complex systems (Gerhart et al. 1977; Thomas and Seibert 1977).

(iv) Bioaccumulation

Even in situations where a toxicant has no effect on algae, the algae may concentrate the toxicant from the water and thereby accelerate its entry into the food chain. Integration of results on algae with general studies of toxicology would be facilitated by measurements of bioaccumulation of toxicants by algae and their passage in particulate form to higher levels of the food chain (Davies 1974; Hart and Scaife 1977).

COSTS AND STAFF REQUIREMENTS

Autoclave (possibly replaceable by domestic pressure cooker)	$5000

Temperature and light control (incubator or controlled environment chamber)	$ 500-2000
Particle counter (optional)	$9000
Compound microscope	$2500
Misc. glassware (volumetric flasks, test tubes etc.)	$ 500

Testing can be carried out by technical staff trained in basic microbiological lab procedures. A standard growth rate test can be expected to run about 5 days, with most of the first day spent in preparation, and about 4 h on each subsequent day in microscope counts, reduced to 1 h per day if a particle counter is available.

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A MICROBIAL PREDATOR-PREY TEST SYSTEM

by S.G. Lawrence and M.H. Holoka

ABSTRACT

A predator (Tetrahymena vorax)-prey (Chlamydomonas reinhardtii) food chain is placed in a chemostat (continuous flow, chemically limited by nitrogen as NO_3, 20°C) such that the alga is generated in one vessel to a density of 10^6 cells/mL and fed to the protozoans in a second dark vessel. The protozoans are maintained at a density of 10^4 cells/mL. No chelators are added to the medium. The following parameters are measured before and after addition of a toxicant: inorganic nitrogen and toxicant concentrations throughout the system, population levels and cell weights.

RATIONALE

The use of a predator-prey system as a bioassay tool, integrating toxicity data obtainable from individual species, is a step toward assessing toxicant effects at the ecological level.

Various protozoa, among them Tetrahymena vorax Kidder, are known to feed on small green algae (Elliott 1973) such as Chlamydomonas reinhardtii Dang in nature. The two organisms then, represent a primary producer and a primary consumer known to form a part of a natural food chain. Taub and McKenzie (1973) and Lawrence and Holoka (in prep.) showed that these two organisms grow compatibly in a chemostat system.

METHOD

(i) Stock Cultures

a) Algae. Axenic C. reinhardtii cultures can be obtained from biological supply houses, from the culture collection of algae at the University of Texas (Starr 1978) or from the authors.

One hundred mL NC-2 medium (Table 1) is placed into 250 mL flasks and autoclaved. About 1 mL of a culture of algae is inoculated into the flask which is placed at 20°C in the light (150-200 $\mu E/m^2/s$). Depending on the age of the inoculum, this working culture will be ready for transfer to the chemostat in 2-5 d. Stock cultures of algae should be transferred routinely every 1-2 weeks to produce working cultures.

b) Protozoa. Axenic T. vorax can be obtained from the authors (see Lawrence et al., in prep.) or from the American Type Culture Collection (ATCC), Bethesda, MD.

1) Stock culture: Ten mL YEP medium (0.25% yeast extract, 1.5% proteose peptone in deionized or glass-distilled water) is placed into test tubes, capped and autoclaved. About 0.2 mL of a culture of T. vorax is inoculated into this medium and incubated in the dark at ~20°C. Depending on the age of the inoculum, the culture will be ready for transfer into an algal culture in 2-4 d. Stock cultures should be transferred to YEP medium routinely every week.

2) Monoxenic culture:

a. Preparation of working culture of algae, see above.

b. When algal working culture is 2-5 d old, transfer 1 mL protozoan culture (2-4 d old) into algae. Incubate at about 20°C in dark.

Table 1. Formulae for stock culture (non-chelated "NC-2") and chemostat medium (modified after Provosoli et al. 1970).

A. NC-2 medium

Ingredient	mmol/L	µg/L
KCl	1.34	
$MgSO_4.7H_2$	0.32	
KH_2PO_4	0.88	
K_2HPO_4	0.68	
$NaSiO_3.9H_2O$	0.141	
$NaNO_3$	0.584	
$CaCl_2.6H_2O$	0.1	
H_3BO_3	0.0005	
$FeSO_4$	0.002	
Thiamine-HCl		100
Ca-Panthothenate		225
B_{12}		1

B. Chemostat medium as above except $NaNO_3$ is 0.146 mmol/L and NaCl is 0.882 mmol/L (to replace Na). The medium is autoclaved in 16 L glass aspirator bottles. All attachments to other parts of the system are made aseptically.

Chemicals are added from stock solutions to distilled, deionized (Millipore "Super-Q" system) water, in order. The pH of the medium is adjusted with 0.1 mol/L HCl and 0.1 mol/L NaOH if necessary.

Table 2. Formula for Lugol's iodine.

Ingredient	gm
KI	10
I (resublimated)	5
Na acetate	5
Water	70

Dissolved KI in 20 mL water, add iodine, dissolve. Add 50 mL water and Na acetate, dissolve.

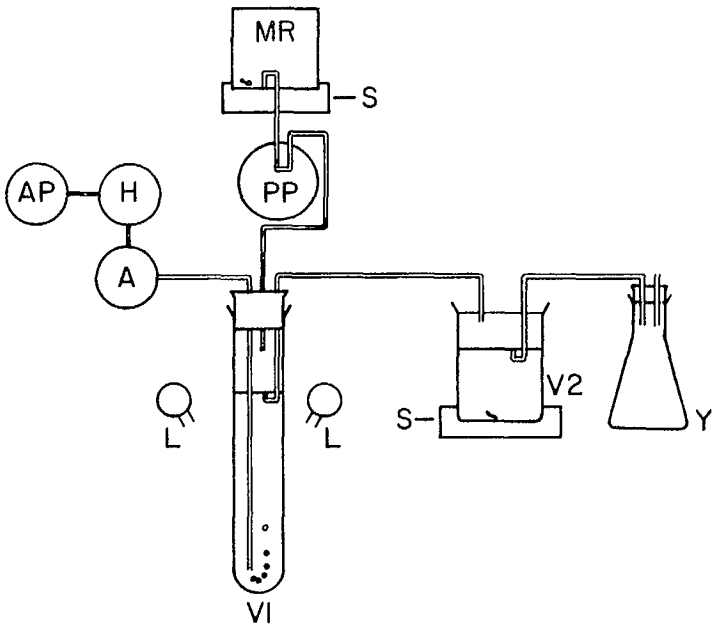

Figure 1. Two-stage chemostat apparatus, prey (algae) - predator (protozoa) system. A, acid air scrubber; AP, air pump; H, humidifier; L, light banks; MR, medium reservoir; PP, peristaltic pump; S, stirrer; V1, algal vessel; V2, protozoan vessel; Y, yield. The system contains only two members; sterilization and sterile technique ensure exclusion of bacteria and mold. MR, V1 and V2 are constantly stirred; V1 by means of the air supply entering at the bottom of the vessel, MR and V2 by means of magnetic stirrers. Algae are forced into V2 by means of the air pressure.

(ii) Chemostat

According to chemostat theory (Málek and Fenel 1966): if during the exponential growth phase of microorganisms, fresh medium is added at a rate sufficient to maintain the population at a fixed value and a like amount of spent medium and organisms removed, growth will not cease, but continue indefinitely. A chemostat is set up such that a culture of fixed volume receives medium at a constant rate. The medium is formulated such that all substances and conditions except one essential for growth (in this case: NO_3^-) are present in excess of growth requirements. The growth limiting nutrient is present at a concentration sufficient to support a given cell number or biomass. The growth rate depends upon the flow rate of the medium and on the dilution rate, i.e. the volume passing through the vessel per unit time. By controlling the dilution rate, the growth rate can be adjusted to a convenient value.

The chemostat (Fig. 1) is set up such that the algae vessel is continuously subjected to 22 $\mu E/m^2/s$ light which is furnished by daylight-simulating fluorescent bulbs, e.g. Vitalite Duro-Test®, set in banks of four. The medium reservoir is connected to the algal vessel only - all fluids are pumped from the algal to the protozoan vessel. The protozoan vessel is wrapped in black plastic to prevent light entry. The medium is detailed in Table 1 (B). Nitrogen as NO_3^- is the limiting nutrient. Operating temperature is 20 ± 1°C; pH is 6.7-6.9. Air, the source of CO_2 for algae and O_2 for the protozoa, is passed through acid to remove ammonia, and a filter to remove bacteria, and then bubbled through the algae culture. Since the protozoan vessel

receives air only indirectly via the algal vessel, the culture must be stirred rapidly to prevent O_2 limitation.

Medium is pumped into the algal vessel until it is about one-half full, and is inoculated with 3-5 mL of a working culture. The vessel is allowed to fill and overflow over 12-18 h at about half the final expected rate of flow into the protozoan vessel. At that point, 3-5 mL of protozoan monoxenic culture is inoculated into the protozoan vessel. After about 24 h, the dilution rate is adjusted to the desired value, in this case D = 0.056/h at 500 mL/vessel, working volume.

(iii) Determining Toxicant Effects

The following parameters are measured before and after toxicant addition:

a) Nitrogen concentrations: NO_2^-, NH_4^{+4}, and total dissolved nitrogen are determined as in Stainton et al. (1977), every second day of the test.

b) Toxicant: Toxicant concentrations are monitored in medium (filtered and unfiltered) and in protozoan and algal cells prior to addition, just after addition and every second day thereafter. Thirty-five mL algal culture and 45 mL protozoan culture samples are filtered onto 0.22 µm pore size Nucleopore® filters which are placed in test tubes or flat plastic containers until analyzed. Should immediate analysis not be possible, filters should be frozen.

c) Population numbers: From each vessel 3-5 mL of cells are withdrawn, killed with a few drops of Lugol's iodine (Table 2), suitably diluted, and placed in a Sedgewich-Rafter counter chamber. Cells are

counted daily.

1) Algae: Using a compound microscope, 24 fields (or more) are counted at 200x magnification. Suitable factors are applied in order to express the results as number/mL.

2) Protozoa: 48 fields at 125x magnification are counted and suitable factors applied to express results as number/mL.

At steady-state conditions, the cell numbers normally vary less than 10% from the mean.

d) Cell weights: 35 mL of algae and 45 mL protozoan cultures are withdrawn from the chemostat and passed through a 0.22 μm pre-weighed Nucleopore® filter which is dried to a reproducible weight at 60°C and reweighed. Weights/cell or weights/mL can be calculated by using cell counts made on the same sample.

Measurement of the four parameters as described above allows one to determine uptake and bioconcentration of toxicants, and their effect on survival and growth of T. vorax and C. reinhardtii as a predator-prey system.

EXAMPLES OF APPLICATION

Stokes (1975) has shown that algae in lakes contaminated with nickel and copper accumulate these metals. Silverberg (1976) has shown that algae grown in the laboratory accumulate cadmium in distinct vacuoles in the cytoplasm.

Several workers (Carter and Cameron 1973; Thrasher and Adams 1972; Tingle et al. 1973) have shown that growth parameters of protozoa are sensitive to heavy metals even in the presence of large concentrations

of organic material which would be expected to chelate metals.

The 96-h LC50 for washed cells of T. pyriformis grown in organic medium is 840 µg/L cadmium (Carter and Cameron 1973). The value for T. vorax is probably in the same range. We have done two types of tests using the chemostat system. In the first test the effects of a step addition of cadmium was examined. The system was allowed to come to normal steady-state; cadmium was then added in steps of 10, 20, and 40 µg/L with the system being allowed to come to a new steady-state after each addition. Under these conditions, the algae concentrated cadmium; the cell numbers and biomass dropped at each addition, but recovered to a new lower steady-state level. The protozoa reacted to 10 and 20 µg/L (two orders of magnitude lower than the 96-h LC50 for T. pyriformis) by concentrating cadmium at lower levels than the algae and by more marked changes in population numbers. However, new steady-state levels were established. The protozoa disappeared at 40 µg/L cadmium under all conditions. In the second test, after steady-state was attained, the system was exposed to 40 µg/L cadmium. Under these conditions both populations disappeared, the protozoa more quickly than the algae.

DISCUSSION

Information on accumulation of toxicants, acclimation of populations to various concentrations of toxicant, effects on cell numbers, biomass and morphology, and on predator-prey interrelationships is readily available from this system. Such information can be of use in planning or interpreting field experiments. For example, in field tests

in which low concentrations of cadmium were used, phytoplankton increased in biomass and crustacean zooplankton decreased, apparently because the zooplankton were directly affected by cadmium at low concentrations and were not available to graze upon the phytoplankton (Schindler, unpubl. data).

This system has been developed to test effects of non-volatile compounds; in order to test volatile compounds some arrangement would have to be made for trapping and analyzing compounds leaving the system.

This system could be adapted to any free living alga which does not form colonies, grow on surfaces or respond negatively to agitation, and which would nourish the predator of choice. The predator organism is limited under the conditons outlined to motile ciliated protozoa of small size known to consume actively the prey of choice (e.g. Tetrahymena, Colpoda and Paramecium sp.) and which have a growth rate near to that of the prey organism.

COSTS AND STAFF REQUIREMENTS

Basic microbiological equipment including autoclave, constant temperature equipment, compound microscope, 5-place balance, glass still		$10,000
Chemostat equipment -	1) Peristaltic pump	$ 900
	2) Stirrers, magnetic (2)	$ 200
	3) Lights	$ 100
	4) Counting chamber	$ 25

Aseptic procedures are applied; basic training in microbiological techniques is necessary. Culture maintenance requires 2-4 h/week.

Tests running over 3-5 weeks require approximately 70-100 man-hours.

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TESTS FOR THE CRAYFISH Orconectes virilis

by S.L. Leonhard

ABSTRACT

The crayfish Orconectes virilis is proposed as a test species in laboratory and field situations. Techniques are described for collecting, holding and determining bioaccumulation, lethal and sublethal toxicity. The sublethal test identifies changes in the duration of the intermolt stage of the molt cycle as indicated by hemolymph calcium levels.

RATIONALE

Crayfish are omnivorous and key energy transformers between various trophic links (Momot et al. 1978). There is a global interest in crayfish culture for table use and as fish bait (Threinen 1958; Huner and Avault 1974). I chose Orconectes virilis (Hagen) as a test species widely distributed in northern North America. The following parameters proved useful to indicate exposure to toxicants: survival of the juveniles and/or adults, delay or failure to progress from intermolt to premolt, and bioaccumulation in various body tissues.

METHOD

(i) Collecting, Holding and Culturing

A complete description of collection, holding and culture methods is given in Leonhard (in prep.).

O. virilis inhabits the stony beds of larger rivers and streams of northern North America. Adults and young-of-the-year are collected

in dip nets or minnow traps. Up to 50-75 adults or 200-500 young may be transported in 20 L of collection-site water in plastic bags layered with local water plants or aquarium polyester filter wool. The bags are kept cool during transport (<20°C).

At the lab, individuals are marked by pleural clips, brands made with a finely-pointed soldering iron, indexed streamer tags, or a colored code painted on the carapace with nail enamel. Sex, reproductive stage, rostral-carapace length, rostral-telson length, weight and molt stage are recorded for each animal. The molting stages are identified following Stevenson (1975).

Animals sorted to size groups are placed in holding tanks (40 L glass aquaria or fibre-glass troughs, 0.5 x 3 x 0.25 m). From 1-50 animals are held in each aquarium and 50-300 in each trough with a flow rate of 200 L/min of dechlorinated water.

Pieces of P.V.C. pipe and mats of polyester filter wool provide suitable cover. Temperature and photoperiod are adjusted to simulate seasonally changing field conditions. The temperature is adjusted at a rate of 2°C/d to 10-12°C for winter conditions, and 18°C for summer-fall conditions. The photoperiod is established at 8L:16D for winter conditions and 16L:8D for summer-fall conditions. The animals are held in total darkness from mid-October to mid-February since they would be under ice-cover in the field for most of this time. Animals are not fed or disturbed during this "dark period". For the rest of the year, the crayfish are fed about 1% of their weight daily, e.g. sinking trout pellets. I found the following standard food preparation in extruded form most successful:

1) Standard food is prepared following the recipe for Daphnia, (Leonhard, this volume).
2) The filtrate is retained.
3) Enough commercial alginate food-binder is added (Keltrol® or Kelset®) to the filtrate to form a paste.
4) The paste is solidly packed into a 50 mL syringe.
5) Strands of paste are expelled onto tinfoil trays.
6) Strands are dried overnight at room temperature.
7) Strands are broken into 1 cm pieces.

This extruded standard food is stored under refrigeration.

(ii) Testing

a) Acute-lethal

1) For in situ mortality tests, crayfish at the same molt stage (from lab cultures) are placed in 0.6 cm mesh wire cages and anchored at test sites in streams, rivers or lakes. A suitable cage size for six adult crayfish is 20 x 15 x 10 cm. One cage from each site is removed at each sample period established, e.g. at 2, 4, 8, 16, 24 h exposure on day 1, and one daily for 30 d following. The sample individuals are placed in plastic bags cooled on ice, returned to the laboratory for inventory records, i.e. the number of deaths are recorded, and weights, lengths, etc. are noted. The criteria for death are: complete immobility, and no flexion of the abdomen upon forced extension. The animals are frozen for residue analysis to identify bioaccumulation at successive exposure intervals.

2) For laboratory tests in static or flow-through systems, juvenile or adult crayfish are used. In static tests, the volume of test solution is established by providing 1 mL/g crayfish/d in well-aerated covered containers. In flow-through tests, 90% replacement/ 10 h is sufficient. The crayfish will survive up to 10 d without

feeding if they have been selected from a well-fed laboratory population. Optimal physical-chemical conditions, monitored daily, are as follows: temperature 18°C; dissolved oxygen 5-9 mg/L; pH 7.2-7.4 calcium 70 mg/L (for Freshwater Institute, Winnipeg Manitoba "Rat River" culture; Leonhard, in prep.).

b) Sublethal

Intermolt period and hemolymph calcium: The intermolt phase of the exposed animals is compared with that of controls. There are cyclical changes in hemolymph calcium throughout the molt cycle, with normally higher levels during premolt than during intermolt, related to calcification needs for the new exoskeleton. Non-elevation of calcium levels as caused by toxicants indicates failure to molt.

Hemolymph samples are obtained as follows: the aerola area of the carapace is dried with cotton and pierced with a 20 G hypodermic needle. The hemolymph is drawn from the heart into a 5-25 µL micropipet. Samples for calcium determination may be preserved for up to three months at room temperature (20°C) when taken as follows: 5 µL of hemolymph are expelled into glass vials containing 2 mL 20% trichloroacetic acid. The protein precipitates (Adegboye et al. 1974). The supernatant is analyzed for calcium spectrophotometrically.

c) Bioaccumulation

After hemolymph samples have been taken, the crayfish can be immediately dissected for tissue samples, or frozen for later analysis. Tissues I found pertinent and practicable are: gill, liver, stomach, intestine, gonad, heart, tail muscle, hemolymph and carapace. The latter can be easily sampled by punching out small "discs" with a common paper punch.

EXAMPLES OF APPLICATION

Orconectes virilis has been tested for the toxic effects of low pH (Malley and Tinker, this volume), fenitrothion (Leonhard 1974, 1976; McLeese 1976), mercury (Hamilton 1972; Doyle et al. 1976), copper (Hubschman 1967), and industrial effluents (Maciorowski 1975).

