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Bio-assays for Microchemical Environmental Contaminants

With Special Reference to Water Supplies *

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A solution of the problem of environmental contamination must be based on accurate measurement of the extent of the contamination and of the resulting hazards. This paper reviews the methods for the estimation of microchemical contaminants in water with the aid of living organisms. The methods are grouped according to the nature of the response of the organism to the contaminant—namely, acute response (usually death), behavioural change, physiological change, biochemical and histochemical change, ecological change, embryological and regenerational change, growth change, histological change and perception by man or aquatic organisms. Finally, the following problems are discussed: selection of appropriate tests and standardization, the dangers of sequential concentration and the need for multi-parametric assays (assays involving several responses of a single organism, or responses of several organisms) for complete characterization of the effects of a contaminant on the environment.

This paper, which is concerned with questions of bio-assay procedures as adapted to the study of actual and potential microchemical contaminants of the environment, was originally conceived as a short review of the recent work in the field, followed by recommendations for possible standardized procedures. However, it very soon became clear that a short and cursory review would prove of questionable use to any serious student of the problem. The relevant work is widely scattered among several disciplines, and contact between workers has been at best slight, with the inevitable result that progress, while substantial, has been fragmentary and lacking a common focus.

However, a number of concepts are emerging to give unity to the diverse and uncoordinated efforts of scientists interested in biological measures of environmental variables. An attempt has been made to define and illustrate some of these concepts in the following discussions, and thus to provide a rational and biologically valid basis for evaluating the myriad bio-assay techniques that have been or are being developed, for examining the feasibility of selecting specific bio-assay procedures to be considered for standardization, and for interpreting the results of such standardized bio-assays so that the findings will contribute as fully as possible to the solution of the theoretical and practical problems of microchemical toxication.

In view of the amount of material covered, detailed accounts of specific procedures will be given only when they are particularly necessary for illustration or are of special interest. Complete information on the procedures mentioned is given in the works cited in the list of references.

MICROCHEMICAL CONTAMINANTS: SOME GENERAL CONSIDERATIONS

Macrochemical and microchemical contaminants

It is useful at the outset to distinguish between macrochemical and microchemical contaminants. The former are compounds which, while having relatively low toxicities, are often released into the environment in large quantities. They often have a degrading effect upon the entire ecosystem. Sewage, many industrial wastes, nitrates, phosphates, and similar materials fall into this category. In contrast

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to this, microchemical contaminants are biologically active compounds capable of exerting adverse effects on living systems at concentrations of the order of 1 mg/l (1 ppm) or less. The most common ones at present are the organochlorine (chlorinated hydrocarbon) pesticides, herbicides, organomercury compounds, and some petrochemical wastes.

Microchemical contaminants may be distinguished from macrochemical contaminants by a number of further characteristics, including:

(*a*) a high degree of physiological and ecological specificity (i.e., they are selectively toxic);

(*b*) resistance to biochemical degradation;

(*c*) a strong tendency towards sequential concentration in living organisms while passing through the trophic web;

(*d*) a capacity for delayed onset of toxication.

Thus a toxicant used in the control of a forest insect may decimate the aquatic fauna of a stream into which it has been carried by drift or rainwater, while leaving the aquatic flora relatively intact (Cope, 1961). Such a toxicant may also persist in the soil of the forest floor or upon the sprayed trees for extended periods of time, owing to its resistance to biochemical degradation (O'Brien & Matsumura, 1964). It may first be concentrated in aquatic plants, then be transferred to and further concentrated by herbivorous fishes, and finally reach lethal concentrations in the blood of fish-eating birds (Hunt & Bischoff, 1960; Hunt & Keith, 1962). If the concentrating mechanisms bind the toxicant in such a way that it is out of contact with sensitive systems within the organism, toxication may be delayed until the organism is subjected to a stressor. The tendency of the organochlorines to accumulate in body fats, and then be released into the blood during periods of cold or food deprivation stress, constitutes such an example of delayed toxication (G. Cooch, D. Mount, P. McKinley—personal communications).

Nature of the problem

The recent publicity and controversy over the real and suspected dangers of the massive use of synthetic organic biocides has served to define the general nature and magnitude of the problem of environmental contamination by microchemical toxicants. That there is a problem should come as no surprise, for the evidence has been clear in its implications. Large numbers of states have been moving rapidly towards a high degree of industrialization, although actual progress has varied widely from nation to nation. With this industrialization has come the production of waste products, and new and exotic chemical compounds. Chemical control of agricultural pests, both plant and animal, is now almost universal. Pesticides are also used on a large scale in health programmes and by civil governments and military services to control disease-bearing or pest invertebrates.

Hence the question is not the presence or absence of environmental contamination, but rather its extent, and the present and potential direct and indirect influences this contamination has on the earth's biota, including man. It should be recognized, however, that the synthetic organic biocides (pesticides and herbicides) are but one source of microchemical contamination. Petrochemical wastes, industrial effluents and mine-drainage waters have on occasion proved extremely toxic and hazardous to aquatic life. The evidence also indicates that there are additional materials exerting deleterious influences, whose specific identities and characterizations remain to be established. For example, there is now appearing in certain domestic water supplies of urban Germany and most assuredly in other countries as well an unidentified toxicant that may originate in automotive exhaust gases or in road asphalts. Dr Helmuth Althaus of Gelsenkirchen states (personal communication) that the Berlin Institute of the Ministry of Public Health, Federal Republic of Germany, is now working on filtering procedures which will permit collection of the material for analysis and evaluation of its toxic properties.

The general nature of microchemical contamination of the environment has been comprehensively treated by several students of the problem (Carson, 1962; Hynes, 1960; Marchetti, 1962; Rudd, 1964). The integrated, holistic treatment of the mass of accumulated evidence by Rudd (1964) is worthy of special attention.

THE ROLE OF BIO-ASSAYS IN EVALUATING MICROCHEMICAL CONTAMINANTS

Historical aspects

The history of bio-assays is a long and chequered one. Probably the first deliberate use of a bio-assay procedure to determine microchemical contamination was the employment of tasters by early monarchs to assure themselves that no evil game was being played with their dinner. During the early stages of

chemistry, pharmacology, and similar fields bio-assays were a standard tool for quantifying the amount of active principle in a compound. This application of the bio-assay, especially in endocrinology and related fields, remains a common practice today.

However, with the evolution of highly sophisticated instrumentation and analytical procedures (chromatography, spectrography, etc.) there has in recent years been a strong trend away from the use of bio-assays for quantitative analysis. One consequence of this shift in emphasis has been an increasing paucity of information on the biological properties of the many new and esoteric compounds being produced by synthesis, or as by-products of the various activities of our modern technologies. A valuable result of the recent " pesticide controversy " has been the clear demonstration it has provided of the universal ignorance prevailing as to the possible consequences of releasing into the environment compounds whose biological characteristics remain undefined. The present swelling research effort directed towards supplying the deficient information has been in large part motivated by a realization of the extent of our ignorance. It is in this area of study—the qualitative and quantitative evaluation of toxic effects on living systems—that bio-assays find their widest application and potential for future development.

Definition of the bio-assay

Defined in its simplest and most meaningful form, a bio-assay is the use of a living system to assess the effects of an environmental variable. The bio-assay may be used to determine:

(*a*) the quantity of active substance or influence present;

(*b*) the kind of influence (quality of change) exerted on the test system; and/or

(*c*) the magnitude of change in the system being employed.