Crayfish were tolerant of saline ground water in continuous flow-through bioassay tests. Only after 10 d exposure in undiluted effluent did 50% mortality occur (Giles et al. 1979).

In a field exposure to a single application of fenitrothion, there was no mortality in adult crayfish for the test period of 30 d post-treatment. Fenitrothion had declined from an initial concentration of 64 µg/L to trace levels of 0.1 µg/L in water and silt a few hours after treatment. Fenitrothion levels in whole crayfish peaked at 1.37 µg/g at 19 d after application and persisted at lower levels for 30 d (Leonhard 1974).

Crayfish were exposed to various levels of cadmium (3-3000 µg/L) in the (accidental) presence of 135 µg/L copper during a long-term static bioassay. Initially, all of the crayfish were C_4 stage (intermolt). The hemolymph calcium concentrations were 0.75 ± 0.1 mmol/L. The control animals (exposed to neither heavy metal contamination) molted within a 10 d period. The hemolymph calcium levels in these crayfish changed cyclically from intermolt values of 0.75 mmol/L to premolt values of 12.5 mmol/L as the animals progressed to molt. The toxicant exposed crayfish did not molt within 100 d. The hemolymph calcium levels did not become elevated for this entire period (Leonhard, unpubl. data).

Cadmium concentration (mg/L) in each tissue (liver, gill, gut, tail,

muscle), expressed as a function of time, for each test concentration, showed that the final concentration of cadmium in the tissue was proportional to the external environmental concentration. The bioconcentration did not continually increase with prolonged exposure. Liver and gill tissue accumulated more cadmium than stomach and intestine. The tail muscle accumulated least.

DISCUSSION

O. virilis has been used in all of its life stages for acute and chronic bioassays to determine the degree of toxicity of a variety of compounds.

One particular drawback in using death as an endpoint with these animals is that a certain amount of handling is necessary to determine (and thereby influences) this "response".

For assaying bioaccumulation, crayfish are useful since the relatively large size of this invertebrate ensures that there is enough wet weight of a tissue from a single specimen to determine even µg/L residue levels. This permits the investigation of localization of effects and mode of action of toxicants as well as determination of persistence of a compound within the crayfish and its consequent availability within the food chain.

The sublethal test proposed determines changes in the duration of the intermolt stage. In the case mentioned, the animals remained in intermolt for 100 d. At that point the test was terminated. It is not known if the animals would have molted subsequently. The control animals did molt within 10 d. The observed extension of the

intermolt is likely to adversely affect the population. Crayfish must molt in order to grow. Moreover, if the males do not molt to the sexually mature form, they cannot copulate. If there should be a recovery and return to the molting stage this would still adversely affect the animals in nature, since it would put them out of phase with their environment. External factors such as water temperature and light conditions determine the onset of molt and exposed populations would have missed these seasonal triggers.

COSTS AND STAFF REQUIREMENTS

Collecting, holding and culturing: traps, nets, coolers, tanks, light and temperature control	$3000
Testing: spectrophotometer (hemolymph calcium)	$3000
cages (*in situ*), tanks (lab), temperature and light control, volumetric flasks, pipettes, dissecting kit, misc. glassware and plastics	$2500

Professional training, or supervision for testing, including spectrophotometric work is desirable. For maintenance of animals and tanks approximately 1 man-day per week is required, and 1 d per month for media and food preparation. Ongoing tests on mortality, molting, hemolymph and bioaccumulation, excluding data analysis, will require about 3 d per week of one person.

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EFFECTS ON SURVIVAL, GROWTH AND REPRODUCTION OF *Daphnia magna*

by S.L. Leonhard

ABSTRACT

Techniques are described for culturing *Daphnia magna* to produce a laboratory stock of uniform age and history. A reconstituted medium is used for both control and diluent. The daphnids are fed a standard food during culture and testing. Survival, growth (molting) and reproduction are used to evaluate toxicants in both static and flow-through testing systems.

RATIONALE

Daphnids have been used as pollution indicators for decades. Their advantages as a test organism are: small size, ease of culture, maintenance and testing, relatively short life span, uniformity of cultures produced through parthenogenesis and sensitivity to pollutants (Anderson 1944). Westlake *et al.* (1978) concluded that tests with daphnids were the easiest and most sensitive in a series of sublethal experiments on trout, flagfish, and daphnids exposed to oil refinery effluent.

Mortality (defined as a cessation of gut contraction and heart beat) is one effect of toxic action.

A more sensitive determination of toxicity is reproductive impairment. Several life history events can be monitored in a uniform age-classed culture generated from parthenogenetic stock of *Daphnia magna* Straus: the age of the female at first clutch, the size of and interval

between broods (Marshall 1978), the total number of clutches per female and/or total young produced. The short life span of these organisms makes them very suitable for such generation studies. A further test is the determination of the number, frequency and success of molts from juvenile to adult stage.

METHOD

(i) Collection

Animals are collected in a 500 µm mesh dip net and transferred to a 3-5 L Nalgene® container half-filled with water from the collection site. Daphnids should be transported at densities $\leq$ 500/L and water temperatures $\leq$ 25°C. The animals should be sorted from the sample within 6-8 h as follows.

(ii) Washing and Separation

The sample, diluted 1:5 using dechlorinated water or reconstituted medium (Table 1) as a diluent at sample temperature, is placed into a 25 L aquarium, aerated with an airstone. The water is allowed to reach 20-22°C over 24 h. The sample is sieved through phytoplankton nets (230-950 µm mesh size) until the Daphnia adults are concentrated. The contents of the net are flushed using sterile diluent, into a 100 mL capacity glass dish containing diluent. Animals are sorted into size categories by removing them with an eye dropper under low magnification of a stereomicroscope.

Table 1. Procedure for preparation of reconstituted medium.

1) Prepare the following 7 stock solutions:

   Add each weight of chemical to 250 mL deionized water.

1.	KCl	12.5 g
2.	$MgSO_4.7H_2O$	10.0 g
3.	K_2HPO_4	1.5 g
4.	KH_2PO_4	1.5 g
5.	Na_2SiO_3	5.0 g
6.	$NaNO_3$	12.5 g
7.	$CaCl_2.6H_2O$	5.48 g

2) Add 1 mL of stock #1 through 6 for each litre of deionized water in the final medium.

3) Reduce pH to 6.65 with 1.2 mol/L HCl, i.e. acidify.

4) Add #7 stock solution (7 mL/L).

5) Confirm pH (it must be < pH 7.0 to avoid precipitation of the calcium).

6) Autoclave to sterilize.

7) Adjust pH (maintain pH < 7.0).

(iii) Mass Culture Initiation

A 25 L aquarium is filled with reconstituted medium as described, aerated gently at a rate of $\simeq$ 200 mL/min with an airstone. Standard food (Table 2) is added at a rate of 0.1 mL/25 animals and the pH adjusted to 7.2-8.0. Two adult parthenogenetic females per litre are added and maintained at a 12L:12D photoperiod.

(iv) Culture Maintenance

The culture is fed every second day (0.1 mL standard food for each 25 animals). The population is maintained at 100/L by culling excess

Table 2. Standard food.

Components are:

1) Algal media - Use either a) or b).

a) FW6

Stocks are all 100 mmol/L	mL/L stock
$NaNO_3$	30
K_2HPO_4	3
$MgSO_4$	4
$CaCl_2.6H_2O$	2
$NaHCO_3$	10
Distilled water	950

Sterilize by autoclave.

b) Bristol's medium

Stock solutions	g/400 mL
$NaNO_3$	10
$CaCl_2$	1
$MgSO_4.7H_2O$	3
K_2HPO_4	3
KH_2PO_4	7
NaCl	1

10 µL of each stock solution are added to 950 mL of double distilled water. To this is added a drop of 1% $FeCl_3$ solution.

2) 10 g trout crumbles, ground fine in a blender (e.g. Victor Fox TF crumbles or #6 ration, Purina).

3) 0.5 g Cerophyl® powder (Source: Cerophyl Laboratories, Kansas City, MO, U.S.A.)

Preparation:

Mix trout crumbles and cerophyl. Add to 150 mL algal media and blend thoroughly. Strain through cheesecloth three times. Store in refrigerator in sterile dilution bottles or sterile serum vials. Shake before using. Must be remade monthly.

daphnids with a net. Evaporation losses are replaced with distilled water weekly. Bimonthly, the water should be siphoned to a depth of 20 mm, debris removed and the volume replaced with fresh reconstituted medium.

(v) Production Schedule of FWI Culture

The strains cultured at the Freshwater Institute were collected from a local sewage lagoon and a "pot hole" lake in the Erickson, Manitoba area.

First clutch occurs 10 (S.D. ± 2) days after hatching. Second and succeeding clutches occur every 2-3 d. Each female has an average of 10 (S.D. ± 2) and a range of 3-36 eggs per clutch, and reproduces an average of 11 (S.D. ± 1) times in her 48 (S.D. ± 9) d life cycle at 20-22°C. (Leonhard and Lawrence, in prep.).

(vi) Cultures for Toxicity Tests

The reconstituted medium is allowed to reach 20°C. Twenty mL are placed into each of 25-30 glass vials (25 x 100 mm) set in a tray. A portion of a reproducing population from mass culture is placed into a glass petri dish. An egg-bearing female is transferred into each vial using a pasteur pipette. On alternate days 0.01 mL standard food is added. Water is changed prior to feeding. The vials are examined twice daily for young. The young are removed and placed singly into vials, or young-of-the-day are placed into a common 600-1000 mL beaker 1/2-3/4 full of reconstituted medium. The time of hatching is recorded. These young are raised to adulthood at a density of 100 daphnids/L and used as brood stock of known age and history for toxicity tests.

(vii) Toxicity Tests

a) Acute-lethal toxicity: The static test does not require replacement of medium and is conducted in constant room light intensity at 20 ± 3°C. Animals are cultured in reconstituted medium. Tests are run in 100 mL pyrex or Dispo® (non-toxic, disposable) plastic beakers pre-rinsed with medium. Four to 10 concentrations of toxicant are assayed. There are five replicates of each test concentration and five control beakers (containing reconstituted medium and daphnids). Five animals are inoculated into each beaker. Four-day old daphnids should be used. To facilitate simultaneous inoculations, five daphnids each are pipetted with culture medium into 5 mL Dispo® plastic beakers pre-rinsed with medium. At time zero, the beakers are decanted and the daphnids poured or washed with test dilution into the test containers. This procedure eliminates problems of trapping air under the carapace or careful inoculation under the medium surface and thus avoids "floaters".

The daphnids in each container are fed 5 µL standard food on alternate days. The animals are checked with a magnifying lens. The criterion for death is complete immobility with cessation of gut contractions and heart beat. Moribund appearing animals are removed with 1 mL of test solution and observed for 30 s in a clean 5 mL Dispo® beaker. Dead animals are discarded and live ones returned to their original test container. Cumulative deaths are recorded on a logarithmic time scale to provide data points for probit analysis. Alternately, the Spearman-Karber method (Hamilton et al. 1977) for estimating median lethal concentrations in toxicity bioassays may be used.

Similarly, daphnids may be used in flow-through dilutor bioassays. The 100 mL beaker test vessel is replaced with a "bioassay basket", constructed simply of a 100 mm long piece of glass cylinder the bottom of which has 950 µm mesh glued to it with silicone, and to which a hanger of galvanized wire has been attached. The "basket" is suspended in each dilutor cell so that the container is 1/2-3/4 full of solution and replacement is continuous as the cell drains. The daphnids are inoculated into the "baskets".

b) Reproductive Impairment: This is a long term (≏ 20 d) static bioassay with replacement. The fluid lost through evaporation must be replaced with double distilled water, or preferably, the medium is decanted on alternate days and replaced with fresh medium. The test vessels are not aerated, and are provided with constant 200 lx light and maintained at a temperature of 20 ± 1°C. Reconstituted medium is used for both the control and diluent. Four to 10 concentrations of test products are tested using 20 mL 2.5 cm x 10 cm glass vials. There are 4-10 (10 is preferable for statistical analysis) replicates of each concentration and controls (reconstituted medium with organisms). A single 24-h old daphnid is pipetted into each concentration and its development observed daily.

Molts are recorded and the exuviae preserved in sugar-formalin (4% formalin to which 40 g/L sucrose has been added; Haney and Hall 1973). The date of birth of broods and interval between broods is recorded. The female is pipetted into fresh media after delivering her clutch. The brood is counted and preserved in sugar-formalin for later measurements of individual length and scrutiny for teratogenesis.

The primiparous female is monitored for a further 10 d and her total number of clutches and size of broods are recorded. The total number of young produced in the 10-d period following first birth gives a measure of fertility. Reproductive impairment is expressed as a % reduction in the total number of young produced or a delay in brood delivery. The failure of a juvenile to molt successfully to a mature adult, and its subsequent death, is also recorded.

EXAMPLES OF APPLICATION

Anderson (1944) used this cladoceran to define toxicity thresholds of industrial waste components. Daphnids have been used since to: assay combinations of inorganic compounds (Freeman and Fowler 1953); define the toxicity threshold of sodium sulfonates (Freeman 1953); assay aquatic herbicides (Crosby and Tucker 1966); test carbamates (Parker *et al.* 1970); DDT (Crosby and Tucker 1971); various metals (Biesinger and Christensen 1972); polychlorinated biphenyls (Nebeker and Puglisi 1974); and more recently to evaluate the acute and chronic toxicity of copper (Winner and Farrell 1976; Andrew *et al.* 1977). Throughout this time numerous screening schemes have been proposed; e.g. Wollerman and Putnam (1955) used daphnids to evaluate the toxicity of herbicides; Anderson (1971) estimated the thresholds of toxicity of various heavy metals; Buikema *et al.* (1976) tested various concentrations of a reference toxicant mixture; Westlake *et al.* (1978) tested liquid oil refinery effluent.

In an application of the acute toxicity tests described in this paper, *Daphnia magna* were extremely sensitive to a saline groundwater

(SGW) effluent from the tar sands area in Alberta, Canada. All test organisms died within 0.5 h exposure to 15.8%, 21.1%, 25.0%, 28.2%, 37.5%, 50% and 100% SGW. (Giles et al. 1979).

The mortality test also has proven to be a reliable, rapid check on the efficiency of the FWI water dechlorination systems. Since daphnids succumb within hours to < 1 µg/L chlorine (Leonhard, unpubl. data), the acute toxicity test gives an indication of water quality for culture and experimental purposes. Such tests are paired with chemical analysis of chlorine levels. We have used daphnids as a biological indicator of the success of several water treatment systems (thiosulfate dechlorination, UV dechlorination, reverse osmosis, ozonation, sedimentation chemical treatments). The survival and reproductive success in the test solutions may be reliably compared to that in synthetic control medium. Controls raised in reconstituted medium consistently yield no mortalities and maintain a production schedule as described in the methods section.

DISCUSSION

The mortality tests provide an aide for defining test levels of the toxicant for the more sensitive growth and reproductive impairment tests. The problems of poor culture success and control survival outlined by Buikema et al. (1976) have been minimized by utilizing a reconstituted culture medium with sufficient calcium content to sustain molting, and regular addition of a standard food. The animals must be fed throughout extended tests if robust test organisms are to be expected.

The user is cautioned to define the life history of the stock culture

to be used in testing. The age-classes must be established and made uniform before evaluating number and frequency of broods, total number of young produced and growth and survival of these offspring. The starting point in these tests is critical. Broods produced after the first to third instar of the adult appear to be more toxicant resistant (Leonhard, unpubl. data). For the FWI stock culture of Daphnia magna maintained as described, the production is reliable and replicable in independent tests performed over a number of years. There continues to be 100% survival of controls in the reconstituted medium and uniform production of broods from generation to generation. Cultures established from different sources yield slightly different life history data in laboratory cultures maintained under identical conditions. Different species of Daphnia, as expected, exhibit variations in this brood production pattern, i.e. different release times, size of brood, frequency of broods, survival (Leonhard and Lawrence, in prep.).

The whole-life cycle approach with Daphnia is economical and provides a simple vehicle for generation studies or monitoring continuous-exposure effects or effects of pre-exposure to the toxicant at single or multiple individual life history stages.

COSTS AND STAFF REQUIREMENTS

Collection:	nets, bottles, cooler, thermometer	$ 30
Culture:	aquarium and fittings, transfer bulbs	$ 100
	Petri plates, vials, medium, standard food	
Testing:	misc. glassware, Dispo® beakers, etc.	$ 100
	formalin, sucrose	$ 10

Instrumentation:	microscope	$2000
	pH meter and probe	$ 600
	O_2 meter and probe	$ 400
	conductivity meter and probe	$ 600
	stir table and bars	$ 100

No professional background is required. About five days of training are required covering collection, culture maintenance, sterile techniques and instrumentation, and actual testing. For holding, one person will spend about 2 d per week on preparation of food and media and general maintenance. For testing(complete test run over 20 d following procedures as described) approximately 12 work-days will be required.

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THE USE OF THE ZEBRAFISH (Brachydanio rerio) IN WHOLE-LIFE-CYCLE TESTS

by W.R. Lillie, S.E. Harrison, W.A. Macdonald and J.F. Klaverkamp

ABSTRACT

The zebrafish (Brachydanio rerio) is a small egg-laying species which has a relatively short life cycle, is easily cultured in the laboratory, and is readily available. For use in a continuous-flow, whole-life-cycle test system, we developed or modified existing methods of egg collecting, counting, and hatching. In an application of these methods, experiments were conducted to determine the effect of acidification on egg production, hatchability and survival.

RATIONALE

Chronic exposure to toxic chemicals throughout stages of a fish's life history has been regarded as the best means of estimating long-term safe concentrations of toxicants for fish (Stephan and Mount 1973). For practical reasons, using fish capable of reproducing in aquaria and having shorter life cycles than our native species is advantageous.

The zebrafish Brachydanio rerio (Hamilton-Buchanan) is a small (approximately 5 cm long) egg-laying tropical cyprinid which has a life cycle of approximately 75 days (Eaton and Farley 1974), matures quickly, spawns readily and often, and is readily available from pet shops (Axelrod and Schultz 1955). Consequently, compared with native species, chronic tests performed with zebrafish require smaller, less

costly facilities and are shorter in duration thus reducing labor costs and decreasing the risk of mechanical failures or fish disease. The embryological development of the zebrafish was well defined by Hisaoka and Battle (1958), and Skidmore (1965) investigated the acute-lethal sensitivity of different life history stages to zinc sulphate. Laale (1977) provided an extensive literature review of the biology and use of zebrafish in research.

METHOD

Glass aquaria 76 x 30 x 30 cm (volume $\simeq$ 68 L) are used. They must be visually isolated from each other to prevent behavioural interactions between fish in different aquaria. A smaller, moveable spawning chamber 7 x 15 x 15 cm, with a screened bottom, is suspended in each aquarium to contain the breeding adults (Fig. 1). This chamber allows the eggs to drop to the bottom of the aquarium, and eliminates predation by the egg-eating adults. A sheet of glass or plastic, installed at an angle below the spawning chamber, deflects the eggs to one side of the aquarium for easy collection. A water-inflow rate of 100 mL/min or greater (a 90 percent replacement time of less than 24 h) is recommended.

Light is provided from fluorescent lights mounted directly above the aquaria (approximately 2000 lx as measured at the tank bottom). The 12-h L:12-h D photoperiod includes simulated dawn and dusk phases, by having the room lights on 1 h before and off 1 h after the aquaria lights.

Ten females and 15 males are held in each spawning chamber. To minimize injuries when sexing and distributing these fish it is necessary

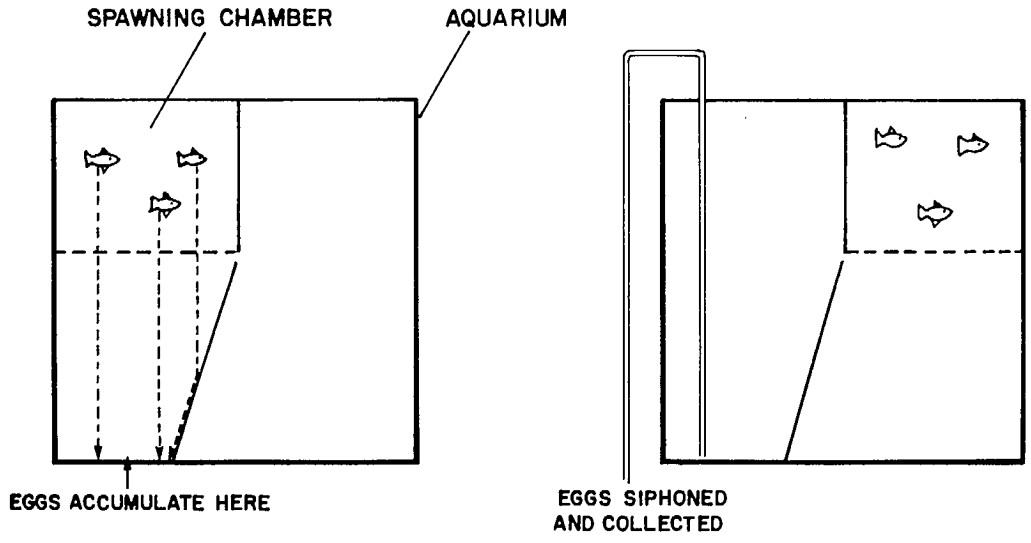

Figure 1. Test aquarium with spawning chamber in egg-laying and collecting positions.

to sedate them with 0.3 mg/L tricaine methane sulphonate (MS-222). Twenty-five adults are fed the following daily: 0.5 g Tetramin® staple food, 0.5 g Tetramin® growth food, and 2.0 mL Heinz® strained beef and liver baby food. Water temperatures between 24 and 26°C appear to be optimal (Niimi and LaHam 1974; Eaton and Farley 1974; Skidmore 1965).

Spawning usually takes place during morning hours, photoperiodicity apparently being the determining factor (Legault 1958). The eggs are collected by scraping the slanted divider (Fig. 1) and siphoning them out. Most of the excess water and debris can be removed by gentle screening. Eggs not used to determine hatchability can be preserved in 5% formaldehyde for counting with a photographic-electrical counter

technique described by Lillie et al. (1975). Briefly, the first step in this method is to photograph the eggs. Then the egg images on the photograph are pierced with a needle which contacts a metal plate below the photograph. This procedure simultaneously marks the photograph and activates an electrical counter, thus recording the number of eggs counted.

Egg hatchability is determined by placing 10 fertilized eggs into incubation chambers (approximately 20 mm diameter PVC pipe with a removable screen bottom). For fungus control, the eggs in these chambers are treated with 10 mg/L malachite green for 1 min, rinsed, then placed in the spawning chambers which are suspended from the side of the test aquaria. The number of embryos hatched after 96 h is used to determine the percent hatch.

EXAMPLES OF APPLICATION

A pH control apparatus described by Lillie and Klaverkamp (1977) has been used to determine the effects of acidification. The zebrafish spawned at pH 6.5 and 5.5 but not at pH 4.5. Fish held at pH 4.5 spawned immediately following an increase in pH, indicating that this effect is at least partially reversible. Mount (1973) noted that although fathead minnows were heavy with eggs, they did not spawn at pH 5.2. Beamish et al. (1975) also observed reduced spawning at pH 4.7 to 5.2 in field studies and postulated that this may be due to reduced serum calcium levels. In our experiments, pH 4.5 did not appear to have a detrimental effect on percent hatch or on adult survival (Lillie et al., unpubl. data).

Kihlström et al. (1971) studied the effect of phenylmercuric acetate on egg production and hatchability of zebrafish in whole-life-cycle tests. Egg production decreased in phenylmercuric acetate concentrations of 1 ng/g water or greater, while egg hatchability decreased in phenylmercuric acetate concentations as low as 0.2 ng/g water.

DISCUSSION

Our method requires refinement in two areas. Although our percent hatch was relatively high (approximately 70%) malachite green has been found to be carcinogenic (Leteux and Meyer 1972), and we recommend that other methods be investigated for fungal control.

Although our work was concerned primarily with egg production, percent hatch, and adult survival, preliminary feeding experiments indicate that the potential exists to complete full life-cycle testing within a period of 4 months. Although a combination of strained beef and liver baby food appears to be a relatively inexpensive, readily available substitute for the infusoria normally required by these fish, further research is necessary to arrive at an optimal diet.

Our acidification experiment (Lillie et al., unpubl. data) and those of Beamish et al. (1975) and Mount (1973) indicate that spawning is very susceptible to acid pollution. Consequently, lack of recruitment could deplete a fish population before mortality or other physiological symptoms were observed. It is therefore essential to assess spawning success when determining "safe" limits for environmental toxicants.

COSTS AND STAFF REQUIREMENTS

12 aquaria @ $25	$300
12 inner chambers @ $10	$120
12 heaters (if a temperature controlled room is not available)	$200
12 x 30 fish/aquaria @ $0.30	$108
Lights and timer	$100
Aquaria stand	$100
SLR camera	$300
Components of the electrical counter (Lillie et al. 1975)	$ 50
Miscellaneous (air stones, glue, pipe glassware)	$100

We estimate that 2 man-weeks to 1 man-month would be required to set up the test aquaria. After set up, approximately 4 man-hours/day would be required to maintain and run experiments. No professional training is required.

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PHYTOTOXICITY TESTS USING THE DUCKWEED Lemna minor

by W.L. Lockhart and A.P. Blouw

ABSTRACT

Axenic cultures of Lemna minor maintained on synthetic media are sensitive to a variety of herbicides. Bioassay procedures described by a number of authors have been adapted to detect phytotoxicity in water and sediment during laboratory and field exposures. Toxicity is expressed mainly as a reduction in asexual frond production, calculated from daily counts of fronds present over a 14-d period. For those herbicides that bleach fronds, percent chlorosis is a sensitive, non-destructive response recorded at each counting period.

RATIONALE

Intensive, mechanized agriculture practised in developed countries relies heavily upon chemicals for control of insect pests, plant diseases, and weeds. This has led to widespread contamination of watersheds with low levels of agricultural pesticides (Gummer, in press). The mode of transport of some of these materials includes an aerial component (Grover et al. 1976); therefore, a toxicity test based upon an organism belonging to the neuston is desirable. We selected Lemna minor because it occurs worldwide (Daubs 1965) and is convenient to culture. Several authors have found it to be a useful bioassay organism (Blackman and Robertson-Cuninghame 1955; Funderburk and Lawrence 1963; Parker 1964)

Responses we have monitored throughout exposure include frond number, and estimates of frond area, and percent chlorosis. After exposure,

wet and dry weights, and chlorophyll content are determined. The simplest responses to monitor are counts of frond number and estimates of chlorosis; both are non-destructive and can be repeated as frequently as desired.

METHOD

Field collected samples of Lemna minor are sterilized by exposure to either 10% hypochlorite or 50% ethanol (Hillman 1961a; Rombach 1976). Individual fronds are isolated in sterile media and clones are developed from them. Cultures are maintained in media prepared by diluting stock solutions of the following (Hillman 1961b):

H_3BO_3	28.6 mg/L	(100 µL)
$Ca(NO_3)_2.4H_2O$	1180 mg/L	(1 mL)
$Cu(SO_4).5H_2O$	8 mg/L	(10 µL)
$FeCl_3.6H_2O$	54 mg/L	(100 µL)
$MgSO_4.7H_2O$	492 mg/L	(1 µL)
KNO_3	100 mg/L	(15.2 mL)
KH_2PO_4	170 mg/L	(4 mL)
$Na_2MgO_4.2H_2O$	12 mg/L	(10 µL)
$ZnSO_4.7H_2O$	22 mg/L	(10 µL)
tartaric acid	30 mg/L	(100 µL)
$MnCl_2.4H_2O$	36.2 mg/L	(100 µL)

Quantities listed in parentheses are those required to make up 1 L of working culture medium with distilled water. All the components except $FeCl_3$ are added to distilled water prior to autoclave sterilization.

The $FeCl_3$ stock solution is autoclaved separately and the appropriate quantity transferred to the working media after cooling.

Cultures are maintained in 250 mL Erlenmeyer flasks with 100 mL of media in a controlled environment room at 25°C under plant growth lights (e.g. Sylvania Gro-Lux®) at a light intensity of about 60 μE/m^2/s. Photoperiod is maintained at 16 h light and 8 h dark.

For test exposures to herbicides, 10 fronds from a stock culture less than one month old, are selected for uniformity in size and color and are placed in a 125 mL Erlenmeyer flask with 50 mL sterile media supplemented with the desired concentration of herbicide. Normally five replicates are made for each test concentration, in the range of 10^{-5} to 10^{-9} mol/L, and an untreated control. Observations are made, usually daily, to determine frond numbers in each flask. Where herbicides cause visible chlorosis, the percent chlorosis is estimated subjectively at the same time. Results are expressed by plotting frond number against time over the 14-d observation period. The response curves at different concentrations can be compared with each other and with the control.

In tests of field persistence of herbicides applied to water, we use the natural water in place of distilled water to dilute stock media solutions. For sediment bioassays a quantity of sediment (ca. 20 g wet weight) is weighed and added (instead of dissolved herbicides) to working media (50 mL). The same procedure, as outlined above, is then followed.

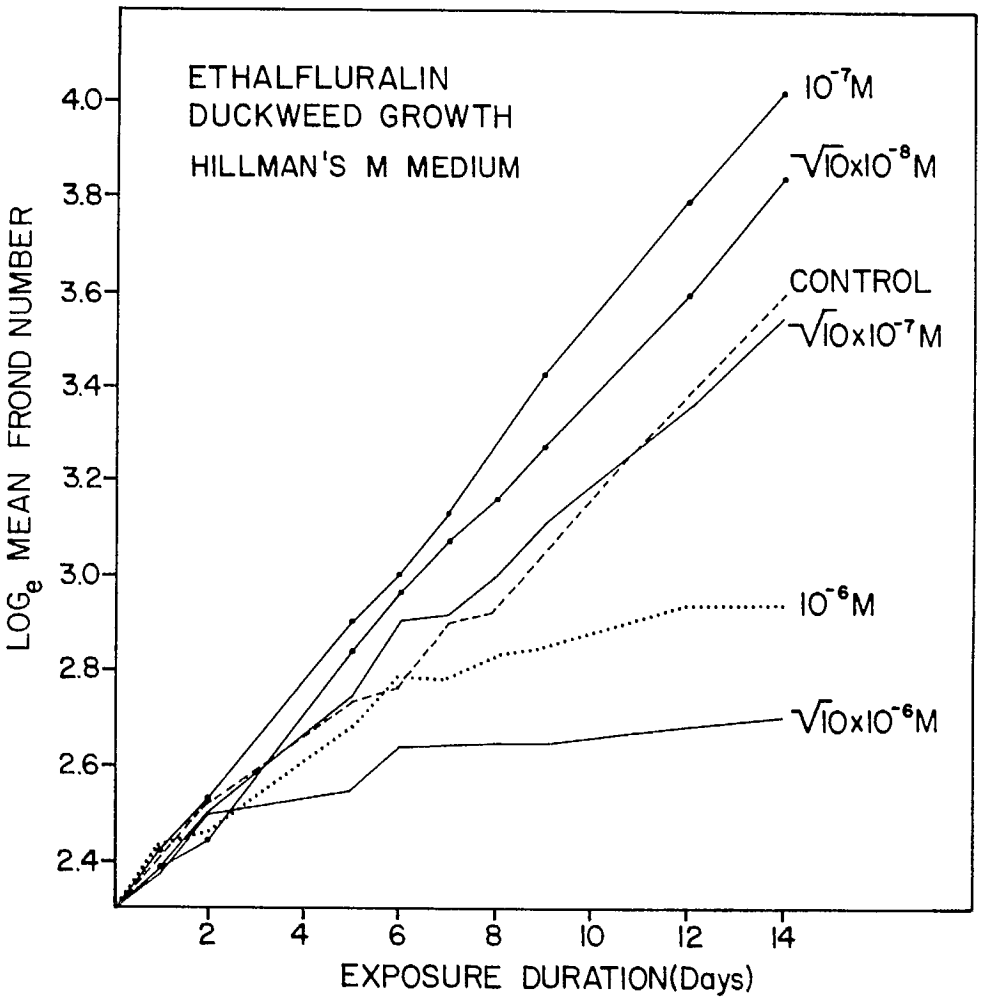

Figure 1. Numbers of fronds produced in various concentrations of the experimental denitroaniline herbicide ethalfluralin, as compared with numbers of tronds produced over the same time period in culture medium with no herbicide treatment (control). Each data point is the mean number of fronds in five replicate flask cultures starting with ten fronds per flask.

EXAMPLES OF APPLICATION

We have applied these procedures to several currently available and experimental herbicides, i.e. paraquat, diuron, diclofop-methyl, 2,4-D acid, fluridone, terbutryn, ethalfluralin (see Fig. 1) and glyphosate. Maximum sensitivity has been to terbutryn, using the frond number response and to fluridone, using the percent chlorosis response (Lockhart *et al.*, unpubl. data). Glyphosate was not toxic to *Lemna minor* when presented dissolved in media. Using the same techniques we have employed radioactively labelled pesticides and other small molecules to estimate the rates of accumulation by *Lemna*.

DISCUSSION

The techniques described here are useful. However, they are being altered in attempts to improve them. For example, the pH of the growth medium described is below 4.5 and we are trying to compare results using media nearer neutral or alkaline for use with compounds with ionizable groups titrating near 4.5, and for compounds susceptible to acid hydrolysis.

The tests are convenient, inexpensive, and there seems to be no reason to confine them to herbicides. They seem particularly well suited to questions of multiple toxicity.

COSTS AND STAFF REQUIREMENTS

Microscope	$2500
Autoclave	$2000
Analytical balance	$2500

Ultrasonic bath	$ 400
Misc. glassware (volumetric flasks, pipettes, etc.)	$ 600

We use commercially available plant growth chambers (starting price about $6000); less elaborate facilities for light and temperature control might be satisfactory.

No professional training is required; however, personnel should be familiar with maintaining sterile conditions. It requires about 0.5 d to prepare a test with 30 flasks, and about 0.5 h per day for maintenance and counting for the duration of the test (usually 14 d).

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BIOCHEMICAL TESTS FOR FISH

by W.L. Lockhart and D.A. Metner

ABSTRACT

Measurements of a number of biochemical responses are used as non-specific indicators of sublethal toxicity. Generally, measurements are carried out on fish exposed to a toxicant and results are compared with those from untreated fish. Methods to determine changes in the following parameters are described: serum glucose, serum protein, total serum lipid, serum and brain cholinesterase. We have used these and other biochemical responses to distinguish between treated and untreated fish following exposures to pesticides, industrial chemicals and fish diseases.

RATIONALE

Biochemical responses provide information of two general kinds, namely, information on the mode of toxic action in cases of exposures to known chemicals, and information that some state of impairment exists in cases of unknown but suspected exposures. A useful approach is to measure a number of biochemical responses (Eisler 1967) on each individual fish, since no single response is always reliable.

A statistically significant ($p \leq 0.05$) difference between mean assay values from treated and untreated fish indicates some response to the treatment.

METHOD

Fish handling prior to blood collection is kept to a minimum (even then, some sampling artifacts may not be avoidable). We use the same procedures on fish trapped wild, as we use in the lab. Since we find anesthetics inconvenient in our field context they are rarely used in either situation. Fish are removed from the water and blood is collected immediately for tests requiring whole blood or serum. Blood is collected through a needle inserted either into caudal vessels through the vental surface beside the anal fin or directly through the ventral body wall into the heart. Blood is drawn gently into a syringe, then the needle is withdrawn and removed from the syringe; blood is expelled into a glass or plastic tube by directing a gentle flow down the side of the tube. Clotting is allowed to proceed for 1-4 h at 0°C (ice bath). Following centrifugation, serum is decanted for analyses.

If brain cholinesterase is to be analysed, the fish is killed with a sharp blow, or by an incision, posterior to the brain, and the brain dissected. If analyses can be done within 24 h, the brain is kept chilled on ice, otherwise it is frozen.

Reagents for most tests can be purchased from a number of suppliers in kit form and we have found them convenient. These prepackaged reagents are intended for use in medical laboratories, and they are based upon standard chemical procedures available in the literature. We have found the test kits prepared by Boehringer Mannheim Corp. to be convenient and readily scaled down to sample volumes available from fish. As quality control samples we use lyophillized human sera prepared either by Boehringer Mannheim or by Hyland.

Many tests require readings of color development after some period of reaction time. Any good spectrophotometer is suitable for this, but those offering multiple sample capabilities are particularly convenient. We use a Zeiss PMQ II equipped with six microcuvettes, an automatic sample changer, and a multipoint recorder.

(i) Serum Glucose (glucose oxidase method)

a) Reagents: Boehringer Mannheim Blood Sugar Test Combination GOD-Period-Method

b) Procedure

1) Deproteinize serum by adding 100 µL uranyl acetate (Cat. No. 15908, Boehringer Mannheim Corp.) and 10 µL serum, vortex mix; centrifuge in 6 x 50 mm test tube.

2) Add 10 µL deproteinized serum supernatant and 500 µL test kit solution No. 2 to 10 x 75 mm test tube; vortex mix; allow to stand 25-50 min at room temperature; read absorbance at 420 nm vs distilled water blank.

3) Repeat 1) and 2) with standard glucose solutions and with a reagent blank containing no glucose. Convenient stable glucose standard solutions are supplied with most kits, or they can be purchased separately or prepared in the laboratory. Glucose in fish serum is calculated from color development with standard solutions, after corrections for any blank absorbance and volume change during deproteinization. Results are expressed as mg glucose per 100 mL of serum.

Note: Fish serum contains unidentified reducing materials (Moule and Nace 1963) and the degree of interference, if any, by these materials in applying the Boehringer reagents to fish serum has not been investigated.

(ii) Serum Protein

a) Reagents: Boehringer Mannheim Test; Total Protein - Biuret Method

b) Procedure

1) Add 10 µL serum to 500 µL solution No. 1 (Boehringer Kit); mix and allow to stand 30 min at room temperature; read absorbance at 545 nm against solution No. 1.

2) Add 10 µL serum to 500 µL solution No. 2 (Boehringer Kit); mix and allow to stand 30 min at room temperature; read absorbance at 545 nm against water.