The living system may be a bacterial colony, a protozoan, a fish, or a circumscribed portion of an ecosystem. The environmental variable may be a highly biocidal chemical (e.g., an insecticide), a compound that alters the physical or chemical characteristics of water (e.g., a detergent), or some other specific environmental variable such as water temperature. The term " bio-assay " loses its meaning only when the living system used in the assay has been so simplified (as, for example, mitochondrial suspensions or enzyme extracts) that there is doubt as to whether the reaction is occurring *in vivo* or *in vitro*.

For the purposes of our present discussion, microchemical contaminants, sometimes accompanied by other stressors such as heat or reduced oxygen supply, become the environmental variables. Despite this, one should not lose sight of the fact that bio-assays are regularly employed in a diversity of circumstances where microchemical contaminants are not involved. Experimental embryology, endocrinology, virology, and histopathology are but a few areas capable of yielding a rich harvest of bio-assay methods requiring only slight modifications to render them useful in questions of microchemical toxicity.

Regardless of the species or system employed in the test, most bio-assays have one thing in common: they utilize *deviation from the norm* in the process being measured as the parameter of effect. Thus, a reduction in rate of O_2 consumption, an increase in sensitivity to external stimuli and a shift in rate of somatic growth all provide quantitative units of change due to environmental variables.

Some further definitions

To complete the frame of reference of this discussion, several other basic concepts are defined in the sense in which they will be used in this article.

Microchemical contaminant. As considered above in some detail, a microchemical contaminant is a biologically active compound capable of having *adverse* effects on living systems at concentrations of 1 mg/l or below. It is generally an exotic compound, new to the evolutionary history of the exposed organisms, so that detoxication and other metabolic mechanisms capable of dealing with it are not well developed.

Toxicant. This is a general term for any environmental variable, usually but not necessarily a chemical, which produces a demonstrable *aberration* in one or more life processes. All microchemical contaminants are thus toxicants, but the reverse is not necessarily the case. That is, oxygen depletion in an aquatic habitat and the resulting anoxia fulfil the criteria for a toxicant, as does excessive heat (heat toxication). It is the quality and quantity of impact upon the organism, the degree of aberration from normal in relation to the magnitude of change in the variable, that characterize a toxicant.

Acute effects. These are the aberrations produced by a toxicant within the first 100 hours or so of

exposure. DuNony (1937) and Gaddum (1953) have both correctly pointed out that biological time is a logarithmic phenomenon. "Time-to-death" curves for various concentrations of microchemical toxicants all show this relationship very clearly. After about 100 hours the slope of such curves rapidly approaches the horizontal, so that a further reduction in toxicant concentration greatly increases the time required to achieve the end-point.

Chronic effects. These are adverse effects that begin to appear after the *circa* 100-hour mark. Such pathological conditions as liver and kidney dysfunction, haemopoietic changes and histological changes in many different tissues may fall into this class.

There are certain functional differences between many "acute" and "chronic" symptoms; acute toxication, for example, is often unaccompanied by any detectable histological change, the aberrations being due to impaired gas-exchange capacity, disorganization of nervous system function, or uncoupling of a critical metabolic enzyme system. The dichotomy between "acute" and "chronic" symptoms is well illustrated by the organochlorine pesticides. Most of these compounds are violent neurotoxins, acting directly in some as yet unidentified way (O'Brien & Matsumura, 1964) upon nervous tissues, to alter their impulse transmission and irritability characteristics. "Acute" symptoms are thus associated almost entirely with nervous system dysfunction; histological changes are rarely detected.

However, in addition to the high initial neurotoxicity, continued sublethal exposure may produce a host of chronic symptoms, including fat and protein dystrophy of the liver and kidneys, thickening of the interalveolar septum, perivasculitis, oedema of the brain, leucocytosis, abnormal numbers of erythrocytes, and modified behaviour patterns (Ulanov et al., 1960). It is noteworthy that chronic effects may require from a few days' to several years' exposure to a toxicant for their elicitation. The findings on long-term smoking and lung cancer clearly show the length of time which may be required for the ultimate expression of a chronic toxic response.

THE NEED FOR ADEQUATE CHARACTERIZATION

In order to evaluate the consequences of releasing a microchemical contaminant into the environment, one must know the effects it will have upon the living things it contacts. This is a deceptively simple point; its apparent simplicity has, as a consequence, often led to gross underestimation of the complexity of the problem. One sanitary engineer, frustrated by the lack of solid and tangible guide-lines available for those dealing with actual pollution situations, concluded that "what remains to be done is for the professional biological fraternity to decide and agree upon what over-all sublethal response [of an organism to toxicants] applicable to many organisms in the food chain is the most sensitive and interpretative; and to develop the most precise methodology to evaluate the response" (Pearson, E.—unpublished).

Unfortunately, as will be shown below, this wish cannot be fulfilled. It is becoming abundantly clear that no single bio-assay, regardless of its sensitivity or sophistication, can provide more than a fragment of information on the consequences of releasing a toxicant into the environment. While all living things have certain physical and chemical properties in common, these similarities must not be permitted to obscure the fantastic morphological, physiological, and biochemical diversity which is found in the biological world. In addition, systems common to the vertebrate animals may be entirely lacking in the invertebrates, and *vice versa.* Plants and animals respond quite differently, as well. Hence, certain toxicants, such as the herbicides, while often relatively innocuous to animal life, may have profound effects on many elements of the flora of an ecosystem. The problem is not hopelessly complex, but it is a grave mistake to underestimate the degree of its complexity.

It is now clear that the best approach is proving to be a multiparametric one based on a quantitatively analytic system utilizing a diversity of bio-assays on a diversity of living systems. Such multidimensional views of a toxicant, furnishing information on what I have termed "toxic response syndromes", offer by far the most comprehensive approach to the effects of microchemical contaminants on living systems. Such a holistic approach to toxication in the environment is essential if we are ever to achieve our goal of predicting the effects of new and empirically untested compounds.

A REVIEW OF CURRENTLY EMPLOYED AND POTENTIAL BIO-ASSAYS FOR MICROCHEMICAL CONTAMINANTS

General considerations

I have, as will be seen, limited my considerations almost entirely to problems of contamination of

water supplies. This explains the preponderance of bio-assay methods described below that utilize aquatic organisms. Nevertheless, many of these procedures can be applied either directly, or with reasonable modifications, to other aspects of the environment, such as soils.

The bio-assay methods currently employed for microchemical contaminants, or those which could with slight modifications be adapted to this purpose, are manifold, and fall into several natural groups. They differ in the amount and kind of information they produce on toxicity, and in the ease with which their results may be extrapolated to field conditions. All, except those involved with sensory perception, utilize deviation from normal as the response parameter. These bio-assays may be classified according to the response parameters utilized, as follows:

(*a*) acute response (usually with death as the end-point);

(*b*) behavioural change;

(*c*) physiological change;

(*d*) biochemical (including histochemical) change;

(*e*) ecological change;

(*f*) embryological and regenerational change;

(*g*) growth change;

(*h*) histological change;

(*i*) perception by man or test organisms.