3) Repeat step 1) with the standard protein solution applied with the kit and calculate serum protein concentration by comparing absorbance from step 1), minus absorbance from step 2), with absorbance for the protein standard. Results are expressed in grams of protein/100 mL of serum.

(iii) Total Serum Lipid

a) Reagents: Boehringer-Mannheim Corp. total lipids test combination - colorimetric method

b) Procedure

1) Into a clean dry 10 x 75 mm test tube, pipet 80 µL reagent grade sulphuric acid and 2 µL serum and mix thoroughly on a vortex mixer.

2) Cap tubes with marbles and incubate samples for exactly 10 min in a boiling water bath. Cool immediately afterwards in cold water.

3) Add 2.0 mL of color reagent (BMC solution No. 2, 14 mol/L phosphoric acid; 13 mmol/L vanilla) to each tube and mix thoroughly in a vortex mixer.

Allow samples to stand for 25-35 min at room temperature, then measure absorbance at 546 nm against air.

Standards consisting of triolein dissolved in chloroform (0-1600 mg/dL) and a reagent blank (2 µL H_2O instead of serum) should be run in an identical manner. Total lipid content is calculated from color development of samples as compared to standards after both are corrected for reagent blank. Results are expressed as mg lipid/100 mL of serum.

Note: Reagents used in this test are both highly corrosive and highly viscous; extra care is necessary when handling and mixing.

(iv) Brain Cholinesterase Extraction Procedure

1) Dissect whole fish brain, weigh, and place in a suitable tube. Add 1 mL of chilled (0°C) phosphate buffer pH 7.2, 0.1 mol/L in the case of large brains (ca. > 100 mg) add approximately 1 mL of buffer for every 20 mg of brain.

2) Homogenize brains using Polytron® homogenizer (speed 7, 30 s) keeping sample tube immersed in ice bath to prevent warming.

3) Centrifuge homogenate at 22,000 x g for 20 min at 0-4°C (e.g. Sorval RC2-B). Transfer supernatant to clean test tube immersed in ice bath and analyse for cholinesterase activity and protein content as described in the following section (v).

Note: The above technique is for situations where the enzyme has experienced no inhibition or irreversible inhibition, e.g. organophosphate poisoning. In this case samples can be done in batch lots since waiting periods of up to two hours do not affect the results. In cases of reversible inhibition, e.g. carbamate poisoning (Winteringham and Fowler 1966), samples should be individually centrifuged and analysed immediately after homogenization. In these cases centrifuging at 5000 x g for 5 min is sufficient.

(v) Serum and Brain Cholinesterase - Assay Procedures

a) Reagents: Boehringer cholinesterase test combination. This test is based upon the procedure of Ellman et al. (1961) and uses acetylthiocholine as substrate.

b) Procedure

1) Into a cuvette maintained at 25°C pipet 300 µL of solution No. 1; 10 µL of serum (or supernatant of brain extract) and 10 µL of solution No. 2.

2) Mix and record absorbance change at 405 nm against air every 30 s for 3 min. From table of values supplied with the test kit the activity can be determined from the absorbance change. If automated equipment is available (PMQ II, automated sample changer, multipoint recorder) the absorbance change can be plotted and the enzyme activity related to the slope of the absorbance change. It is necessary to determine a reagent blank, using water as a sample, to correct for spontaneous substrate hydrolysis.

Serum enzyme activity is usually expressed in terms of milliunits/mL of serum, or as activity/mg of protein from the biuret assay. Brain tissue enzyme activity is expressed in terms of milliunits/mg of protein determined by the methods of Lowry et al. (1951).

EXAMPLES OF APPLICATION

Blood sugar has been one of the most studied components of fish blood, often in efforts to understand the comparative biochemistry of diabetes. Early reports indicated that glucose became elevated during asphyxia (Menton 1927), but more recent suggestions indicate a hormonally mediated response to "stress" rather than a direct response to hypoxia (see discussion by Chavin and Young 1970, and by Hattingh 1976).

Sampling procedures can induce changes in blood glucose (Wardle 1972); this is frequently the case with fish.

We have applied the glucose assay to fish from a number of experimental exposures to toxicants. The response observed can be either to elevate glucose concentrations or to depress them relative to untreated fish. For example, we have observed significant increases in blood sugar following an outbreak of a fish disease due to Chondrococcus columnaris. Mean serum glucose increased to 211 mg/dL from 74 mg/dL (unpubl. data). Conversely, we observed a depression in blood sugar concentrations in fish exposed to an insect growth regulator, methoprene. Mean serum glucose fell from 65 mg/dL to 34 mg/dL at a treatment level of 10 mg/L (Madder and Lockhart 1978).

We have applied the protein assay to fish from a number of experimental exposures and found it to be relatively insensitive. It is useful, however, in conjunction with other assays of serum enzymes. Serum enzymes are generally assayed to yield activity per volume of serum, and we find it somewhat more precise to use the protein measurement to express enzyme activity per weight of serum protein.

Bouck and Ball (1966) have drawn attention to the possibility that protein changes may be artifacts of sampling methods. Protein in rainbow trout serum was found to fall steadily in the interval between time of capture by seining or electroshocking and time of blood sampling.

We have less experience with applying the lipid assay than with others reported here. We noted decreased levels of lipid and protein in a small group of white suckers captured from a river several weeks after treatment with methoxychlor. These decreases may have been consistent

with starvation. Methoxychlor, in this instance, was found to cause large increases in invertebrate drift (Flannagan et al. 1979), and loss of invertebrates presumably could have limited fish food supplies.

Cholinesterase enzyme assays have been applied to detect sublethal poisoning after both experimental and operational applications of organophosphate and carbamate pesticides. Application of these assays to fish was described by Weiss (1958, 1959, 1961) and more recently by Coppage and co-authors (Holland, Coppage and Butler 1967; Coppage 1971, 1972; Coppage and Matthews 1974). Generally, the brain has been the organ analyzed, but serum has also been used (Hayama and Kuwabara 1962).

We have used cholinesterase analyses to monitor field applications of the organophosphorus insecticides fenitrothion and malathion, and the carbamate propoxur (Baygon®), using fish, birds, and honey bees as sources of enzyme preparations (Lockhart et al. 1973; Findlay et al. 1974; Lockhart et al. 1977; Lockhart, unpubl. data).

DISCUSSION

Ideally, differences in biochemical measurements should be related to doses of toxicants, but this is seldom reported. Biochemical testing of fish lacks the years of clinical records to assist in correlating the biochemistry to other indicators of poisoning or disease. The approach will be useful although somewhat empirical, until the metabolic bases for specific responses are understood. An additional statistical limitation is that fish seem more variable than mammals in their normal ranges, and hence, it is difficult to distinguish a pathological from a normal value.

COSTS AND STAFF REQUIREMENTS

A reliable spectrophotometer, a constant-temperature water bath, centrifuges, balances, and a homogenizer at a total minimum cost of about $12,000 are required in addition to standard laboratory glassware. Reagent kits cost less than $100 each and are sufficient to make 100-200 analyses if scaled down to volumes described here. Specialized micropipetting systems such as the Eppendorf® are convenient for these small volumes.

The analyst should be familiar with analytical chemistry, clinical chemistry and microtechniques. Time required for assay varies, depending upon the test being done. Most serum tests required only a few minutes after separation of serum. Tissues such as brain require dissection and homogenization, then extraction for variable periods with buffers. Buffer preparation is quite time consuming if prepared kits are not used. Still, time required per test will usually not exceed 30 min.

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TESTING EMBRYONIC AND LARVAL LIFE STAGES OF FISH

by W.A. Macdonald

ABSTRACT

Fish embryos and larvae are sensitive and convenient subjects for testing environmental contaminants. Death and a variety of sublethal responses including respiration rate, fin and body movements, heart-rate, response to light or touch, rates of development, growth, occurence of anatomical abnormalities, yolk utilization, chorion strength, the timing and duration of hatch, and the initiation of larval feeding behaviour can be used to detect toxic effects. Two simple test methods, one using rainbow trout and one using whitefish, have yielded satisfactory results with several toxicants.

RATIONALE

Early life stages of fish are sensitive indicators of environmental stress. Rosenthal and Alderdice (1976) indicate that gonadal tissue, early embryos, and larvae are particularly sensitive among marine species investigated. McKim (1977), in a review of partial and complete life cycle toxicity tests, recommends the use of fish embryo-larval tests to help in establishing water quality criteria. Responses used towards this end are:

(i) Acute-Lethal

The most commonly recorded response in fish embryo-larval tests is death. A variety of criteria have been taken to indicate mortality,

but the most common criterion is the loss of transparency. With appropriate lighting conditions, large numbers of eggs can be screened for mortalities using this criterion. In some tests cessation of heart beat or blood circulation, absence of respiratory, fin, or body movements, or lack of response to tactile stimulation can be useful criteria of death. Choice of a suitable response to represent lethality requires careful consideration of the species, the toxicant, and the test conditions.

(ii) Acute-Sublethal

Acute-sublethal responses also are recorded in some fish embryo-larval tests. Respiration rate and specific biochemical or histological tests may be applicable to particular toxicants. Late embryo and larval stages can be monitored for rates of fin and body movements, heart-rate, and response to light or touch.

(iii) Chronic-Sublethal

Chronic-sublethal responses can be monitored in long-term toxicity tests. Rates of development, growth, yolk utilization, chorion strength, the occurence of abnormalities, the timing and duration of hatch, and the initiation of larval feeding behaviour are indicators of toxicity, in addition to biochemical and histological tests relating to specific toxicants.

METHOD

Techniques I have found suitable for rainbow trout (Salmo gairdneri) and lake whitefish (Coregonus clupeaformis) are outlined below. Other

commonly tested freshwater species, with representative references giving methods, are: fathead minnow, Pimephales promelas (Mount 1968; Till 1977); brook trout, Salvelinus fontinalis (McKim and Benoit 1971; Trojnar 1977); walleye, Stizostedion vitreum vitreum (Siefert and Spoor 1974); zebrafish, Brachydanio rerio (Creaser 1934; Niimi and LaHam 1974).

(i) Petri Dish Method for Rainbow Trout

Newly fertilized rainbow trout eggs are obtained by stripping Freshwater Institute brood stock, or from commercial suppliers as later-stage "eyed" eggs. The eggs are held in a flow-through incubator (Heath Techna Corp., Washington) at 10°C. For toxicity testing, samples of ten eggs or larvae are transferred to petri dishes (90 mm I.D.) containing 40 mL of reconstituted water (Table 1). Temperature at 10°C is maintained in an incubator or controlled environment room. The water is renewed daily and clean dishes are used every second day. Larger dishes are recommended during and after hatch. Light intensity is kept low (2 to 20 lx, Kwain 1975). Toxicant exposures are conducted generally following the recommendations of Sprague (1969) for static bioassays.

This method is useful for acute tests using small numbers of eggs or larvae, and large numbers of treatments. Dissecting-microscope observations of heartrate, fin and body movements are possible.

(ii) Basket Method for Whitefish

In this method, which is more suitable for larger sample sizes and longer exposure times, the eggs or larvae are held in baskets (Fig.

Table 1. Formula for reconstituted soft water (Marking and Dawson 1973).

$NaHCO_3$	48	mg/L
$CaSO_4.2H_2O$	30	mg/L
$MgSO_4$	30	mg/L
KCl	2	mg/L

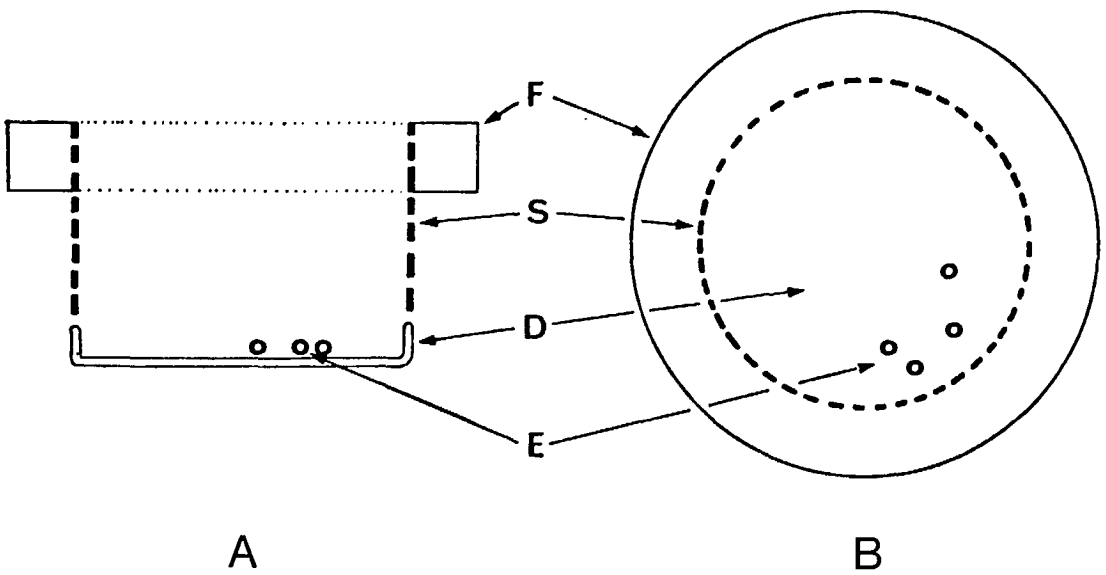

Figure 1. Floating basket for use in embryo and larval toxicity tests. Sectional side view (A) and top view (B) show a styrofoam ring float (F), a petri dish bottom (D) holding the eggs (E), and a nylon screen (S) affixed to the float and dish with epoxy cement. Both 90 and 50 mm diameter models have been used. Mesh opening suitable for whitefish is 0.8 mm.

1) floating in aquaria or tanks used for culturing and testing. Tests are conducted in accordance with standard bioassay principles (Sprague 1969).

Whitefish embryos and larvae are cultured and tested by this method at 3.5°C using reconstituted water and static conditions with aeration. Two to four baskets, each containing 20-50 embryos or larvae, are held in 20 L of culture or test water. Water is renewed and baskets are cleaned weekly.

The basket shown in Fig. 1 allows transfer or removal of eggs or larvae for observation in a small amount of the test water in the petri dish.

EXAMPLES OF APPLICATION

A review of results obtained with fish eggs and larvae is found in McKim (1977). I used the petri dish method to detect the development of sensitivity to the organophosphate pesticide fenitrothion and to follow development of the enzyme cholinesterase in rainbow trout embryos (Klaverkamp et al. 1977). The criterion of death was cessation of heart beat. A concentration-dependent depression of heartrate was also observed (Macdonald, unpubl. data). For copper and vanadium, 96-h LC50's of 0.4 mg/L and 118 mg/L, respectively, were determined for late embryo stage rainbow trout, using similar methods. Vanadium also initiated premature hatching (Giles, in prep.). Zinc toxicity to whitefish was studied using the basket method, with loss of transparency as the criterion of death; 96-h LC50's of 2.5 mg Zn/L for embryos and 2.3 mg Zn/L for larvae were determined (Macdonald, unpubl. data).

DISCUSSION

McKim (1977) demonstrated the feasibility of testing fish embryos and larvae to estimate maximum acceptable toxicant concentrations (MATC). For such work the floating basket method described above is useful because it can be adapted to various species and to static or flow-through tests in either the lab or the field. The petri dish tests are useful for preliminary acute tests and for acute tests involving microscopic examination of the living subjects.

COSTS AND STAFF REQUIREMENTS

Dissecting microscope	$3500
Low temperature incubator (Petri dish method)	$1500
Other temperature control (floating basket method)	$1500

Technical staff with basic training in embryology and bioassay techniques will be able to run these tests. Petri dish tests can be set up in a day and will normally run for less than a week; one person (full time) can handle up to 100 dishes. Basket exposures can be set up within a week to a month (depending on facilities available and testing details); one person working 2-3 d per week can handle up to 100 baskets.

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TESTING EFFECTS ON CARDIOVASCULAR AND RESPIRATORY FUNCTIONS IN FISH

by H.S. Majewski, J.F. Klaverkamp and M.A. Giles

ABSTRACT

The effects of toxicants on cardiovascular/respiratory functions in fish are determined by changes in buccal pressure amplitude, cough frequency, and heart and ventilation rates. A surgical (catheter), and a non-surgical (electrode) method for measuring these parameters is described. Applications of these tests using organophosphate insecticides, organic solvents, and heavy metals are discussed.

RATIONALE

Variations in cardiovascular/respiratory parameters in fish have been measured by numerous authors to determine the sublethal effects of chemicals in the aquatic environment. A toxicant which acts primarily on the gill surface and consequently affects gas exchange would greatly hinder a fish's chances for survival, especially if subjected to further environmental stress such as an increase in water temperature and/or a decrease in available oxygen. Hughes and Adeney (1977) found that sublethal concentrations of zinc increased ventilation and cough frequency, and decreased heart rate in rainbow trout (indicating interference with oxygen uptake), and consequently affected the ability of these fish to cope with the additional stress of environmental hypoxia. We describe techniques for measuring some cardiovascular/respiratory parameters in rainbow trout (Salmo gairdneri), and discuss results from exposure of this species to selected toxicants.

METHOD

(i) Surgical

Adult fish, generally weighing from 0.3-2.0 kg, are anesthetized using an approximate ten minute exposure to a 1:3000 solution of 2-phenoxyethanol or a 1:15000 solution of MS 222®. A buccal catheter (Clay Adams PE 60) is implanted for the purpose of obtaining cough frequency, ventilation rate and pressure amplitude (Saunders 1961). An opercular catheter may also be implanted if differential pressure amplitudes are desired. To obtain electrocardiogram data, needle electrodes are inserted just under the skin and sutured into place. The recording electrode is inserted midventrally, slightly anterior to the pectoral fins, and the reference electrode is inserted midventrally slightly anterior to the pelvic fins. After these procedures are accomplished (~10 min), the fish are placed into restraining chambers, constructed of green acrylic sheeting, equipped with a water jacket for temperature control, and movable partitions to restrain the fish (Smith and Bell 1967).

An intermittent-flow toxicant delivery system (Harrison et al. 1975) is employed to maintain toxicant concentrations and flow rates. Dissolved oxygen is maintained near saturation levels by the use of an air stone inserted in the restraining chamber. Fish are allowed to recover for 3 h after being placed into the restraining chambers. Toxicant is then introduced and maintained at the desired concentration for a 24-h period, and cardiovascular/respiratory responses are observed.

Physiological data are recorded with a thermal-tip recorder. Ventilation rates and pressure amplitudes are recorded with a pressure

transducer and amplifier. ECG data are obtained by utilizing a bioelectric amplifier, patient cable, and a set of needle electrodes. The recorder is set up in series with an iso-transformer to eliminate electrical artifacts. For heart rate data, a 5-way switchbox is used, so that for every five fish, only one bioelectric amplifier is required. For pressure recordings, a 5-way stopcock manifold serves the same purpose. Recorder tracings obtained with this system are presented in Fig. 1.

(ii) Non-surgical

An alternative to the surgical technique has been utilized in our laboratory to measure ventilation rate, heart rate, partial ECG waveform and cardiac-respiratory coupling. The apparatus (Fig. 2) consists of a pair of telescoping, plexiglass tubes fitted with three stainless steel electrodes which interface with a bioelectric amplifier and a thermal-tip recorder. Fish are placed or allowed to swim voluntarily into a large diameter tube. A small diameter tube is then inserted so that the back and forward movement of the fish is restricted to a distance equal to the length of the caudal fin. The chamber can be placed in an aquarium or in a stream of flowing water with or without toxicant. Although a large diameter chamber may be employed for both large and small fish, more consistent recordings are obtained if the diameter is adjusted for the size of fish. In our experience, 7 cm diameter chambers work well for 20-60 g rainbow trout whereas 9 cm diameter chambers are required for 100-250 g fish.

A combined ECG-ventilation recording (Fig. 3A) can be obtained between electrodes E1 and E3 (Fig. 2). For visual examination of the

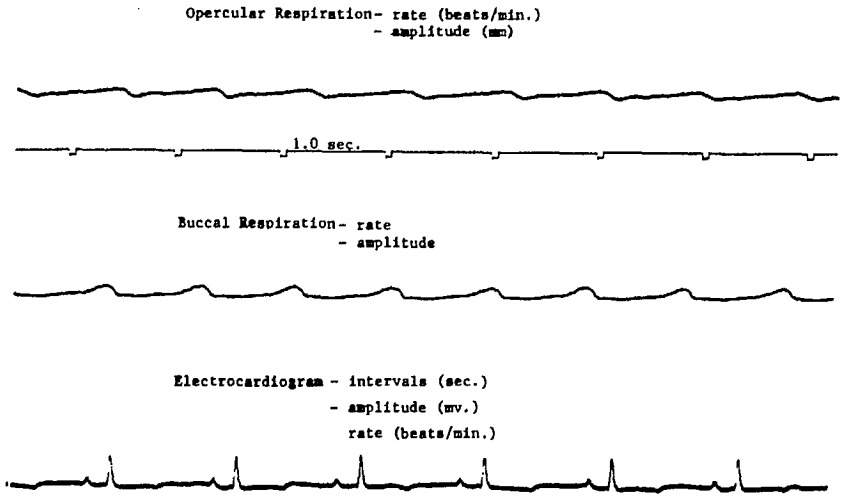

Figure 1. Opercular, buccal and ECG recordings obtained from a non-exposed rainbow trout (Salmo gairdneri) employing the surgical method. Parameters obtained from each tracing as listed.

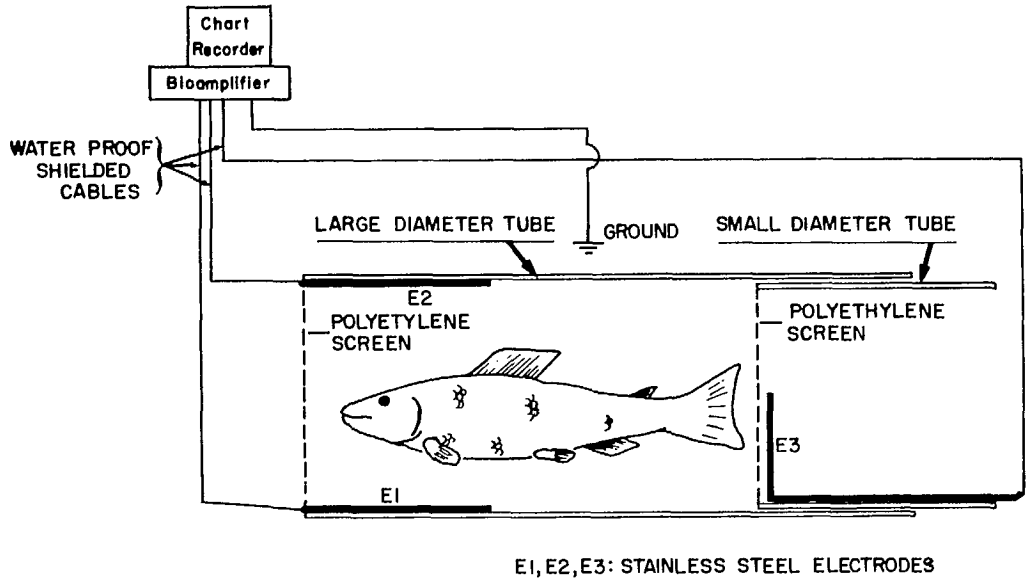

Figure 2. Schematic of electrode chamber for recording bioelectric potentials arising from cardiac and ventilatory musculature activity.

traces these recordings provide all relevant data on cardiac and ventilation rates and on coupling. If a rate computer is employed to process the bioelectric data it would be necessary to dampen one or the other of the signals.

A relatively interference-free respiratory signal (Fig. 3B) can be obtained between electrodes E1 and E3 (Fig. 2) by lowering the frequency cut-off of the lower band filter on the bioamplifier. The bioelectrical signal corresponding to ventricular contraction (Fig. 3C) is most readily obtained between electrodes E1 and E2 (Fig. 2) but can also be recorded between E1 and E3 by increasing the frequency cut-off of the lower band filter on the bioamplifier.

EXAMPLES OF APPLICATION

The threshold concentration of bleached kraft mill effluent causing cough frequency and buccal pressure amplitude increases in sockeye salmon (Oncorhynchus nerka) appeared to be about 20% of the static 96-h LC50 (Davis 1973; cf. Walden et al. 1970). Pesticides, such as DDT at 0.38 of the 96-h LC50, and fenitrothion at 0.15 of the 24-h LC 50, have produced a concentration-dependent cough response in coho salmon and rainbow trout (Schaumburg et al. 1967; Bull and McInerney 1974; Lunn et al. 1976; Klaverkamp et al. 1977). Figure 4 illustrates the cough response and an ECG recording (surgical method) from a rainbow trout exposed to 1.0 mg/L fenitrothion for 21 h. Free chlorine (Bass and Heath 1977) and heavy metals (Skidmore 1970; Drummond et al. 1974) have also elicited a pronounced increase in cough frequency.

An increase in ventilation rate and an elevation in buccal pressure

Figure 3

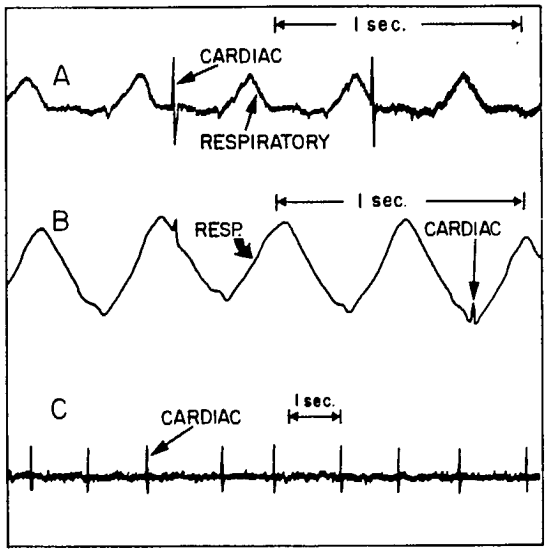

Figure 4

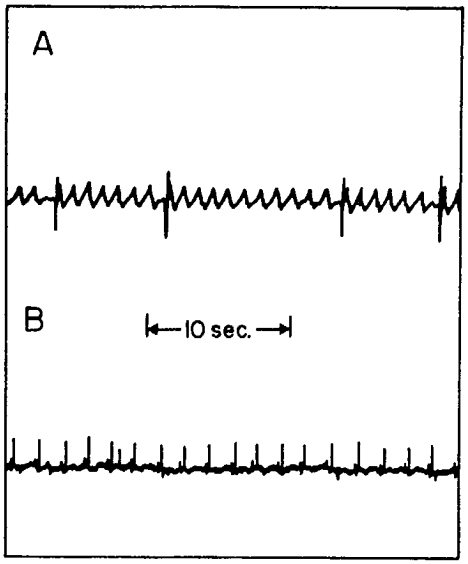

Figure 3. Cardiac and ventilatory biopotentials recorded in electrode chambers with different electrode and filter combinations: (A) E1 to E3, lower cut-off filter $\leq$ 1.5 Hz; (B) E1 to E2, lower cut-off filter $\leq$ 1.5 Hz; (C) E1 to E3, lower cut-off filter $\leq$ 5 Hz.

Figure 4. Buccal ventilatory trace including four coughs (A), and ECG trace (B), from a rainbow trout (Salmo gairdneri) exposed to 1.0 mg/L fenitrothion for 21 h.

amplitude, associated with an increased water volume across the gills, are commonly observed responses to a variety of chemicals, such as zinc, bleached kraft pulp mill effluent, and acetone (Skidmore 1970; Davis 1973; Majewski et al. 1978). In trout, these responses are often accompanied by a decrease in heart rate (Lunn et al. 1976; Bass and Heath 1977; Klaverkamp et al. 1977). Hughes and Adeney (1977) have demonstrated an increased coupling between heart and ventilatory rhythms in rainbow trout exposed to 40 mg/L zinc. This type of reaction occurs in response to hypoxic conditions at the gill surface.

Changes in heart rate, and ECG waveform aberrations in fish usually occur in response to relatively high concentrations of chemicals (Bahr 1973; Majewski and Klaverkamp 1975). Bradycardia is often present in rainbow trout subjected to hypoxic conditions (Weintraub 1975).

DISCUSSION

Several difficulties may be encountered in applying the surgical procedure to monitor sublethal responses of various species of fish to toxicants. In many cases it may be difficult to determine the time required for postoperative recovery so that normal (resting) values of the various parameters may be recorded prior to exposure to the toxicants. Although parallel comparisons of unexposed and exposed fish may be conducted, cardiovascular/respiratory parameters in a population of fish can be highly variable, both under normal and toxic conditions. The impracticality of using large numbers of surgically treated fish further complicates the variability problem. These difficulties can be partially offset by an experimental design which uses each fish

as its own control so that the individual relative responses to toxicant stress can be quantified.

The problems associated with variability due to surgical procedure and recovery from anesthesia can be eliminated by testing unanesthetized fish using the non-surgical procedure. Heart rate, ventilation rate, cardiac-respiratory coupling, and cough frequency can be measured on large numbers of fish for extended periods of time. This procedure is not ideal for the study of ECG waveform, or for the measurement of relative buccal pressure amplitudes, which are better obtained by the use of ECG electrodes and a buccal catheter. However, the two procedures can be employed in a complementary manner to provide an assessment of toxicant effect upon the cardiovascular/respiratory systems in fish.

On occasion, fish appear to acclimate or adapt to toxicant exposure. This may be due to reflex mechanisms capable of maintaining homeostasis (Davis 1973; Majewski and Klaverkamp 1975).

The cough response has been extensively investigated, but underlying mechanisms which elicit this response are not fully understood. Hughes (1975) has demonstrated that the cough response is essentially a reversal of water flow across the gills and may serve a cleansing function. This reaction may be stimulated by suspended particles, or chemicals affecting the gill surface, creating a decreased diffusion capacity. Lead, zinc, copper, and mercury are associated with mucous secretion at the gills, perhaps inducing fish to cough in order to clear the mucous.

COSTS AND STAFF REQUIREMENTS

The cost of this testing system, including recorder, amplifiers, transducers, electrodes and toxicant delivery, amounts to approximately $20,000.

After initial set-up and construction, one biologist (or one well-trained technician under the supervision of a biologist) can operate equipment and analyse output. Evaluation of one chemical, determining the four parameters described, would require approximately 75 man-hours.

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CALCIUM UPTAKE: A SUBLETHAL TEST FOR CRAYFISH

by D.F. Malley and L.J. Tinker

ABSTRACT

Calcification of the new exoskeleton following molting in crustaceans depends upon the uptake of Ca^{++} from the aquatic environment. Changes in the rate of postmolt $^{45}Ca^{++}$ uptake can be used to assess the effects of toxicants on the transport of Ca^{++}, and thence to predict whether there will be effects on the molting process and on ionic regulation. Exponential rate of disappearance of $^{45}Ca^{++}$ from a known volume of water containing a postmolt crayfish is measured and compared over several hours before and after application of the toxicant.

RATIONALE

Molting is a universal and obligatory process for growth and development in crustaceans. The process involves laying down a partial new exoskeleton under the existing one, splitting and escaping from the old exoskeleton, taking up water in order to expand the body volume, and calcification (hardening) of the new enlarged exoskeleton. An essential source of Ca^{++} for calcification of the new exoskeleton is the aquatic environment (Robertson 1937, 1960; Schurr and Stamper 1962; Greenaway 1974) although some Ca^{++}, resorbed from the old exoskeleton, is conserved in freshwater crustaceans in the gastrolith, and as Ca^{++} bound to haemolymph proteins (McWhinnie 1962). During postmolt, the influx of Ca^{++} is larger than that of other physiological ions such as Na^{+}, K^{+}, Mg^{++}, SO_4^{--}, or Cl^{-}, and relatively easy to measure using radiotracers. Changes

in the rate of $^{45}Ca^{++}$ uptake by postmolt crayfish can be used to assess the effects of toxicants on the transport of Ca^{++}.

Toxicants effective in this manner can be expected to interfere with the molting process in crustaceans and should be studied further for effects on transport of other ions and on ionic regulation in general.

METHOD

The following is mainly based on experience with Orconectes virilis (Hagen), but likely to be applicable to related species as well.

(i) Collecting and Holding

Crayfish are obtained by collecting from field populations (or by culturing them in the laboratory; see Leonhard, in prep.). Methods of capture include baited wire minnow traps, or hand capture after disturbing these animals in a stream. Crayfish in lake habitats can be collected by S.C.U.B.A. divers. Postmolt individuals are present in field populations from late May to September in Manitoba and Ontario. Stages of the molt cycle are identified after Stevenson (1975); crayfish in stages A, B, C_1 and C_2 are suitable for $^{45}Ca^{++}$ uptake studies.

Crayfish can be maintained outdoors in plastic tubs, 30 cm x 45 cm x 30 cm (preferably not brightly coloured; see Leonhard, in prep.) containing about 30 L of lake water, at densities of up to 25 animals per tub. Tubs are fitted with covers of rigid plastic sheeting on wooden frames to exclude rain and litter. Plastic flower pots, with a 7.5 cm mouth diameter, and weighted with stones, polyester aquarium filter wool or PVC

tubing of 12 to 15 cm length and 10 cm diameter, are provided for cover. Crayfish are fed commercial soft pellet dog food, such as "Gaines Burger Bits"®, or a laboratory-prepared extruded pellet (Leonhard, in prep.). Temperature should be maintained below 25°C, preferably between 15°C and 17°C, for example, by placing the tubs on a water table through which cool water at 10-15°C flows, or by placing them on a wooden frame floating in the littoral zone of a lake.

Following collection, crayfish maintained in a laboratory should be exposed to long-day light conditions such as 16 h light:8 h dark. Temperature should be 15-17°C. Feeding is described above. Water in the holding tubs, unless it is continually flowing, should be renewed every 2-3 d.

(ii) Testing

Uptake of $^{45}Ca^{++}$ is measured by placing postmolt crayfish individually into 250 mL beakers containing 370 or 740 kBq of $^{45}Ca^{++}$ in 100 mL of water (medium) from the same supply as that used for their maintenance. For convenience, one run may involve setting up 12 such beakers. One crayfish of known weight, carapace length, molt stage, and sex, is placed in each of 10 beakers. Seven of these are experimental and subjected to the toxicant. Three crayfish serve as controls to confirm that the rate of disappearance of $^{45}Ca^{++}$ is a constant exponential rate over the experimental period. The two beakers without crayfish are included to confirm that evaporation or adsorption to the glass has an insignificant effect on $^{45}Ca^{++}$ concentration. Temperature is maintained

at 16 ± 1.0°C by placing the beakers on a water table or in an environmental chamber.

At the beginning of the test and at 0.5-1 h intervals thereafter the beakers are gently swilled and 50 µL aliquots of the medium are removed and added to 15 mL of PCS solubilizer fluor (Amersham) for scintillation counting. After a minimum of four aliquots have been taken, i.e. after 2-4 h, the toxic agent is added to give the desired concentration in the medium. Measurement of the rate of disappearance of $^{45}Ca^{++}$ from the medium is continued by removing at least an additional four 50 µL aliquots from the medium over a 2-4 h period. If all the aliquots containing $^{45}Ca^{++}$ from a single medium are counted within a few hours of each other, the counts are relative to one another and no corrections are required for radioactive decay. Quenching is assumed constant for all samples since the volume of the medium added to the fluor is insufficient to affect its characteristics.

The concentration of Ca^{++} in all beakers at the end of the experimental period is analyzed by atomic absorption spectrophotometry as a check that the Ca^{++} shown to be taken up by the disappearance of $^{45}Ca^{++}$ is retained by the crayfish. Also, a sample of medium is taken upon addition of the toxicant, to ascertain that its concentration is at the theoretical value.

Natural logarithms of CPM of $^{45}Ca^{++}$ (Y) are regressed against time in hours (X) and a straight line ($Y = a + bX$) is fitted by the method of least squares. The slope, b, is calculated separately for the initial control period, $b_{control}$, and for the period in the presence of

of the toxicant, b_{tox}. These slopes are compared and expressed as % inhibition:

$$\text{percent inhibition} = \left(1 - \frac{b_{tox}}{b_{control}}\right) \times 100\%$$

Statistical testing becomes necessary when the slope is shallow, the variance of points about the regression line is relatively high, or two slopes are very similar. Student's t-test as described by Steel and Torrie (1960)is used to test statistically whether a slope is equal to zero and to test differences between slopes b_{tox} and $b_{control}$.

EXAMPLES OF APPLICATION

This test has been applied to adult crayfish, Orconectes virilis (Hagen), collected from a Precambrian Shield lake in the Experimental Lakes Area (ELA), northwestern Ontario with about 70 µmol/L Ca^{++}, 30 µS/cm conductivity, and 40 mg/L total dissolved solids.