Acute-response bio-assays

The most commonly employed bio-assay is the TL_m (median tolerance limit) test, giving the time-concentration relationship for which 50% of the test organisms expire or otherwise cease to function in a gross way. Standard methods for this procedure have been published by the American Public Health Association (1960) and the American Society for Testing Materials (1959). This method has been applied, in one form or another, to a great variety of aquatic organisms, including fish (Carpenter, 1927; Doudoroff et al., 1953; Henderson et al., 1959, 1960; Jones, 1938; Weiss & Botts, 1957; Workman & Neuhold, 1963), arthropods (Anderson, 1944, 1945, 1950; Bushland, 1951; Loosanoff, 1960), molluscs (Loosanoff, 1960; Wurz, 1962) and other forms (Anderson et al., 1948). Doudoroff & Katz (1950), Rudd & Genelly (1956) and others have contributed useful reviews of the findings so developed.

Since the lower limit of toxicity indicated by such tests is known to be higher than the true biological threshold of response to the toxicant, " application factors " have been employed to extrapolate down from the TL_m values into presumably safe concentration realms. Henderson (1957) and Warren & Doudoroff (1958) have discussed these " application factors " in terms of industrial wastes.

It is of interest that different regions have adopted widely varying criteria for applying the results of acute-response bio-assays. In America a 96-hour TL_m with an application factor of $\div 10$ is generally used. In Holland the usual practice is to accept a 20-day TL_m with an application factor of $\div 10$. In Germany, followed recently by Switzerland, the maximum permissible concentration is determined by a 20-day TL_m with an application factor of $\div 20$. The USSR, in contrast to the above countries, gives the impression of virtually rejecting the TL_m concept as a result of the excellent progress being made there with behavioural and other measures of sublethal toxication. It should be stressed that these are not " official " standards set by the respective governments, but rather standards which seem, from numerous discussions and reviews of the current literature, to be widely accepted by those concerned with problems of environmental contamination.

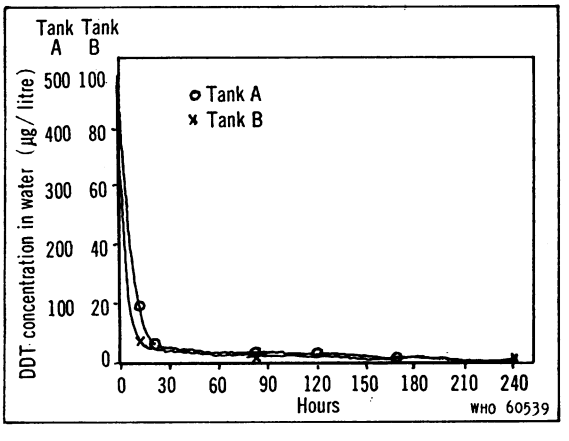
Lloyd (1961), Neuhold & Sigler (1962), Gaines (1962), Lloyd & Herbert (1962), Adlung (1957) and others have successfully employed the acute-response method in examining the effects of multiple-toxicant exposures and the influence of a variety of environmental parameters such as temperature, water hardness, and so forth upon the toxicity of microchemical contaminants. The method has also been used to determine the toxicity of run-off water from pesticide-treated fields (Tarzwell & Henderson, 1956).

It should be recognized that the acute-response method, employing as it generally does death or immobilization of the organism as criterion, is not a biological measure in the sense that other types of bio-assays are. Death is, by definition, the cessation of biological activities. Hence, it is asymptomatic; that is, no information relevant to sublethal effects can be obtained from the response. The acute-response assay has the advantage of being fast (as fast as the experimenter wishes to make it simply by manipulating the concentration), easy to interpret, and simple and cheap to employ. While in some disrepute at present, it may ultimately prove a highly valuable tool for certain monitoring purposes when used in conjunction with, or after base lines have been established by, multiple bio-assay procedures.

Acute-toxicity tests have as a rule been conducted in static systems; i.e., aquaria where the water is changed only periodically, generally once every 24 hours and sometimes not at all during the test. The findings of Holden (1962), based on a study of ^{14}C-labelled DDT, are of great importance in evaluating the results of such static tank tests. He found (see Fig. 1) that over 95% of the DDT had been removed from solution in static bio-assay tanks within 24 hours; up to 90% was removed within the first 10 hours. In addition, of the DDT removed from the water during the test period, an average of only 57% was absorbed in any manner by the test fish, the remaining 43% being absorbed or adsorbed by or on to the detritus and the tank surfaces. The situation is in part caused by the insoluble nature of many organochlorines in water. For example, DDT at a particle size of <41 Å has a solubility of 1.2 µg/l at 25°C (Bowman et al., 1960). It is therefore clear that great care must be exercised in interpreting the results of any static tests, including those employing acute response.

FIG. 1

RESIDUAL CONCENTRATIONS OF ^{14}C-LABELLED DDT IN AQUARIUM WATER OVER A 240-HOUR PERIOD [a]



[a] Reproduced, by permission, from Holden, A. V. (1962) *Ann. appl. Biol.*, **50**, 474.

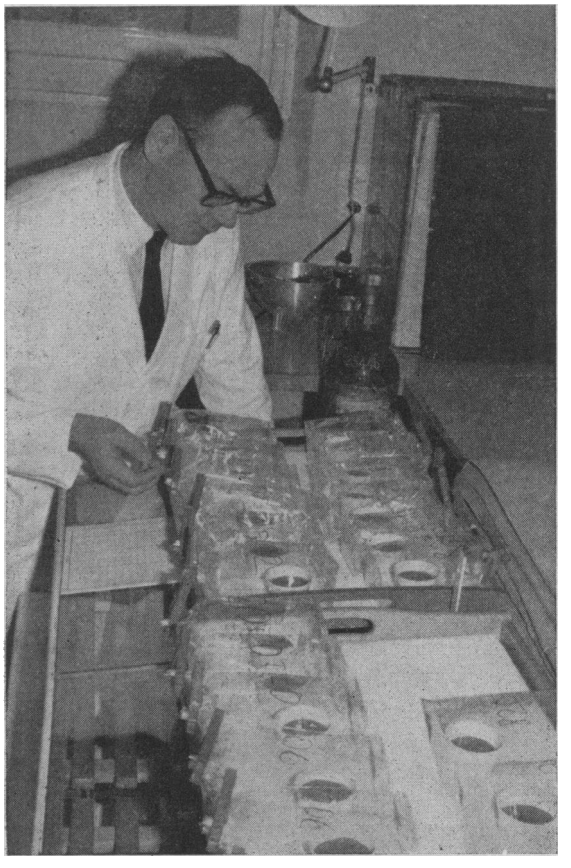
Many of the difficulties inherent in static test procedures can be overcome by using continuous-flow exposure systems. The manifold problems of loss through absorption and adsorption, secondary toxication through accumulated metabolic wastes and so forth are either eliminated or vastly reduced. However, until recently building and maintaining continuous-flow systems was a major problem, involving considerable time and expense. Fortunately, several practical continuous-flow apparatuses have now been developed, such as that of Mount & Warner (1965). Their continuous-flow device, which was developed especially for microchemical contaminants, can be constructed in a few days by laboratory personnel using readily available laboratory glassware. Total cost for the materials is less than US $50. It has proved highly dependable over testing periods of up to one year.

Other innovations, such as the simple, inexpensive, continuous-flow pump developed by Symons (1963), provide a variety of possibilities for future workers. The Water Pollution Research Laboratory (1965) in Watford, England, has also contributed some useful ideas to this subject.

A highly successful technique employed by Hasselrot (1964, 1965) which more or less falls within the present category involves placing fish in cages and exposing them *in situ* to the contaminated environment. By determining the currents and water-flow patterns, and siting the cage in such a way that it is exposed to a variety of environmental conditions in the aquatic habitat to be studied, he has what amounts to a perfect continuous-flow system. Using this scheme, he has been able to evaluate both short- and long-term toxicities of lake waters contaminated with pulp mill effluents, and of river waters contaminated with heavy metals from mine drainage. In one such study (personal communication) of pollution of the Swedish river Dalälven in which caged salmon (*Salmo salar*) fry were used, he obtained, in addition to the usual mortality data, evidence of extreme depression of haematocrit values after 14 days' exposure. This approach has much to recommend it and warrants further development.

Several acute-response assay methods have been developed for use with the fruit fly, *Drosophila melanogaster*. Dr H. R. Weilenmann (a Cantonal Chemist for Zurich, Switzerland) among others has found it practical to expose fruit flies held in transparent, air-filled plastic bags to pesticide-contaminated samples. Fig. 2 shows such a test in progress. Known numbers of flies are placed in the bags with small dishes of sample mixed with a small amount (normally 4%) of honey to induce feeding by the flies. The number of dead flies and the number with abnormal behaviour are recorded at predetermined intervals. The method is inexpensive and simple to employ.

FIG. 2
PESTICIDE RESIDUE TEST WITH PLASTIC BAGS AND *DROSOPHILA MELANOGASTER*



A similar procedure, but one that permits testing of extracted or concentrated toxicants, uses small vials or glass jars. Solvent-extracted toxicants are placed in the vials or bottles, and then rotated or swirled until the solvent has evaporated, leaving a film of toxicant coating the inner surface of the container. Again, known numbers of *Drosophila* are introduced and their condition is periodically recorded. Mortality curves and evidence of sublethal behavioural pathology from contact with the deposited toxicant can then be obtained.

These systems have both advantages and failings. The use of easily-reared *Drosophila* is an important point in their favour. However, it has been found that many solvent extracts of plant materials have intrinsic toxicities of their own, making it difficult to ascertain with certainty the toxic contribution of the microchemical contaminant. The systems have none the less been reported to be successful in actual use, and should therefore be given careful consideration where bio-assay data of this sort are desired.

Still another system, designed to measure the toxicity of the vapour-phase of toxicants, employs *Drosophila* held over, but out of direct contact with, a toxicant which has been deposited on a filter-paper. This method is reported (*Chem. Engng News*, 1961) to have been originally developed by Sun & Sun (1952), who for some years have addressed themselves to bio-assays for microchemical toxicants. The system is shown in Fig. 3. Two Petri dishes are used, the toxicant-treated filter-paper being placed in the bottom dish. A filter-paper is placed between the two dishes, the second one being inverted and placed on top. After the dishes have been clamped together with rubber bands, flies are introduced through a small hole previously drilled in the centre of the top dish. It is important to maintain a high relative humidity and a source of food in the upper chamber, so a small dish of water and a vial containing sugar water are placed in the unit.

After the flies have been introduced, the Petri dishes are placed in an incubator at a pre-set temperature, usually 25°C and never over 30°C. The toxic vapour diffuses upward through the filter-paper floor of the fly chamber. Mr S. L. Wit of the Utrecht group reports (personal communication) that 0.05 mg/l of dichlorvos (DDVP, a high-vapour-pressure pesticide) is detectable in treated cereal when extracted with methylene chloride and the residue dissolved in petroleum ether.

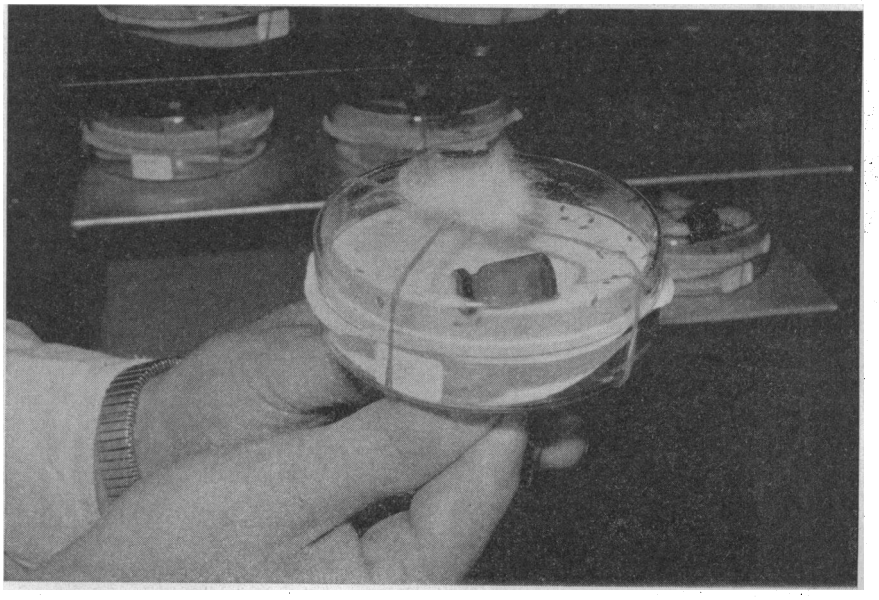
The amount of residual oil in the extract is reported to have an important influence on the vapour pressure of many microchemical contaminants, and may therefore interfere with the sensitivity of the test.

A *Drosophila* collecting pipette has proved useful for adding flies to the test chamber, according to the Utrecht group. A glass tube, modified as shown in Fig. 4, is attached to a vacuum line, and the requisite number of flies are sucked into the tube, the amount of vacuum being controlled by a finger-covered orifice on the side of the tube. The flies are then blown from the pipette into the top chamber through the small hole drilled in the top.

Bio-assays using behavioural change

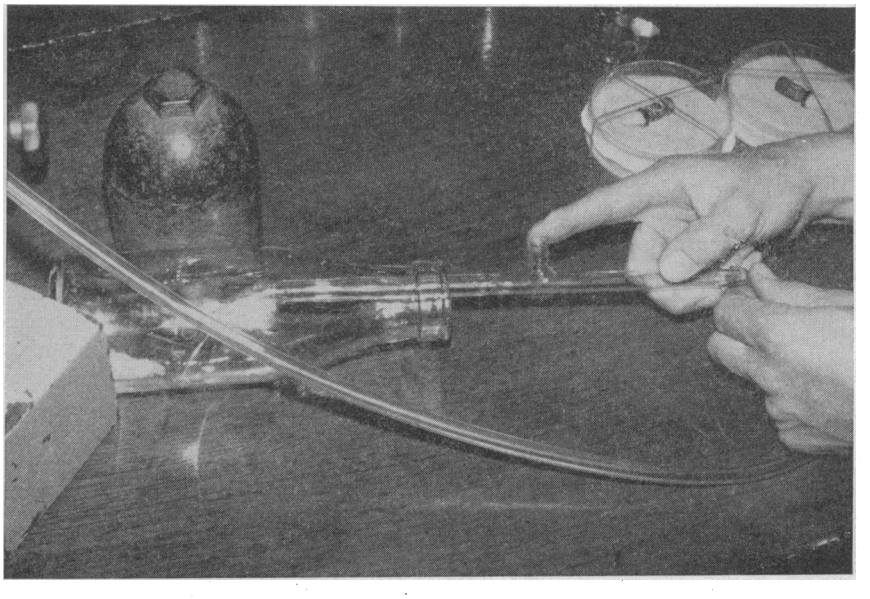
Despite the very substantial progress in basic studies of animal behaviour (ethology) and the widespread use of behavioural measures in the study

FIG. 3
VAPOUR-PHASE BIO-ASSAY USING *DROSOPHILA MELANOGASTER*



of drugs and pharmaceuticals, relatively few attempts have been made to develop behavioural measures of sublethal toxication for microchemical contaminants. Even today the behavioural bio-assays of chemical effects upon aquatic organisms are focused principally on pharmaceuticals and other similar compounds, which are rarely, if ever, encountered as environmental contaminants.