In one experiment, the effect of lowered pH was investigated. After a 4.67 g molt stage B crayfish (carapace length 2.72 cm) was placed in 100 mL of lake water containing 725 kBq of $^{45}Ca^{++}$, a constant exponential rate of disappearance of $^{45}Ca^{++}$ was observed over the first 10 h (Fig. 1A). Amount of Ca^{++} in the medium at the end of experiments closely approximated the value predicted from $^{45}Ca^{++}$ disappearance. Thus $^{45}Ca^{++}$ accurately traces Ca^{++} uptake. $^{45}Ca^{++}$ uptake, by a 3.80 g C_2 molt stage crayfish (carapace length 2.53 cm) ceased when the pH was reduced from pH 6.9 to 3.6 and was partially restored when the pH was returned to near the initial value (Fig. 1B). More work is required

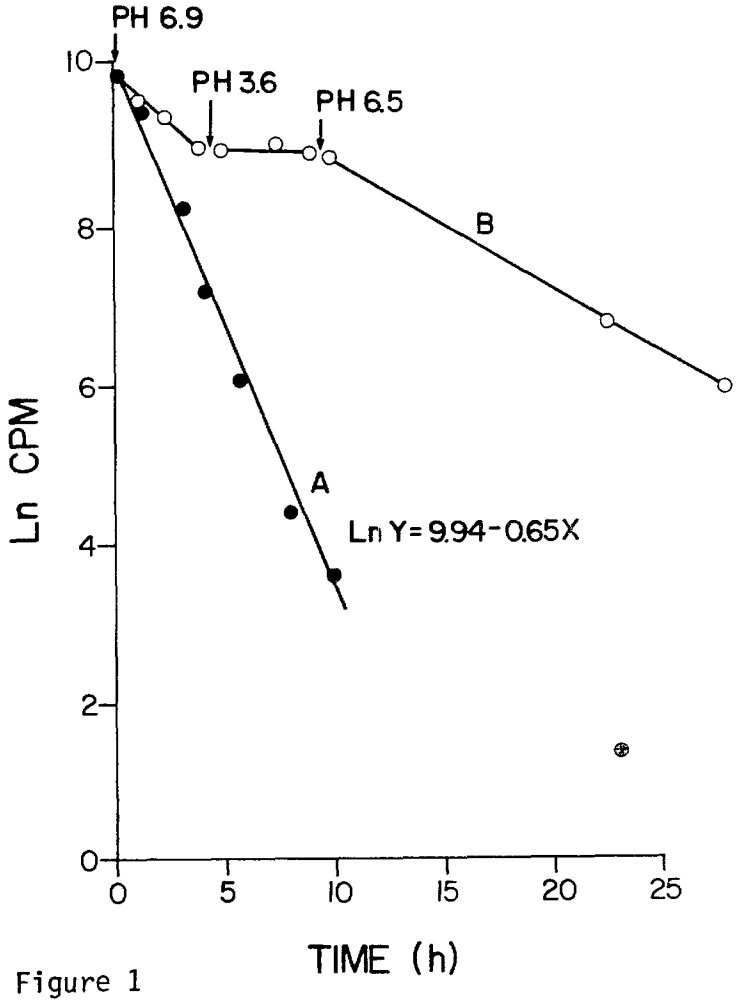

Figure 1

Disappearance over time of $^{45}Ca^{++}$ from 100 mL of medium into a crayfish in B molt stage (A) and effect of low pH on disappearance from the medium of $^{45}Ca^{++}$ into a C_2 stage crayfish exposed to three different pH values consecutively (B).

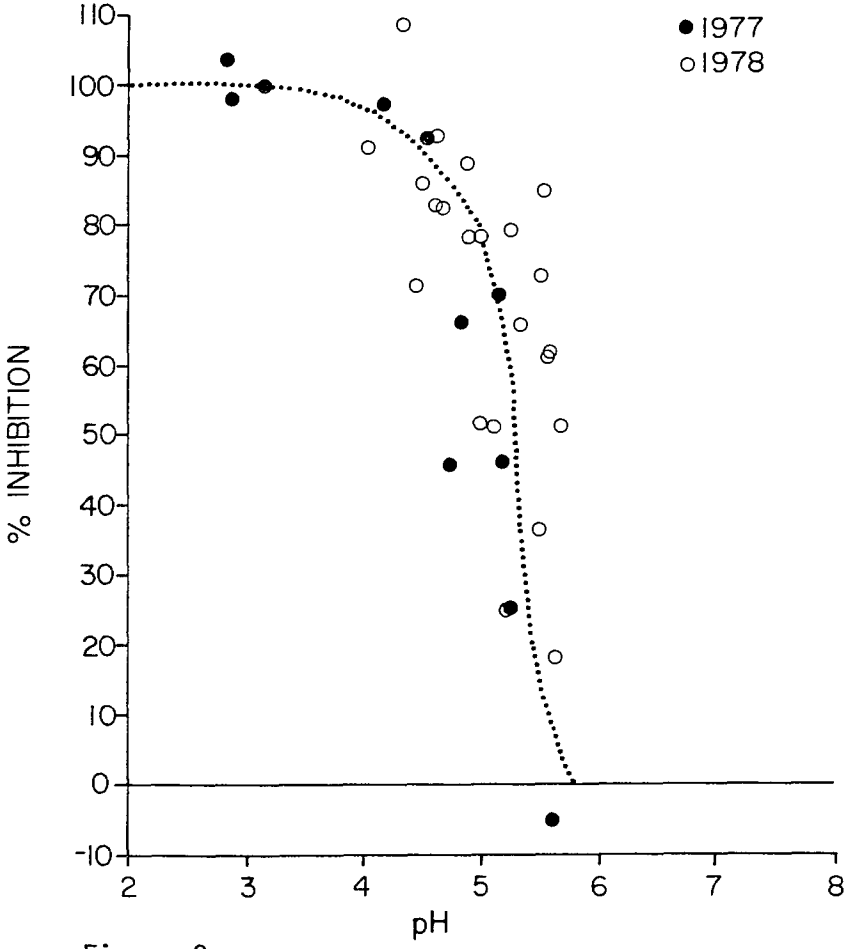

Figure 2

Influence of low experimental pH on Ca^{++} uptake. Percent inhibition is plotted against the mean pH of the medium during the period of measurement. Each point represents a single determination on one crayfish.

to establish the degree of reversibility of uptake inhibition by low pH. The effect of decreasing the pH below 6.0 on $^{45}Ca^{++}$ uptake is shown in Fig. 2 for 33 individuals studied in the summers of 1977 and 1978 (Malley, in press). Between pH 5.75 and 4.0, $^{45}Ca^{++}$ uptake is partially inhibited; below pH 4.0 it is completely inhibited.

This test has also been used to measure the effects of certain heavy metals on Ca^{++} uptake. Cadmium at levels 20, 32 and 50 µg/L and selenite at 0.1, 1.0 and 10 mg/L caused no consistent inhibition of $^{45}Ca^{++}$ uptake in postmolt O. virilis from ELA (Malley, unpubl. data).

DISCUSSION

The uptake of Ca^{++} by a crayfish is highly dependent on the stage of the molt cycle (Greenaway 1974; Malley, in press). Other variables such as origin and age of animal, feeding and other aspects of previous history, experimental temperature, water chemistry, and amount of handling possibly also affect Ca^{++} uptake by crayfish at a particular time. In addition, there is considerable physiological variation between individuals in rates of ion transport, other factors being constant. Therefore, in our test the animal serves as its own control. Thus, toxicants can be screened using crayfish captured from the field without too much concern for their past history. Nevertheless, in order to predict the impact of the toxicant on natural populations, the interaction between toxicity and these variables will need to be examined. The minimum requirements for this toxicity test are that the crayfish be in early to middle postmolt stages and that temperature be constant over the

entire experimental period. Crayfish in molt cycle stages C_3, C_4 and D do not take up Ca^{++} sufficiently rapidly to measure a change in rate over a few hours.

The test can be adapted for use in a field station since the basic equipment and method are simple. A water table can be used for temperature control. Scintillation vials can be transported to a laboratory for counting.

The procedure described here applies to adults of the crayfish O. virilis, but $^{45}Ca^{++}$ uptake has also been measured in first to third instar stage crayfish, in postmolt Daphnia magna (Malley et al. 1979), and in the opossum shrimp, Mysis relicta (Malley, unpubl. data). It is expected that the method can be modified for use on a number of freshwater crustaceans, in which individuals in early postmolt stages are recognizable and available.

COSTS AND STAFF REQUIREMENTS

Water table (outside)	$ 100
or (optional) controlled environment chamber, minimum size ≃ 0.5 m^3	$ 2100
Scintillation counter	$15000
pH meter	$ 400
Plastic tubs, aquaria, misc. glassware (scintillation vials, pipettes, volumetric flasks etc.) chemicals	$ 800

Maintenance of holding facilities and feeding of crayfish requires 3-4 h per week by a non-professional. Running of tests including data analysis will require a minimum of 2-3 d of training. Work input

for a 8-h test run including scintillation counting, Ca^{++} and toxicant analysis, and data treatment amounts to about 15 h for one person.

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AVOIDANCE TESTING FOR FISH AND INVERTEBRATES

by E. Scherer

ABSTRACT

A technique to determine whether a species can detect and avoid an environmental toxicant is described. A choice between two kinds of water is provided, "pure" vs. "contaminated", separated by countercurrents of low velocity (< 1 cm/s). An integrated viewing and recording device yields continuous and accurate tracings of the test organisms on a strip-chart recorder. The method proved to be applicable to motile aquatic invertebrates as well as fish.

RATIONALE

Choice experiments have been a classic tool to determine the ecological requirements of a species, for instance, with respect to temperature, substrate, food, etc. With their sensory organs, animals are constantly scanning their environment; in combination with their locomotor apparatus, they are able to select areas with favourable factor configurations. While these mechanisms may work well enough under natural conditions, with sensory organs "calibrated" and responses genetically pre-programmed to meet the requirements of the species, the advent of man-made pollutants in the environment posed some new problems. Considering the relatively short history of exposure to man-made toxicants, we cannot assume pre-programmed avoidance responses to exist. Fish may very well not be able to detect or avoid certain toxicants, thus rendering them all the more hazardous - analogous to the human

predicament with regard to carbon monoxide. If avoidance occurs, reducing the risk of exposure for the fish, changes in spatial distribution and migration patterns are likely to be detrimental to local fishing industries (Sprague et al. 1965; cf. Scherer 1977).

METHOD

(i) Apparatus

a) Test chamber (Fig. 1A). Water is pumped into a rectangular tank of clear acrylic plastic from both ends at the same rate, regulated by PVC valves. Baffles of perforated PVC reduce water turbulence. The water drains from the bottom plate and side walls at the center of the tank (measurements of prototype: 120 x 18 x 15 cm). Flow velocities of less than 1 cm/s yield a distinct separation between the two bodies of water at the center. A stock solution of the compound to be tested is kept at the same temperature as the water, aerated if appropriate, and injected into either side of the tank by a dosing pump. A row of rheostat-controlled incandescent bulbs and a diffusing screen provide equal illumination of variable intensity. Infra-red lighting may be used in the case of turbid or coloured pollutants, to distinguish chemical from visual stimulation. The tank is enclosed and viewed through a one-way mirror to reduce visual stimulation of the fish.

b) Recording (Fig. 1B and 2). An open sight viewer, connected to the shaft of a power-supplied potentiometer, is mounted so that an observer can track a specimen in the test chamber. Varying the position of this viewing device changes the output voltage from the potentiometer. This voltage is simultaneously fed (1) to a strip chart

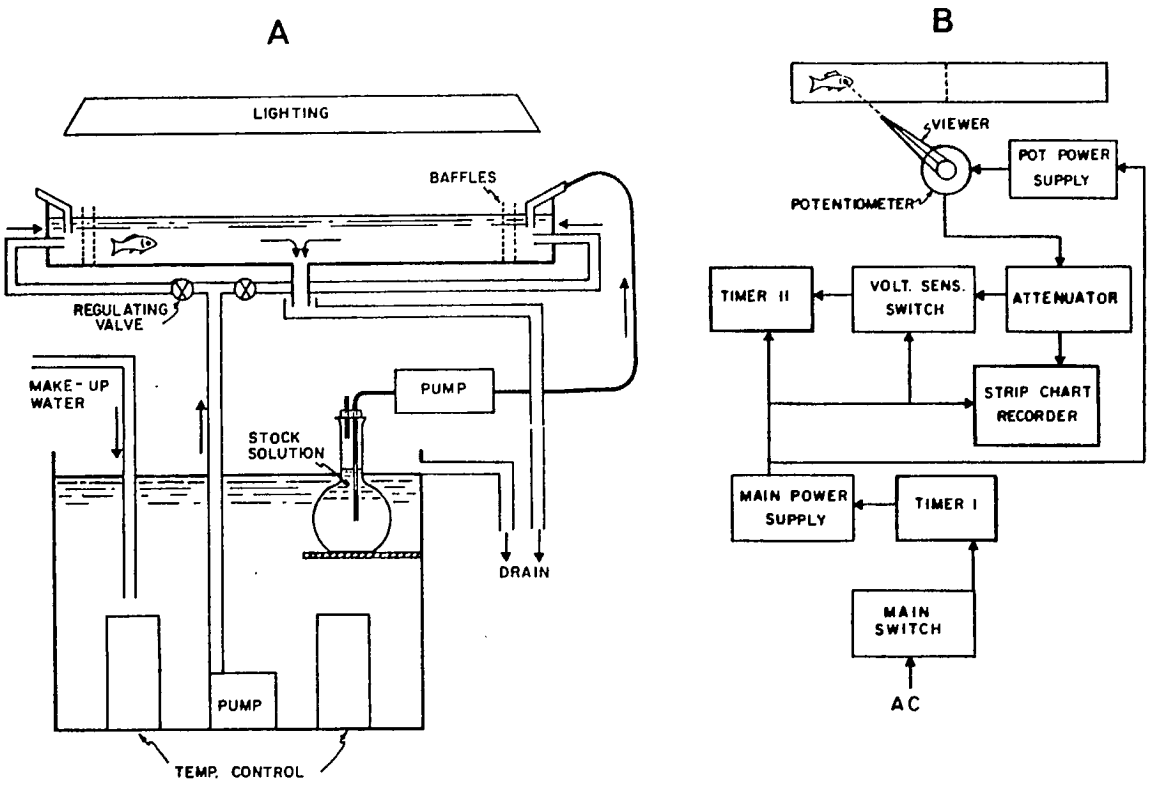

Figure 1. A, test chamber with accessories, schematic; B, block diagram of recording assembly. From Scherer and Nowak (1973).

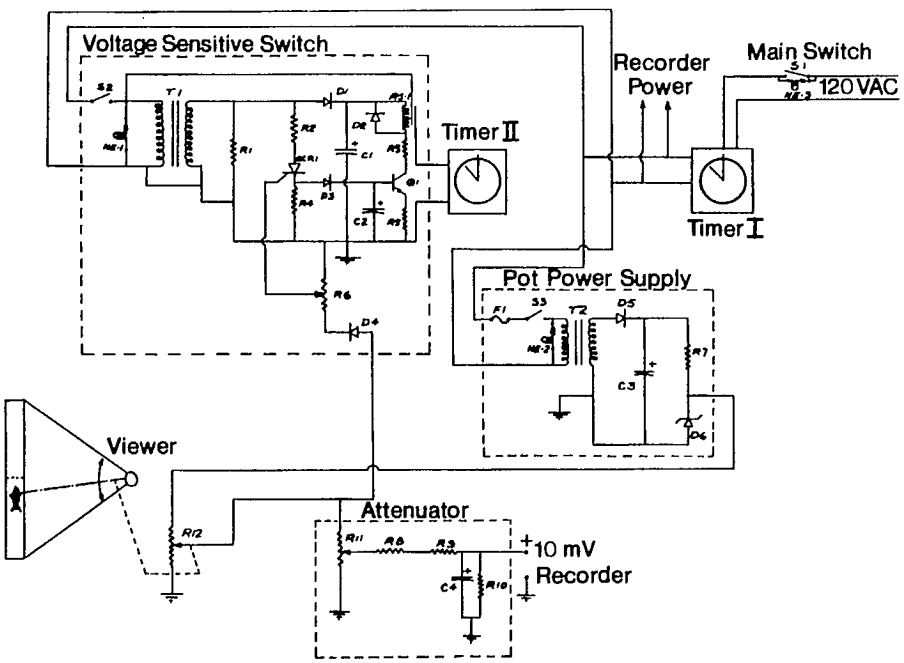

Figure 2. Circuit diagram of recording assembly. From Scherer and Nowak (1973).

recorder, giving an instantaneous, permanent record of fish position and movement, and (2) to a voltage sensitive switch. The latter activates a timer whenever the fish enters one half of the tank, thus registering the cumulative time of exposure to the conditions in that section. A second timer is used to record overall elapsed time of the experiment. The viewing device should be mounted far enough away from the tank to keep perspective distortion small (minimum distance from the 120 cm long prototype is about 80 cm); otherwise, correction factors can be applied. An isosceles triangle of tank ends and viewer is recommended.

(ii) Testing

A basic requirement for this test is motility of the organism. The test specimen, well acclimated to the test temperature, should habituate for several minutes to the chamber, pure water entering from both ends, before testing commences. Some species, e.g. whitefish (Coregonus clupeaformis), tend to assume a rather consistent swimming pattern moving back and forth from one tank side to the other. Others, e.g. some strains or batches of rainbow trout (Salmo gairdneri), show phases of inactivity during which they tend to remain motionless for several minutes in one area of the test chamber, thus not encountering any choice situation. A specimen exhibiting this behaviour may be exchanged for a consistently active one, provided there is no evidence of a correlation between locomotor activity and sensory-behavioural function (cf. Discussion). Testing is always preceded by a control recording (pure water in both sides) of the same duration as the following tests (minimum of 5 min). A test time of 15

min is commonly used in our lab. The number and level of toxicant concentrations to be tested is determined in exploratory experiments, with the 96-h LC50 value as a (high) reference point. Only if the supply of test specimens is limited, several concentrations should be tested on the same individual. In this case, it is preferable to proceed in ascending order of concentration, to reduce the likelihood of toxic effects occurring during the testing procedure. Nevertheless, sensory adaptation and behavioural habituation may occur. Clearer results can be expected when using a specimen only once, for one concentration.

Recording the movements of a test specimen with the apparatus described, provides an immediate read-out of time spent in one half or the other of the tank, usually expressed as percent of total time; deviations from 50% indicating either avoidance or preference (see Fig. 4). Arcsine or logit transformation is recommended for statistical analysis (de March and Scherer, in prep.).

Strip chart tracings allow determination and statistical analysis of parameters underlying these time proportions, e.g. swimming speeds, depths of penetration and duration of individual visits into either side (Fig. 3). This information is essential since the integrative percent-time measure alone can be misleading; e.g. time accumulated in the toxicant half may be due to reduced motility caused by the toxicant (Shelford and Allee 1913; Sprague and Drury 1969).

EXAMPLES OF APPLICATION

This method and its precursors have been successfully applied to

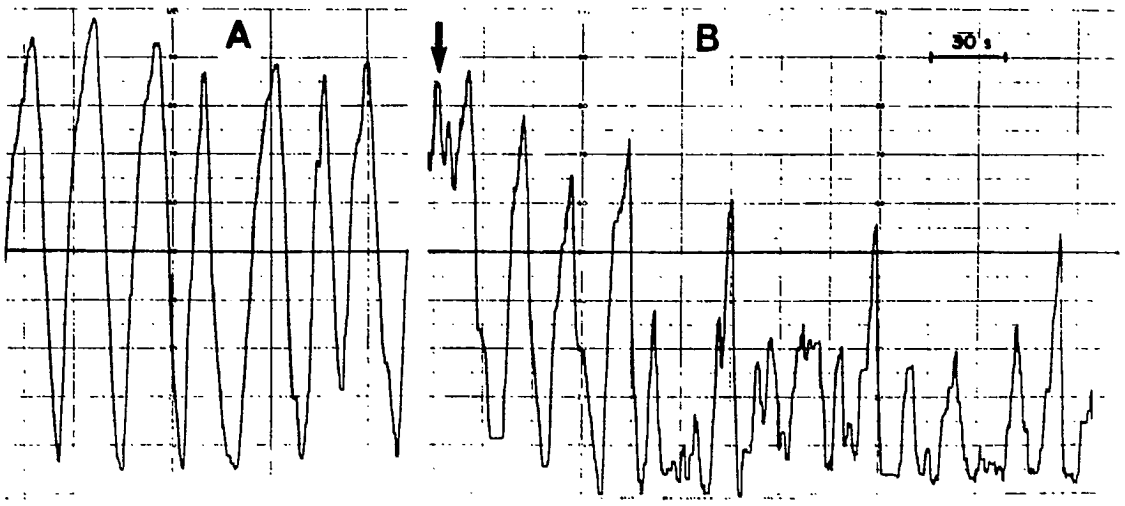

Figure 3. Examples of strip chart recordings: Avoidance response of a whitefish (Coregonus clupeaformis). A, pure water in both halves of the test chamber; B, 0.5 mg/L cadmium (administered as $CdCl_2$) in one half of the tank (= upper half of strip chart); arrow marks toxicant injection.

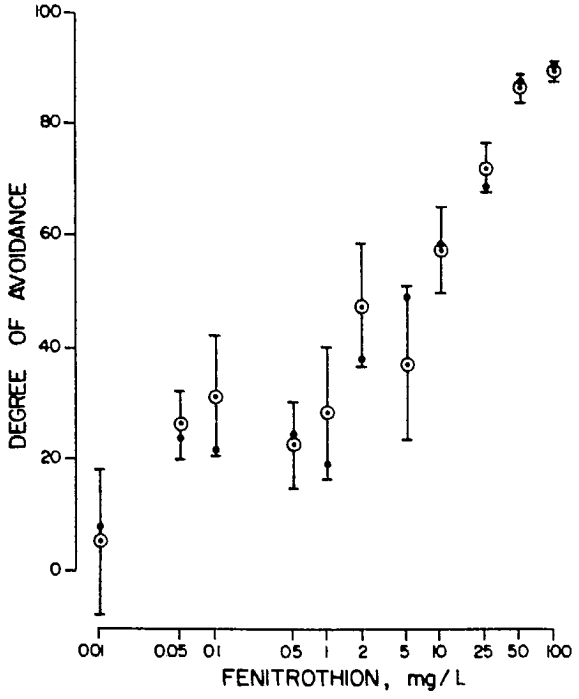

Figure 4. Avoidance of the organophosphate pesticide fenitrothion by goldfish (Carassius auratus). Degree of avoidance: 0 = 50% time accumulated in pure water; 20 = 60%, etc. ⊙ = response mean with standard error; • = response median. From Scherer (1975).

numerous fish species and toxicants including dissolved gases and effluents (e.g. Shelford and Allee 1913; Jones 1947; Sprague et al. 1965; Sprague and Drury 1969; Lawrence and Scherer 1974; Scherer 1975; Tatsukawa and Hidaka 1978; Scherer and McLean, in prep.). Moreover, a scaled-down version of the same apparatus proved practical in tests with Gammarus (Maciorowski et al. 1977; de March and Scherer, in prep.); application to other motile invertebrates appears equally feasible.

DISCUSSION

A number of different paradigms for avoidance testing exist (Scherer 1977; this volume). The technique described here and its precursors have been used so far mainly to determine the direct response to toxicants by naive fish. While avoidance responses with thresholds well below 96-h LC50 values have been found in such experiments (e.g. Jones 1947, 1948; Sprague et al. 1965; Scherer 1975), cases of non-avoidance or apparent preference (attraction) were also reported and their likely detrimental consequences discussed already by Shelford and Allee (1913) and Shelford (1917), later by Jones (1947, 1948). Sprague and Drury (1969), investigating responses to free chlorine, and Lawrence and Scherer (1974), testing the supernatant fraction of drilling mud suspensions, described shifts from avoidance to preference and back to avoidance over ranges of increasing concentrations. Underlying physiological mechanisms are unknown.

As mentioned under "Method", test specimens of some species or strains, e.g. of rainbow trout (Salmo gairdneri), may show considerable differences in their motility (level of locomotor activity) before test.

In such a case, a test schedule based on an equal number of choice opportunities at the tank center seems more appropriate than a pre-set limit for the test duration.

In any field situation and laboratory set-up, the possibility of (sensory) adaptation, learning, and/or toxic effects during the time of observation cannot be excluded, even during this short-time test. It is recommended to check for possible changes by comparing the response at the beginning with that at the end of the strip-chart recording.

With the view on "real-life" factor interactions, the basic test procedure can be easily modified and further developed (cf. Scherer 1977), e.g. to determine the response after pre-exposure to the same or another toxicant (Scherer and McLean, in prep.), to detect and quantify impairment of an existing conditioned or unconditioned preference or avoidance (Kamchen and Hara, in prep.; see Scherer, this volume); or by putting a toxicant in competition with another or with a non-toxic agent.

COSTS AND STAFF REQUIREMENTS

Precision dosing pump	$750
Temperature control	$650
Strip chart recorder	$400
Timers	$150
Misc. materials for test chamber and recording assembly	$200

Time required for constructing and assembling the set-up is estimated at 30 man-hours.

One test, including habituation time, will take about 30 min. Ten replicates per concentration are recommended. Professional training is not required for running the tests, but desirable for analysis and interpretation.

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TESTING RESPONSIVENESS TO OVERHEAD-LIGHT STIMULATION

by E. Scherer

ABSTRACT

The spatial distribution of a negatively phototactic species like the walleye (Stizostedion vitreum vitreum) is largely determined by responses to varying light intensities. The avoidance of high illumination levels by this species is utilized in a laboratory test, presenting three overhead light intensities (viz. 2, 20, 200 lx as measured on the tank bottom) in ascending and descending order, and recording the resulting changes in vertical position of the test fish. Toxicant-caused deviation from a baseline response pattern thus established is used as a measure for sublethal impairment of visual and/or related neural and motor functions.

RATIONALE

In principle, this test on responsiveness uses a preference/avoidance paradigm. There are basically two different aspects to preference/avoidance responses with regard to toxicants and, consequently, two different experimental approaches (Scherer 1977). First, the direct response to the toxicant(s) can be investigated (Scherer, this volume). Second (the approach chosen here), we can determine the effect of toxicants on existing (learned or "innate") response patterns, e.g. food preferences and avoidances, and responses to abiotic factors of the environment, such as temperature or light. Such genetically preprogrammed or facilitated responses are usually adaptive mechanisms of

high survival value (Tinbergen 1951); thus, their change by toxicants may significantly reduce survival. Ogilvie and Anderson (1965) and Opuszyński (1971) observed shifts in temperature selection caused by DDT and copper, respectively. With regard to light intensity discrimination and selection by fish, little is known in terms of baseline data (see reviews by Woodhead 1966; Blaxter 1970), and less about toxicant effects. The author (Scherer 1971, 1976) described and quantified the response of the walleye (Stizostedion vitreum vitreum Mitchill) to a sequence of three light intenstiies. This visually orienting, negatively phototactic (Ali and Anctil 1968) predatory fish is widely distributed and economically important in Canada and used here as a model for other potential test species.

METHOD

A temperature-controlled reservoir feeds water into two identical glass aquaria (40 x 45 x 90 cm) mounted side by side for test and control, each equipped with an overhead light panel consisting of six fluorescent, dimmable bulbs. After transfer of three randomly selected walleyes to each aquarium, fish are given 2 h for habituation and adaptation to 200 lx before testing commences.

A fixed sequence of light intensities is presented in each test: 200, 20, 2, 2, 20, 200 lx as measured directly under the glass bottom of the aquaria (Table 1). Each light phase lasts exactly 15 min. In conjunction with Luxtrol® dimmers, Vitalite® fluorescent bulbs are recommended for their good daylight-simulating spectral energy distribution (color rendering index R_a = 0.91 at 5500°K). Spectral changes

Table 1. Light intensities (lx), at upper limits of positions 1-6, and at ≏ 2 mm beneath water surface, measured along the water column above light cell (= reference point). In parentheses: ranges observed in three profiles laid through tank, providing eight columns additional to the one above reference point. From Scherer (1976).

Surface	4.4 (3.5 -4.74)	46 (31.8-46.0)	355 (275-355)
6	3.28 (2.73-3.75)	32 (22.4-33.8)	275 (228-285)
5	3.15 (2.65-3.65)	30 (21.8-39.5)	267 (225-278)
4	3.0 (2.65-3.50)	29 (21.0-38.7)	258 (222-270)
3	2.9 (2.4 -3.36)	28.5 (20.0-38.4)	250 (217-260)
2	2.7 (2.31-3.27)	25 (20.8-37.6)	245 (221-267)
1	2.6 (2.18-3.32)	25 (21.8-36.7)	245 (215-259)
Tank bottom	2	20	200

through dimming are not noticeable to the human eye, nor substantiated by spectroradiometric measurements.

During tests, the vertical positions of three fish in each aquarium are recorded manually and simultaneously by two experimenters every 10 s, using a timer with audio signals through earphones, and a grid at the front plates of the aquaria marking 13 equal layers (3 cm wide) over 39 cm of total water column. Five minutes are left unrecorded between each change of light intensity; this interval proved sufficient to let fish quiet down after some excitation occasionally observed during

and immediately after changing the light level, or after removing and reinstalling the one-way mirror between tank and experimenter before and after the 2-lx levels. The latter procedure is necessary because the fish are not clearly visible behind the mirror at this low light intensity. The experimenters sit as quietly and as motionless as possible during these 2-lx phases; as always, no illumination other than that emanating from the tanks is provided. There is no indication of fish reacting to the observers in that situation.

Fig. 1 illustrates the response pattern achieved with unexposed, apparently healthy walleyes.

EXAMPLES OF APPLICATION

The response described above was applied in an experiment investigating the effects of mercury-contaminated diet on walleyes (Scherer et al. 1975). The controls, after a diet of shredded pike for more than six months, did not react "perfectly" (Fig. 2). By comparison, however, the experimentals showed a nearly complete loss of the response (Fig. 3).

DISCUSSION

Other species, including invertebrates, could possibly be tested in a similar test arrangement, by modifying test light intensities, adaptation time and aquaria dimensions. For instance, planktonic crustaceans are known to show distinct light-dependent vertical distribution patterns that may lend themselves to similar tests. In fish, the discrimination of different light intensities and the resultant

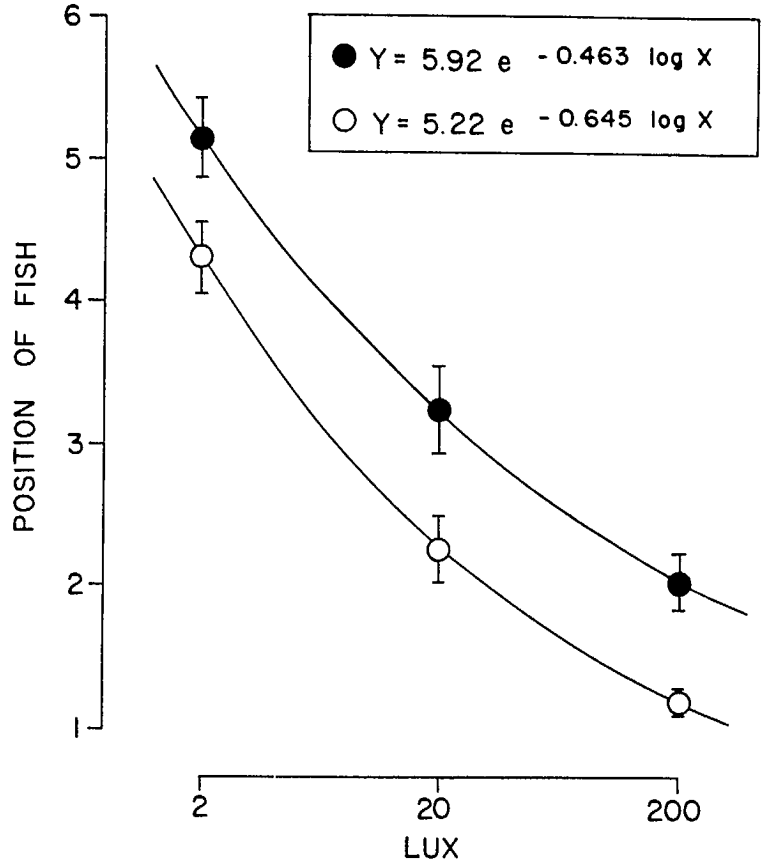

Figure 1. Vertical positions of walleyes at three different light intensities presented in a fixed sequence. Data points represent means with standard errors (n = 48). Open circles, first half of test schedule, from 200 to 2 lx. Closed circles, second half of test schedule, from 2 to 200 lx. (From Scherer 1976).

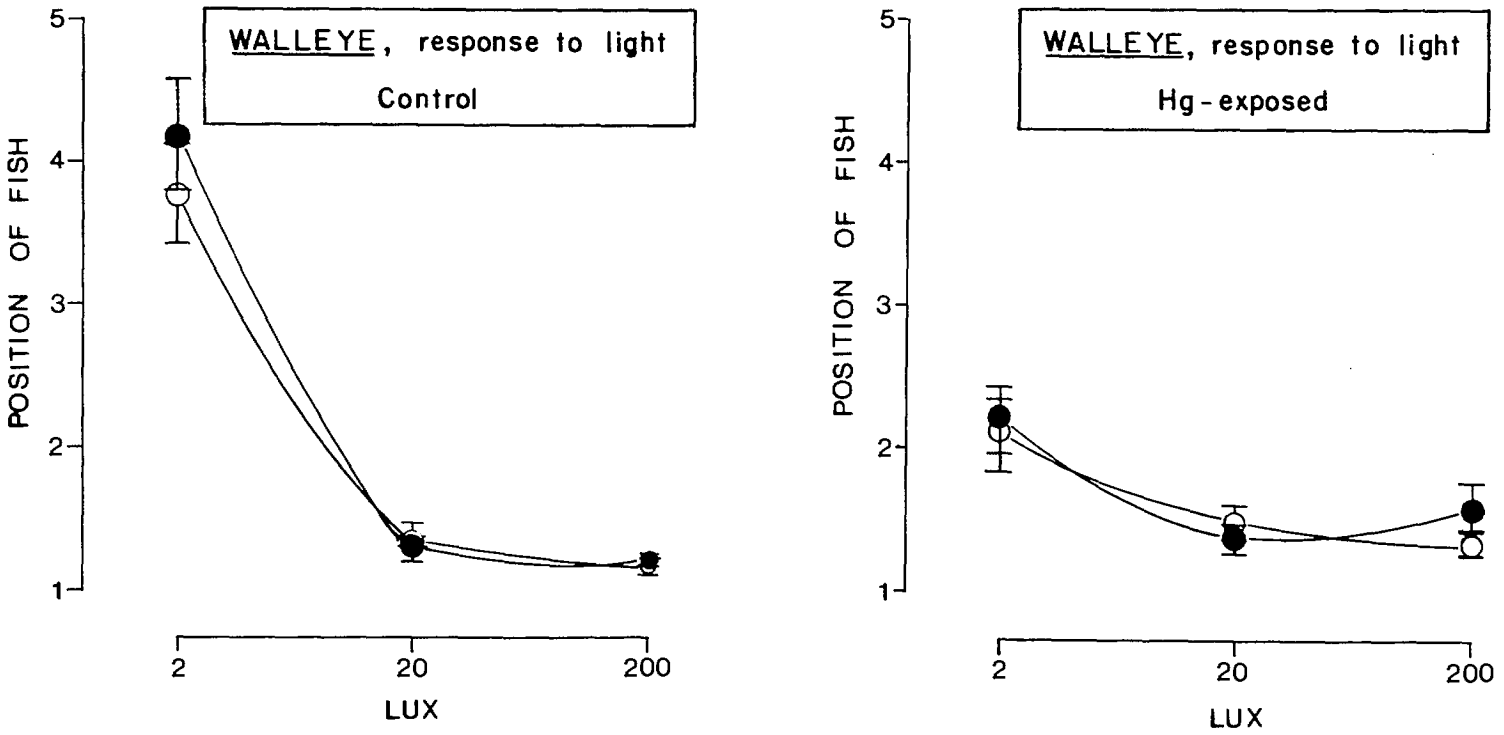

Figure 2 and Figure 3. Effect of mercury uptake through diet on light response of walleyes. Open and closed circles as in Fig. 1. From Scherer et al. (1975).

locomotor response is based on retinal function, stimulus transfer to, and processing in, the optic tectum, and transfer to a functioning locomotor system. Toxicant-caused impairment at any of these sites is likely to become evident in the resultant response, as measured by the test procedure described (see also Scherer 1971).

The manual recording technique may well be replaceable by automated methods, e.g. by time-lapse photography with subsequent data transcription. In that case, a greater number of fish at a time could be monitored. However, technical simplicity and direct visual observation offer some advantages.

COSTS AND STAFF REQUIREMENTS

Temperature-controlled reservoir tank	$850
2 aquaria with one-way mirror panes, fluorescent lights, ballasts and dimmers	$350
Custom-built audio timer with 2 earphones (design details on request) or 2 stopwatches (less convenient alternative)	$ 50

To simultaneously monitor the test and the control aquaria, two experimenters are required. No professional training is necessary. One test, including habituation and adaptation of the fish, takes about 4 h.

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THE OPTOMOTOR RESPONSE TEST

by E. Scherer and S.E. Harrison

ABSTRACT

Described is a technique to elicit the optomotor response in fish and invertebrates, by rotating circular screens marked with black and white vertical stripes around a cylindrical test chamber. Illumination level and screen speed can be varied, the direction of movement reversed; water temperature is controlled. The optomotor response, widespread throughout the animal kingdom, is considered essential for maintenance of position within the habitat, and for schooling in fish. We propose to use this response for a test to detect impairment of visual-orientational functions.

RATIONALE

The optomotor response was first described for terrestrial insects and later investigated in aquatic insects, crabs and fishes (Prosser and Brown 1961; Marler and Hamilton 1966). Arnold (1974) reviewed much of the data available on fish.

In essence, the response is defined as a movement of the animal in the direction of moving reference points in the field of vision. In aquatic species, this behaviour contributes to the rheotactic response, and prevents or compensates for displacement by water currents. Commonly referred to as unconditioned and reported to exist in fish embryos (Fishman 1976), the optomotor response appears to be relatively

strong in pelagic, schooling, and stream-dwelling species, but much weaker or absent in bottom dwellers (Protasov 1970; Harden Jones 1963).

METHOD

(i) Apparatus

Various optomotor test designs have been used for fish over the past 3-4 decades, frequently using the response to determine physiological parameters such as spectral sensitivity, visual acuity, threshold illumination, and flicker fusion frequency (Grundfest 1932; Protasov 1970; Wolf and Zerrahn-Wolf 1936).

In principle, all these experimental devices are similar, consisting essentially of screens with conspicuous striped patterns being moved mechanically or by a variable-speed motor past or around a stationary tank containing the test fish.

Our design (Fig. 1) consists of a circular plexiglass trough 90 cm in diameter, 12 cm wide, and 15 cm deep, on both sides of which is a moveable striped drum. Water is aerated in the water reservoir and the temperature controlled by a portable refrigeration unit similar to that described in Harrison et al. (1975). A submersible pump directs the water into the center of the apparatus from where it radiates outward through the trough and out the drain or back to the reservoir if desired. The drum speed is infinitely variable in both directions (i.e. clockwise and counterclockwise) within the range 0.5 to 26.3 cm/s. An overhead fluorescent light panel controlled by a dimmer switch provides uniform illumination. Convex mirrors mounted in suitable locations above the trough allow observation of the fish without disturbance.