FIG. 4
DROSOPHILA PIPETTE IN USE



For example, very many papers [1] have recently appeared on behavioural changes in fish (principally the Siamese fighting fish, *Betta splendens*) due to toxication with LSD-25 (lysergic acid) and other drugs. Despite the fact that LSD-25, reserpine, chlorpromazine, etc., in no way constitute microchemical contaminants (it being virtually inconceivable that appreciable quantities of these compounds should ever reach aquatic ecosystems), the experiments using these toxicants with aquatic organisms can none the less be very instructive.

If we take the case of LSD-25, we find that a variety of changes in behaviour have been recorded. Unfortunately, most of the studies were only qualitative in nature, leaving unsolved the important but difficult problems of quantifying the responses. For example, at a concentration of " 2 gamma/ml " (*sic*; presumably 2 mg/l of test water) LSD-25 produced surfacing behaviour in goldfish after 10 minutes of exposure, the same response in carp (average weight 2 lb, or 900 g) after 45 minutes, but no change at all in brook trout (Abramson et al., 1961). The frequency of the nose-up, tail-down position (typical LSD-25 reaction in Siamese fighting fish) was markedly reduced, and the initial excitatory phase was absent, when 2.0 g/l of crude brain extract was added to the test tank (Abramson et al., 1957). The angle assumed by the toxicated fish (deviation from the normal horizontal position) proved a moderately sensitive measure of LSD-25 toxication (Baron et al., 1958). In other investigations, use was made of elements of the normal social behaviour of the Siamese fighting fish—namely, chasing, fin display, biting and tailwhips. After exposure (as opposed to *during* exposure), fish became markedly more aggressive. Low-ranking individuals rose in the social hierarchy as a consequence of increased hostility towards other, previously more aggressive fish (Evans et al., 1958). Recordings of muscle action potentials in Siamese fighting fish subjected to a " controlled stress " (hypoxia) (Trout, 1957a) showed that LSD-25 depressed the intensity of activity due to the stress, whereas serotonin increased it. Both compounds reduced the duration of the " almost-continuous-activity " phase of the toxic response. When added simultaneously, serotonin potentiated the effect of LSD-25 on " intensity " and increased its effect on the duration of " almost continuous activity ". The same author (Trout, 1957b) also reported aberrations indicating " perceptual or mood changes ". A chromatic (darkening) response was noted in *Fundulus*, the change being interpreted as due to an effect on the control mechanism of the chromatophores (Wilber, 1958).

From this array of observations, the following behavioural bio-assay parameters can be identified:

(*a*) surfacing behaviour (including its latent period);

(*b*) quantity of tail-up, nose-down response;

(*c*) altered social behaviour (aggression or hostility);

(*d*) duration of almost continuous activity;

(*e*) intensity of almost continuous activity;

(*f*) perceptual or mood changes;

(*g*) chromatophore response (darkening);

(*h*) species-specific characteristics (goldfish, carp, trout).

At the moment, the only highly quantified parameter of the group is that used by Trout (1957a, 1957b) where the changes in muscle action potentials are recorded on a Grass encephalogram. The counting of defined elements of social behaviour (chasing, fin display, biting, tailwhips) also produces very useful quantitative data (Evans et al., 1958). It is clear that development of means of quantifying the other parameters (and possibly adding still more parameters to the repertoire) could give a meaningful multiparametric behavioural bio-assay for compounds of the type of LSD-25. But more important, these sublethal toxic responses may have considerable potential if properly quantified and applied to microchemical water contaminants.

Barbiturates, hypnotics, tranquillizers and other psychotrophic drugs have been subjected in some detail to behavioural bio-assays using aquatic organisms. In most cases the bio-assays were on fish and were designed, not to determine the quantity of material present, but rather to demonstrate (sometimes quantitatively) qualitative changes in behaviour due to sublethal toxication. A few workers (e.g., Ivanova, 1961a, 1961b) were attempting to explore the nature of behavioural mechanisms using drugs as biochemical manipulators of the nervous system.

A review of the different approaches applied to the question of sublethal behavioural pathology caused by these compounds produces a variety of functional behavioural parameters. For example, BTBH (a 3,4,5-trimethoxybenzoyl derivative) at a

[1] Abramson, 1959; Abramson & Evans, 1954; Abramson et al., 1957, 1958, 1961; Baron et al., 1958; Bernstein, 1960; Evans et al., 1956, 1958; Loeb, 1962; Müller, 1959; Smith & Moody, 1956; Trout, 1957a, 1957b; Turner, 1956a, 1956b; Wilber, 1958.

concentration of 5-10 mg/l in water reduced the "combat drive" of Siamese fighting fish (Barth et al., 1962). Secobarbital sodium produced in minnows (*Pimephales promelas*) a variety of symptoms, including "sluggishness", "rolling from side to side", and "retarded breathing" (Bezdek, 1957). In the green crab, *Carcinus maenas*, physostigmine and cocaine (as well as phenol and picrotoxin) caused an "augmentation of reflex action" which according to Blume (1930) was central in origin and located in the abdominal ganglia. Chlorpromazine depressed both "conditioned activity" and "motor activity" in fish, the pattern of depression being complex. In the case of chain motor reflexes, chlorpromazine successively shut off the individual components of the motor reflex. First, it inhibited the components most remote from the time of reinforcement by food, and last of all those components that were directly connected with the food (Ivanova, 1961a, 1961b). Camphor, caffeine, pentetrazol and amphetamine had a "stimulatory" effect on *Betta splendens*, while ephedrine and cocaine had both stimulating and depressant effects (Ketusinh et al., 1962). The arousal response of the Siamese fighting fish was used in a study of the effects of amphetamines (Kraft et al., 1962). Centrophenoxine and its derivatives caused excitation in fish, to the point of producing convulsions. They also induced dilation of the chromatophores (Nakajima et al., 1961). Pentobarbital was found to induce narcosis in *Lebistes reticulatus* (Onkst et al., 1957). Changes in motility and colour (melanization or chromatophore response) were produced in *Phoxinus phoxinus* by imipramine, LSD-25, and another psychotrophic drug (Thuillier et al., 1961a). The same group studied the effects of melatonin, biogenic amines and acetylcholine in relation to the responses just described (Thuillier et al., 1961b). Chlorpromazine reduced the hypoxic stress response in *Betta splendens* and had an opposite effect to that of reserpine on the chromatophores (Trout, 1957a). The fighting response of the Siamese fighting fish was used to evaluate the properties of certain tranquillizers (Walaszek & Abood, 1956).

From this pot-pourri of interactions between drug and aquatic organisms, the following behavioural parameters can be gleaned:

(*a*) strength of combat drive (= fighting response);

(*b*) general movement (tendency towards sluggishness);

(*c*) side-to-side rolling (= loss of equilibrium?);

(*d*) breathing rate;

(*e*) augmentation or depression of reflex action;

(*f*) alteration of conditioned responses;

(*g*) change in motor activity;

(*h*) sequence of interruption of chain motor reflexes;

(*i*) stimulation or general excitation;

(*j*) dilation of chromatophores;

(*k*) narcosis;

(*l*) alteration in hypoxic stress response.