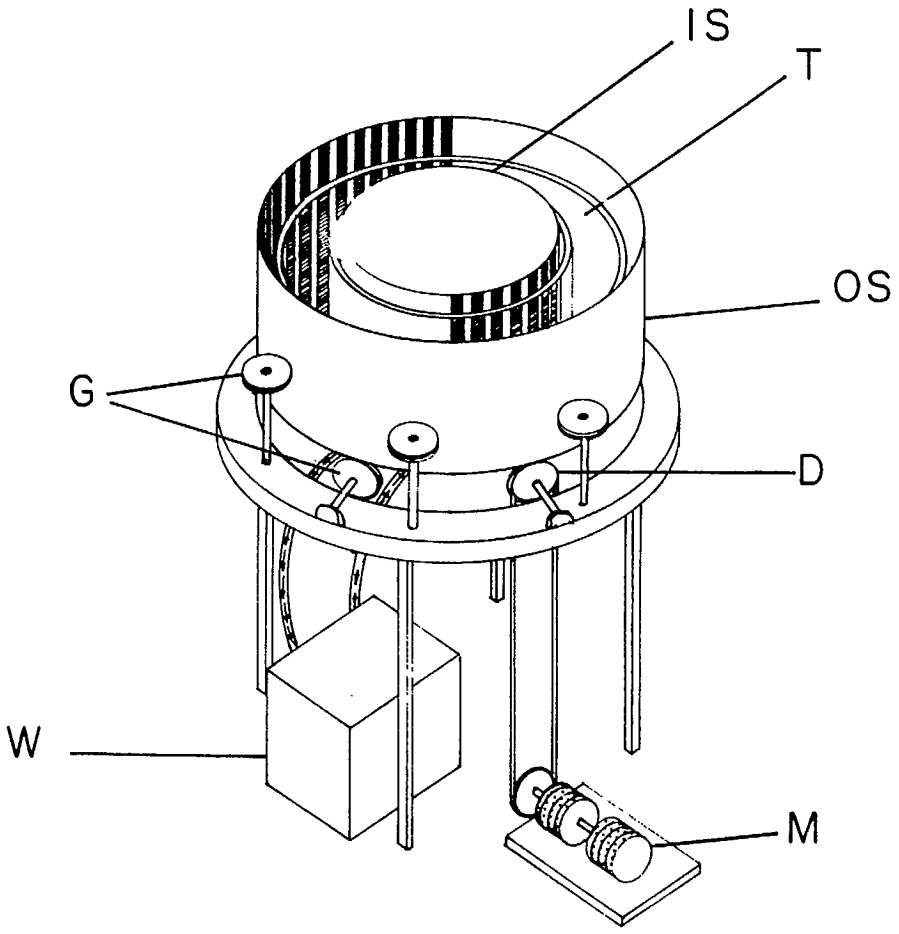

Figure 1. Schematic of apparatus with major components. T, test tank; IS, inner screen; OS, outer screen; M, variable speed motor; D, drive pulley; G, guide pulleys, W, water reservoir with temperature control. Overhead light panel and most stripes omitted for clarity.

(ii) Testing

The test procedure should be optimized for each species by varying test conditions (mainly: drum speed, light intensity, width of black and white stripes) until the maximum positive response is elicited. For rainbow trout (Salmo gairdneri), whitefish (Coregonus clupeaformis), and creek chub (Semotilus atromaculatus), we found the following procedure to produce optimum results:

A fish is placed in the trough and allowed to habituate for 10 min at a mean drum speed (= mean of inner and outer drum speed) of 15 cm/s, illumination 20 lx, with black and white drum stripes of equal width (2 cm). During this time most species will, after some erratic or orientational movements during the first few minutes, display a positive response (swimming with and at the speed of the drum movement) for various lengths of time. After a 5 min pause (drum stopped) follows the actual test period, lasting again 5 min. Using stop watches, the times are monitored during which (1) the fish follows the drum movement (positive response), (2) the fish is motionless (zero response), and (3) the fish is moving opposite the drum direction (negative response). The percent positive response is calculated as the portion of the test period during which the positive response was displayed, and compared for control and toxicant exposed fish.

EXAMPLES OF APPLICATION

In our lab, efforts so far have been concentrated on selecting species and conditions for optimal control responses. A series of

experiments with rainbow trout and creek chub is underway. While we were preparing the final draft of this paper, impairment of the optomotor response of rainbow trout by two herbicides was reported by Dodson and Mayfield (1979). The apparatus and method used by these authors is essentially identical to ours.

DISCUSSION

Literature data (Prosser and Brown 1961; Pavlov 1969; Protasov 1970) suggest that satisfactory control response consistency can be achieved by suitable selection of test species and test conditions, e.g. drum speed, light intensity, width of stripes, temperature, oxygen, pH, acclimation/habituation schedules. However, interaction of these factors has never been thoroughly analysed, i.e. more research on these aspects is needed.

Since the response is based on visual perception, integration and well-coordinated locomotor output, we may expect toxicant effects at low levels similar to those affecting receptor sites and swimming performance (cf. Scherer 1977); this assumption is supported by Dodson and Mayfield (1979).

COSTS AND STAFF REQUIREMENTS

Costs of materials for construction including drive motor with speed control and temperature control unit amounts to about $1200.

Testing is carried out by one person and requires no professional training. At 20 min per test, approximately 20 fish can be tested per day.

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TESTING LOCOMOTOR ACTIVITY RESPONSES WITH ULTRASONIC BEAMS

by E. Scherer, S.H. Nowak, S.E. Harrison and D.L. Kripiakevich

ABSTRACT

Locomotor activity of fish is automatically and continuously monitored by apparatus registering the incidence of fish swimming through inaudible sound beams, the frequency of beam interruptions providing a measure of activity. The method allows a quantitative determination of changes in pattern and level of locomotor activity caused by toxicants. Under laboratory conditions, punched paper tape is used for data recording and efficient computer processing. For field application, a simpler battery-operated numerical printout system is described.

RATIONALE

Motile organisms, with the possible exception of some protists, show rhythmic circadian locomotor activity patterns, thought to result from the interaction of endogenous processes and environmental factors (Bünning 1964; Marler and Hamilton 1966; Brown et al. 1970). Circadian activity patterns ("diurnal", "nocturnal", "crepuscular") and their species-specific modification are linked with the organism's requirements for biotic and abiotic elements of its environment (e.g. light or temperature conditions, prey availability, etc.), while levels of activity reflect metabolic states of the animal. Schwassmann (1971) reviewed aspects of fish activity governed by rhythmicity; overall, quantitative information on freshwater species with regard to their natural activity patterns is still rather sparse. Changes in activity

caused by environmental toxicants have been repeatedly reported (Waller and Cairns 1972; Bengtsson 1974; Weis and Weis 1974; Percy and Mullin 1977; Ellgaard et al. 1977); techniques to record and analyse activity of aquatic organisms are not yet well developed.

METHOD

A classic method for activity recording deploys light beams (preferably infrared or ultraviolet, i.e. invisible to test organisms) in the test area. Their disruption by moving organisms, registered through photocells connected to event recorders, provides the number of beam interruptions per time unit as a measure of activity. This approach is of limited use in our context, because of high absorption and scatter of light in water. By contrast, ultra- (high frequency) sound is known to penetrate water very well. Moreover, technical developments over recent years have made ultrasonic transducers, over a wide range of specifications, available at moderate cost. It appeared promising, therefore, to develop an actograph based on the interruption of ultrasonic beams. In the following, we describe the principle of our technique and its modification in three experimental designs. For more technical details, including circuitry diagrams, see Scherer et al. (1977) and Scherer et al. (in prep.).

The sound frequency selected is 200 kHz, transmitting at 63 dB (re 1 µbar) on the beam axis. Two hundred kHz is considered to be 1-2 orders of magnitude above the maximum frequency perceived by fish (Popper and Fay 1973). A drive level of 2 mW is sufficient to detect fish passages without producing excessive reverberation. The beam in

such a double-ended system (separate receiver and transmitter facing each other) is essentially cylindrical and approximately 3 degrees wide. Long-term stability, as measured by the voltage at the receiving transducer, is ±10% over two weeks, with additional short-term (1-2 s) fluctuations of ±10%. About half of the short-term fluctuation is attributable to "noise" caused by air bubbles in the tank, causing tuning into and out of standing wave modes. The electronic system is set to respond only to on-axis beam interruptions, while rejecting noise effects and standing wave variations. For instance, rainbow trout (Salmo gairdneri) 20-60 cm long cause signal levels to drop by 90 to 99% when crossing the beam (Scherer et al. 1977). Counts are recorded on a digital counter, and automatically transferred in ASCII (American Standard Code for Information Interchange) onto punched paper tape. Data are plotted using a computer or a simpler calculator system (e.g. Hewlett Packard 9100B), interfaced with a paper tape reader.

The timing diagram (Fig. 1) illustrates typical waveforms for two beam interruptions by fish. At t_1, we observe a clear pass through the beam. At t_2, a fish enters the beam, leaves partially and returns, then swims completely out. The programmable Schmitt trigger (comparator type) has adjustable high/low thresholds to protect against such events registering as two counts. With our trout (20-60 cm) we used a 'low' reference at 85% signal reduction, and a 'high' reference at 65% reduction (e.g. 0.5 and 1.5 volts respectively for a 3.5 volt steady-state signal). The Schmitt trigger is capacitively OR-coupled with additional channels to a falling-edge-triggered monostable multivibrator, which drives the counter. This allows interruptions of any beam to register

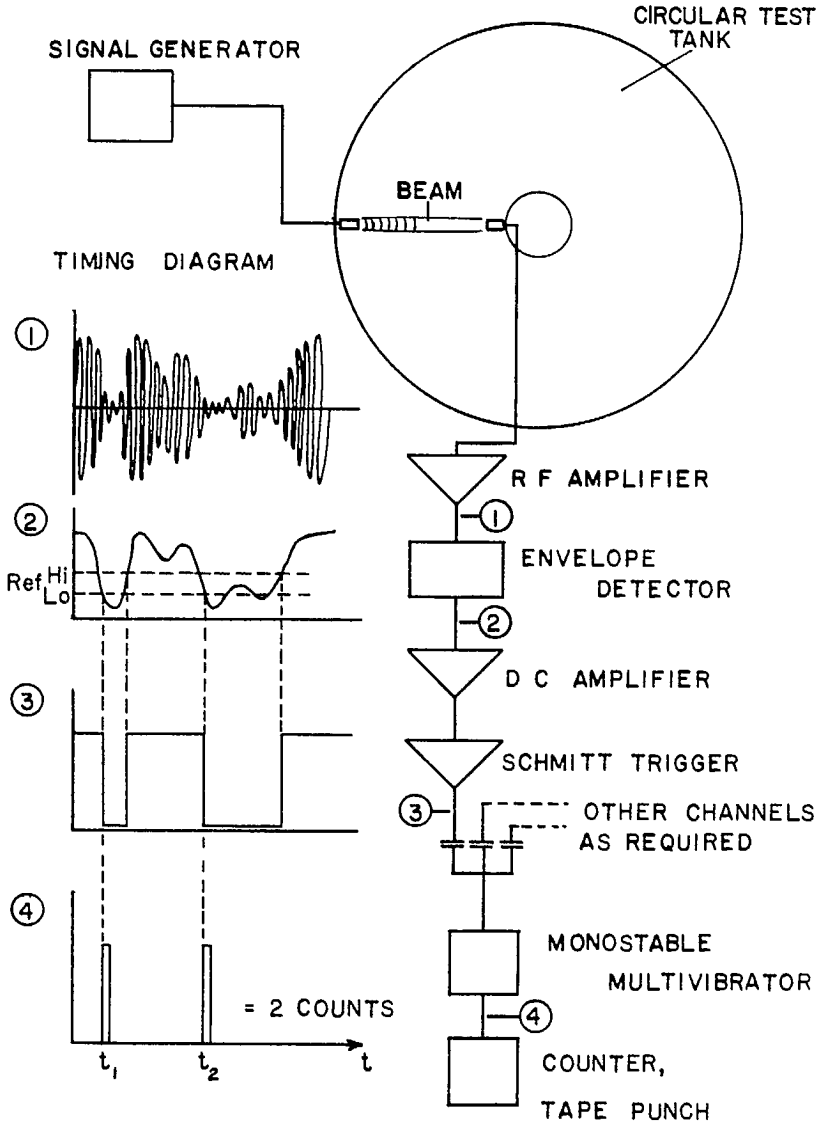

Figure 1. Schematic of ultrasound-beam interruption technique used for activity recording (after Scherer et al. 1977).

counts while another beam is still blocked by a fish.

This technique has been successfully applied in three different experimental set-ups: 2 for lab, 1 for field use (Fig. 2). One lab design uses a 5 m diameter circular tank equipped with two beams transmitting in continuous-wave mode (see Scherer et al. 1977). As no control tank of equal size is available, activity before and after exposure is compared. A second lab prototype employs two identical aquaria (40 x 45 x 90 cm) side by side, one for control and the other for testing, each equipped with two beams. To prevent reverberation effects in such small tanks, sound is emitted in a pulsed rather than continuous mode. A third system, designed for field use, is battery-operated. To reduce power consumption in this case, it is necessary to use a different recording system. It essentially consists of a timing circuit and a small modified "hand-held" type printing calculator which prints out the number of beam interruptions at preset (e.g. hourly) intervals (Scherer et al., in prep.)

EXAMPLES OF APPLICATION

The battery-operated prototype was successfully used to determine the circadian activity pattern of yellow perch (Perca flavescens) in an unpolluted northern Manitoba lake. Six beams were installed in a flow-through cage, anchored to the lake bottom, containing 20 fish (Scherer and Harrison 1979). Experiments are planned to apply this technique to pollution investigations in situ. The two lab designs are currently being used to determine effects of cadmium and an organophosphate, fenitrothion, on activity. Preliminary results indicate that both

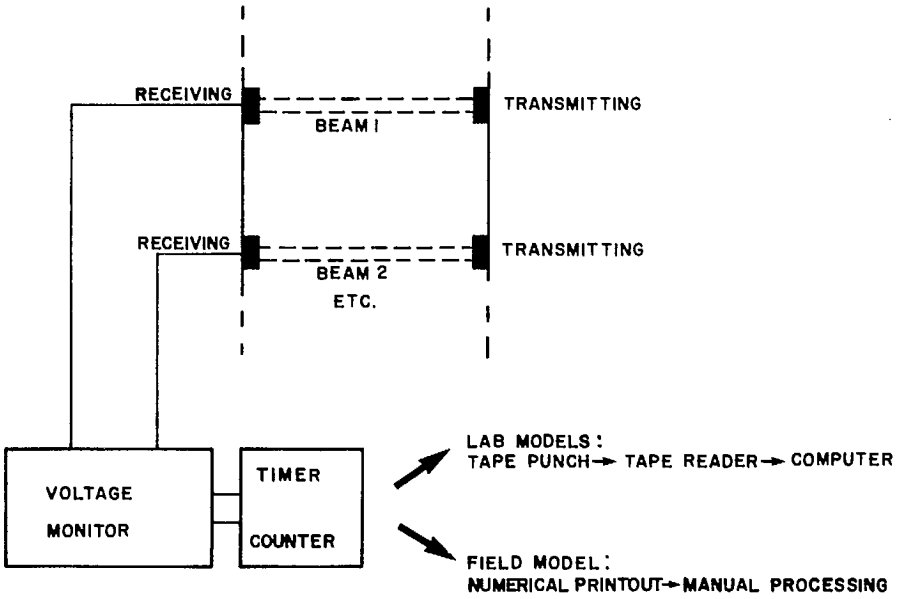

Figure 2. Schematic of activity data recording and processing in laboratory and field versions of ultrasound-beam actograph (after Scherer and Harrison 1979).

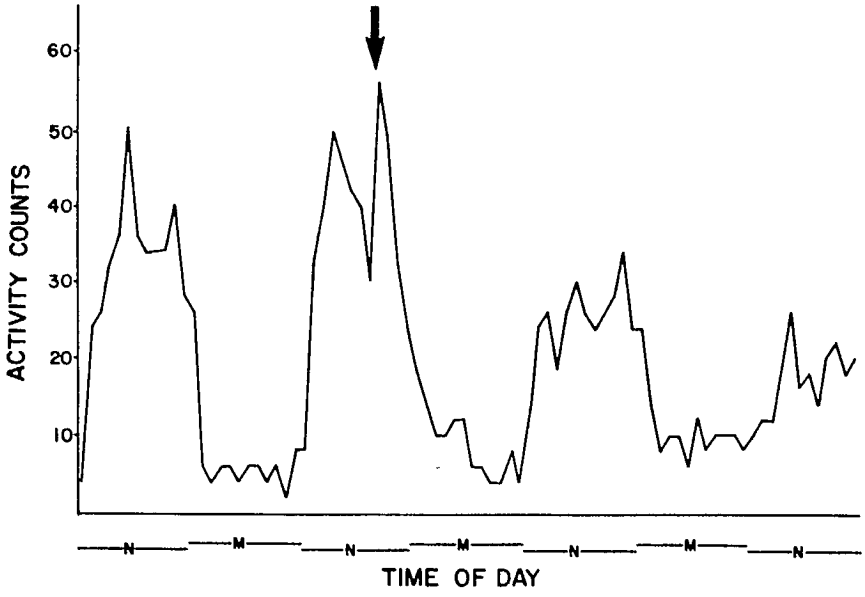

Figure 3. Effect of fenitrothion on locomotor activity of 5 whitefish, Coregonus clupeaformis. M = midnight; N = noon (12L:12D); flow-through technique; single "spike" exposure to 2.5 mg/L (arrow); water replacement rate 50%/1.5 h.

toxicants cause significant changes of activity patterns and levels (Fig. 3).

DISCUSSION

The advantage of this test is the ability to monitor a biological response automatically and continuously (day and night) under both lab and field conditions. As mentioned above, reduction of activity level is one of the sublethal effects known. One of the more conspicuous sublethal effects of mercury-poisoning observed in our lab (at that time not recorded due to lack of apparatus) was increasing sluggishness and reduced escape behaviour of walleyes, Stizostedion vitreum vitreum (Scherer et al. 1975). Such an effect would reduce survival chances of this species under field conditions. Changes in activity patterns, i.e. displacement of activity peaks and lows over a circadian cycle, together with activity level reductions, have also been observed in ongoing experiments with fenitrothion. On the other hand, hyperactivity has been reported as an effect of chlorinated hydrocarbons (e.g. Weis and Weis 1974; Ellgaard et al. 1977). The high level of integration underlying locomotor activity makes a variety of different responses to different toxicants likely, depending on their mode of action at the physiologic-metabolic level. It is hoped that our method will help to define and quantify these effects.

COSTS AND STAFF REQUIREMENTS

Ultrasonic transducers, each	$ 25-50

Electronic circuitry for 2 beams including amplifiers, voltage monitors, timers, etc. (lab models) approximately	$ 400
Tape punch system consisting of tape punch and punch controller (for lab models)	$1800
Electronic circuitry for up to 6 beams including calculator/printer and 2 car batteries (for field models)	$ 600

Electronic workshop help (costs not included above) will be needed for assembly of apparatus. To run the tests, knowledge of and access to modern data processing methods is helpful. Using a Hewlett Packard Model 9100B calculator and plotter, interfaced with a paper tape reader, approximately 6-8 man-hours are required to process and plot the results of two weeks continuous monitoring per tank. Presently, programs for utilizing an IBM 370 terminal are being developed and tested, with the aim to speed up data handling, and to facilitate statistical analysis.

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