As with the group of drug-behaviour interactions discussed above, this gives a good basis for a multiparametric behavioural bio-assay. However, much effort still needs to be devoted to proper quantification of the responses to the drugs. It should be stated, even though it has already been implied, that it is far easier to identify a *qualitative* change in behaviour due to a microchemical toxicant than it is to *quantify* that same response. Unfortunately, before any bio-assay can be of maximum usefulness, the inherent problems of quantitation must be solved. For only then can the dimension of *magnitude* be added to the toxic response equation.

There is also an abundant literature on sublethal effects of drugs upon terrestrial animals. This literature has been excluded from the present discussion because it is of only limited application to the question of microchemical contaminants in water.

Another series of investigators have addressed themselves directly to behavioural aberrations produced by microchemical contaminants. The evidence developed from these studies will seem to many to be more immediately applicable than the above examples to the present question. None the less, one should not forget that there are basic similarities in all behaviourally-oriented bio-assays, whether they be first worked out for organochlorines or amphetamines.

Allison & Cole (1934) and Cole & Allison (1931, 1933) studied the "stimulation" of *Fundulus*, a sunfish, *Euponotis* sp., and a catfish, *Schilbeodes* sp., by various acids and their sodium salts. They found differing degrees of "stimulating efficiency" in the different compounds. Belding (1927), in one of the early studies where behaviour was used as a parameter, recorded loss of equilibrium in fish exposed to small amounts of nitric and hydrochloric acids, and "head balancing motions" in solutions of calcium hypochlorite.

Eichenberger (1960) has perfected a technique using the light-response of mosquito larvae which is both ingenious and practical. The unit, which is essentially a light box with a built-in magnifying glass, is placed over a glass dish containing mosquito larvae of known age in a toxicant solution. The responses of the larvae to a small light at one side or the other of the dish, which is turned on as desired by the observer, are recorded. Directionality, extent and types of movements have all been found useful response parameters. The system is reported to be very sensitive to the type and degree of toxication, and is now regularly used in the Cantonal Laboratory at Zurich, Switzerland, and probably elsewhere.

Along the same general line, Mellanby (1958), having successfully analysed the normal alarm reactions in mosquito larvae, is now adapting this syndrome to a behavioural bio-assay procedure for pesticides (Mellanby, personal communication).

Philip Butler, Director of the Sabine Island Biological Laboratory of the US Fish and Wildlife Service (personal communication), has developed a very ingenious procedure using the movement of the shells of oysters as an indicator of their toxication by microchemical contaminants. When a deleterious substance such as a pesticide is introduced into the water, the bivalves react in a variety of ways, such as violent cleaning movements, closure of the shell, etc. These movements are recorded and subjected to quantitative analysis.

Kaminski & Kisielinski (1962), Klock (1960), Klock & Pearson (1961) and Hopkins et al. (1931) have also clearly shown that invertebrates can be successfully employed as bio-assay organisms using behavioural parameters. Klock, for example, has used the pumping rate of the mussel, *Mytilus edulis*, the rate of clearance of a suspended fine clay by the tubeworm, *Mercierella enigmatica*, and the irrigation rate of the isopod *Sphaeroma pentodon* as measures of toxication by phenol. His work stressed quantification of the response rate and examined the threshold concentrations necessary to produce a detectable change in deviation from the norm.

The significant work of Kaminski (Military Institute of Hygiene and Epidemiology, Warsaw, Poland) is still in the developmental stage and most of it has not yet been reported in the literature. He is currently developing a multiparametric analytical procedure using a variety of vertebrate and invertebrate species. By employing an oligochaete (*Enchytraeus albidus*), a mollusc (*Planorbis corneus*), an isopod (*Asellus aquaticus*), a fish (*Lebistes reticulatus*) and on occasion other species, and by using a variety of behavioural measures, he is finding it possible to provide a comprehensive, multidimensional picture of toxicant effects. He has selected organisms which can be reared in the laboratory, assuring a continuous supply of test organisms of known background, avoiding potential problems of pre-exposure to pesticides and other contaminants. In contrast to my own work, discussed below, he is striving for measures which require a minimum of apparatus, and which can be employed by technical assistants having a minimum of special training. At present he is working with rather high toxicant concentrations, but it is believed that, as his analytical procedures are perfected, the sensitivity of the methodology will be markedly increased.

My own group (Warner, 1967; Warner et al., 1966) has successfully developed a method for simultaneously measuring a variety of behavioural parameters in fish. These parameters include, among others, total activity level, response to a novel stimulus (light), habituation to stimuli, rate and amount of avoidance-response acquisition, memory, rate of reversal learning of conditioned responses, sensitivity to temperature change and so forth. Comparison with the behaviour of healthy, untoxicated fish as norms allows a multi parametric profile of toxicant-induced behavioural aberrations to be rapidly generated. The raw data developed during a test are transferred to IBM punch-cards and subjected to a variety of statistical analyses on the IBM 7040 and 7090 computers. As a consequence, studies of sublethal toxication using an array of behavioural parameters can be conducted extremely rapidly and with virtually no risk of the subjective bias that often plagues behavioural studies.

One conclusion that emerges from these studies is that quantified behavioural change is the most sensitive indicator yet developed of toxicant-induced change in living systems. For example, parallel studies using the most sensitive histochemical and biochemical analytical procedures currently available clearly show the behavioural measures to be not only faster but at least 10 times more sensitive to microchemical contaminants such as the organochlorines and organophosphates (Warner, 1967).

While the behavioural bio-assays which have been worked out to date show considerable promise, it is clear that the potential for future advances is very great. A wide variety of existing behavioural measures await adaptation to problems of micro-

chemical toxication. Conditioned responses, some of which were employed by ourselves, offer a wide spectrum of possibility. Bianki & Demina (1963) and Lester Aranson (personal communication) have used them in examining the effects of brain lesions on fish behaviour. Other workers [1] have employed conditioned responses in a wide variety of ways, their various approaches and methods having varying potential usefulness as microchemical bio-assays. French (1942) and Jones (1945) examined the effect of temperature on retention in the goldfish, and Warren (1961) the effect of telencephalic lesions on learning in *Macropodus opercularis*. Others have employed methods using operant conditioning (Haralson & Bitterman, 1950), configurational learning (Perkins & Wheeler, 1930) and second-order olfactory and visual learning (Sanders, 1940) in fish.

Even such behavioural parameters as diurnal activity rhythms (Spencer, 1939) and precisely quantified components of social behaviour studied in the cichlid fish *Pelmatochromis* by Heiligenberg (1965a, 1965b), and swimming ability (Fry & Hart, 1948; Katz et al., 1959) are readily amenable through simple modifications to toxicant bio-assays.[2]

Bio-assays using physiological change

Owing to the difficulties inherent in such experimentation with aquatic organisms, bio-assays involving physiological measures of change have been only infrequently attempted. For the purposes of this discussion, it should be noted that the term " physiological " refers to those internal processes (e.g., muscle contractility, nerve impulse transmission, glandular secretion or excretion, and so forth) which function directly in the maintenance of the *milieu intérieur* and/or provide the immediate substrate mechanisms for behaviour. Obviously, then, there is no distinct line between " physiological " and " behavioural ", nor between " physiological " and " biochemical ".

None the less, the paucity of what would normally be called physiological measures which so far have been developed for aquatic organisms is striking. Of those workers interested in physiological mechanisms and their aberration due to microchemical toxicants, many have ended up relying largely upon behavioural, histological or biochemical measures to evaluate the changes. For example, the important work of Weiss on the effects of organophosphate insecticides on fish (1959, 1961), while entitled " Physiological effect... ", utilized a biochemical procedure involving sacrificing the test fish to determine the residual concentration of acetylcholinesterase in tissues following exposure to the toxicants.

Of the few physiological measures successfully applied to aquatic organisms, oxygen consumption (Halsband, 1955, 1957; Halsband & Halsband, 1954; McFarland, 1954; Weiss & Botts, 1957) and haemopoeietic changes (Dawson, 1935; Jakowska et al., 1958; Schiffmann & Fromm, 1959) seem most easily approached. E. and I. Halsband and Meyer-Waarden have pursued several interesting lines of approach, utilizing the conductivity (or specific resistance) of fish bodies, and thresholds of sensitivity to shock, as indicators of toxicant-induced changes (Halsband, 1965; Halsband & Halsband, 1965; Halsband & Meyer-Waarden, 1960, 1963). Fellman et al. (1962) recorded a qualitative inhibition of the chromatophore control mechanism in the guppy (*Lebistes reticulatus*), and Ogilvie & Anderson (1965) found a DDT-dependent shift in temperature preference in young Atlantic salmon (*Salmo salar*), low doses producing a shift towards a higher preferred temperature, high doses a shift towards a lower preferred temperature. There was some evidence that the minimum lethal temperature may have been raised. Whether these measures may be defined as " physiological " is perhaps open to question. But it is clear that they are at least quasi-physiological, and can prove very useful as toxic response parameters when used in bio-assays.

Fujiya (1961, 1962, and personal communication) has successfully demonstrated sublethal toxication in aquatic animals exposed to pulp mill wastes and other toxicants, using a variety of measures which include:

(*a*) change in haemoglobin content;

(*b*) fragility of red blood cells to change in osmotic pressure;

(*c*) specific gravity of blood;

(*d*) red blood cell count;

(*e*) rate of calcium absorption.

It is, moreover, expected that considerable progress will be made in the development of functional

[1] E.g., Bull (1928, 1930); Chernova (1958); Geller (1963); Prazdnikova (1962); Scarborough & Addison (1962); Turner (1956a, 1956b); Vanderplank (1938); Wodinsky et al. (1962).

[2] Other publications containing useful measures which should be noted are those of the Food and Agriculture Organization of the United Nations (1960), Ballard et al. (1956), Behrend & Bitterman (1963), Best (1963), Cole & Caldwell (1956), Cutting et al. (1959), Lehrmann et al. (1965), Prevost (1960), Reventlow (1961), Russel (1958, 1960), Wai & Hoar (1963), and Westphal (1965).

physiological bio-assays. Certainly, much has already been accomplished with mammals in this respect. The study of Gowdey & Stravraky (1955) on autonomic effects of acute aldrin and dieldrin poisoning in cats illustrates this. Using vagotomized and adrenalectomized animals under anaesthesia, they found the following physiological effects due to aldrin:

(*a*) slowing of the heart;

(*b*) potentiation of effects of electrical stimulation of the vagus nerve;

(*c*) augmentation of secretory effect of the chorda tympani on the decentralized submaxillary salivary gland;

(*d*) reduced rate of destruction of acetylcholine added to the blood.

With dieldrin, they found only a marked central excitation, and none of the peripheral effects.

There is, of course, no reason why such parameters cannot ultimately be used with aquatic organisms. The growing variety of physiological and quasi-physiological measures now becoming generally available should hasten the trend. The use of electroencephalographic (EEG) and electroretinographic (ERG) techniques on fish and other aquatic organisms is now possible. Other procedures, such as the measurement of body chloride, density, and water content (Black, 1951) of fish, have been found meaningful. The mechanisms of physiological adaptation and adjustment, such as temperature acclimatization, heat and cold tolerance (Ogilvie & Anderson, 1965; Warner, 1967) osmoregulation and so forth (Brett, 1946; Fry et al., 1942; Roots & Prosser, 1962; Tsukuda & Ohsawa, 1958) have all been demonstrated to have definite potential as determinants in physiologically oriented bio-assays.

Bio-assays using biochemical (and histochemical) change

In principle, bio-assays using a biochemical change as the parameter of toxic response function in precisely the same manner as do behavioural or acute-response assays. That is, the biochemical mechanism or system under examination is disturbed by the toxicant, the degree of aberration being determined by some direct or indirect method of measurement. Were these methods of measurement normally very simple ones, for example, a change in colour or shape of the affected part, then the issue would be clear. Unfortunately, most biochemical mechanisms require elaborate procedures and sometimes instrumentation for their demonstration. Hence, the bio-assay aspect of the exercise is often lost in the complexity of the demonstration. Despite this, the role of the organism or system remains the same: providing a suitable substrate for the reactive processes inherent to the microchemical contaminant.

To date, relatively few biochemically oriented bio-assays have been applied to aquatic organisms. Arteberry et al. (1961) employed quantitative changes in blood cholinosterase level and *p*-nitrophenol excretion from the kidney following exposure to parathion. Fujiya (1961) has successfully used several biochemical methods in his attempts to characterize the sublethal toxic responses of aquatic animals to pulp mill wastes, including:

(*a*) paper electrophoresis of serum for protein composition;

(*b*) RNA content of the pancreas;

(*c*) glycogen content of hepatic cells;

(*d*) dehydrogenase activity (with 2,3,5-triphenyl tetrazolium chloride, TTC);

(*e*) degeneration of polysaccharides in the kidney.

Weiss (1958, 1959, 1961) evaluated the effects of anticholinesterase compounds by biochemical determinations of the amounts of residual tissue acetylcholinesterase (AChE) following exposure of the test fish to sublethal concentrations of organophosphates. Following the work of Koelle (1950, 1957), Gerebtzoff (1959), Holmstedt (1959), Holmstedt & Sjöqvist (1960) and others, I found it possible to determine semi-quantitatively the distribution and abundance of AChE in fish tissues *in situ* following exposure of the test fish (*Carassius auratus*) to sublethal amounts of organophosphate pesticides (Warner, 1967). The method involves a thiocholine histochemical staining procedure applied to thin sections (6μ-10μ) of fish tissues obtained with a freezing microtome.

It should be noted that in all the cases discussed above, the biochemical determination provided the basis for a direct measure of toxic response. This circumstance may be contrasted with the more usual situation where biochemical or other analytical procedures are used to determine the toxicant residues present *per se* in exposed organisms. In this latter case, the determination *does not* lead directly to a measure of toxic response but indicates the quantity of toxicant which the organism has absorbed during the period of exposure. The use of DDT-residue

determinations by Allison et al. (1964) is an example of the non-bio-assay use of such methods.

Perhaps the major drawback to currently available biochemical bio-assays is the complexity of their execution. Generally a reasonably sophisticated laboratory facility is required for their use, and in some cases a high degree of expertise with the procedure. It is to be hoped that simplified procedures will ultimately be developed for at least some of these techniques, rendering them more suitable for studies of microchemical contamination.

Bio-assays using ecological change

There are some who would argue that ecological changes do not fall within the definition of bio-assays. None the less, it seems to me that the use of selected ecological measures is not carrying the case of bio-assay too far, for we have simply substituted discrete units of the natural environment for the test-tube or aquarium of the laboratory. Certainly the use of indicator organisms as developed by Kolkwitz & Marsson (Bick, 1963), and later enlarged by Goodnight & Whitley (1960), Patrick (1957) and Shrivastava (1962) shows the use of a bio-assay in an environmental setting, although it appears to have more relevance to macrochemical than microchemical contaminants. I would further submit that the studies of Cope (1961), Kallman et al. (1962) and Cushing & Olive (1956) contain most, if not all, of the essential elements of bio-assays, albeit exercised on a grand scale. This point may be made clear by comparing their work with other ecological studies of micro-chemical contaminant effects on the environment which are of a different nature (Galtshoff et al., 1938; Huish, 1961; Stringer & McMynn, 1960; Warner & Fenderson, 1962; Young & Nicholson, 1951).

The use of artificial streams, while embodying the same principles as the studies referred to above, is a more readily appreciated demonstration of ecologically oriented bio-assays. The pioneer work of Warren & Doudoroff (1958, and personal communication) has shown that the semi-environmental conditions of artificial streams can be used for quantitative evaluation of biotal changes due to microchemical contaminants. One such artificial stream, shown in Fig. 5 and Fig. 6, has been constructed at Zurich, Switzerland, and has to date been employed primarily in assays of the effects of macrochemical contaminants. This approach may prove useful in bridging the gap between laboratory bio-assays and field conditions.

Bio-assays using embryological (and regenerational) change

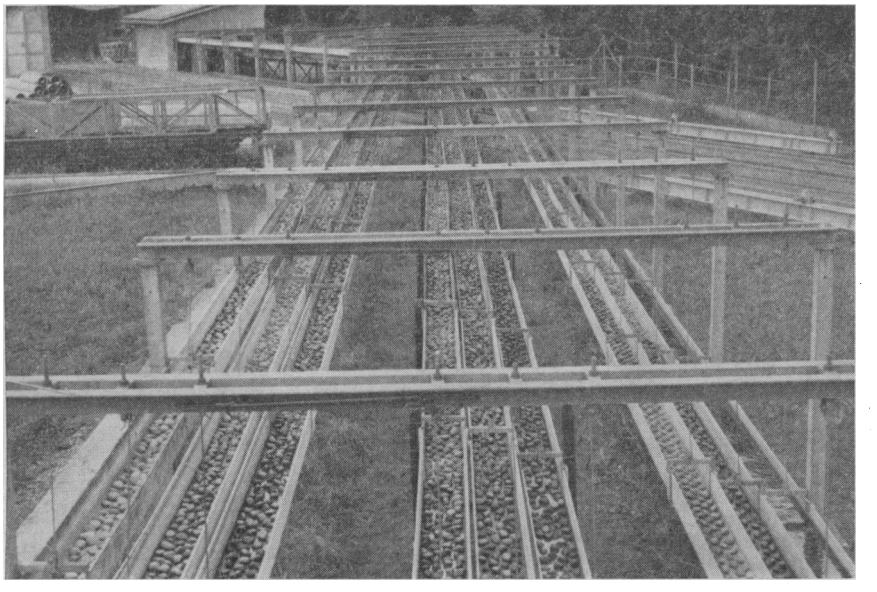
Embryological pathology, that is, the aberrations in early post-fertilization developmental patterns, has for many years been the subject of intense study. The experimental embryologist has commonly employed a variety of means for disturbing the rate and sequence of early developmental stages, including the use of chemical toxicants. A well-known and classical test organism for these purposes has been the sea urchin, whose embryos have suffered uncountable aberrations in the hands of both students and research workers. Sea-urchin embryos have proved useful for determining not only how much of a substance is required to induce abnormal development, but often how little (negative *versus* positive quantity); for it must be borne in mind that hypo- as well as hyper-sufficiency can produce a state of toxication.

These principles of ontogenetic pathology have been successfully employed for microchemical bio-assays by a number of workers, including Okubo & Okubo (1962), Davis (1961), Ruggieri et al. (1960) and Burke (1960a, 1960b). The work of Okubo and Okubo is especially interesting, in that they were attempting to develop a bio-assay methodology based on embryological changes especially for microchemical contaminants. They found, among other things, a very high degree of sensitivity in larvae of the sea urchins *Hemicentrotus pulcherrimus* and *Anthocidaris crassispina* and in larvae of the bivalve molluscs *Crassostrea gigas* and *Mytilus edulis* to sodium cyanide, mercuric chloride and picric acid. In comparative toxicity tests with brine shrimp (presumably *Artemia salina*) and *Balanus*, two rather common bio-assay organisms, the urchin and bivalve embryos proved 10 times as sensitive as *Balanus* nauplii, and 100 times as sensitive as *Artemia*.

On the basis of these and other studies, they concluded that the sensitivity range for fertilized sea urchin and bivalve eggs is approximately equal to that of the littoral fishes. In addition, the ready (potentially year-round) availability of test organisms, especially if one follows the culturing methods perfected by Loosanoff (1960) renders this approach even more useful.

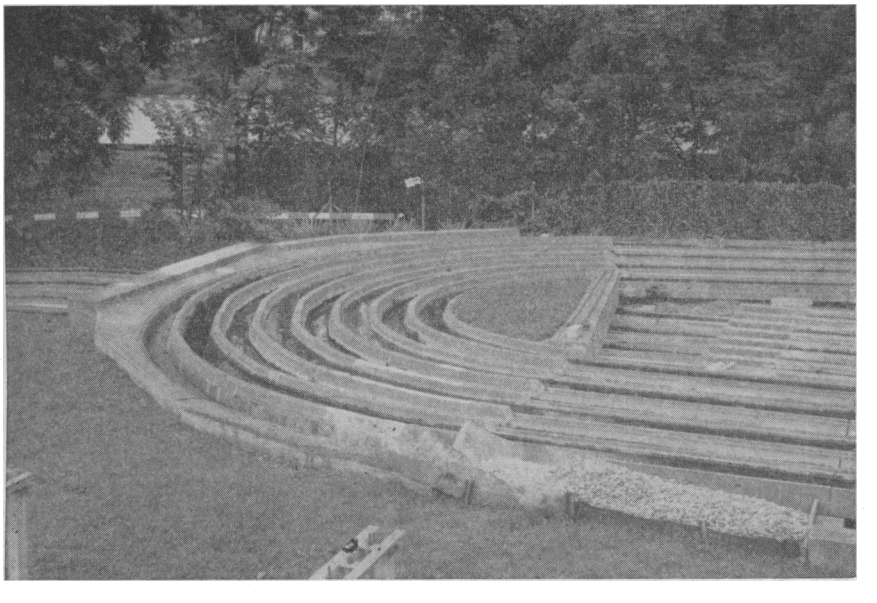
One important advantage of embryological-change methods, virtually unused at present in bio-assay work, is the light which they can throw on the mechanism of action of the toxicant employed. For example, the toxicant holothurin produced in sea-urchin embryos what is called " animalization ",

FIG. 5
ARTIFICIAL STREAM FOR ECOLOGICAL STUDIES, ZURICH, SWITZERLAND



a syndrome in which gastrulation failure is accompanied by hyperdevelopment of the ciliary tuft and a thickened apical plate (Ruggieri et al., 1960). This syndrome is reportedly due to specific action of the toxicant on the protein structure of the cell, in contrast to "vegetalization", in which the carbohydrate metabolism is affected. Sufficient knowledge of such causative mechanisms in the toxic responses

FIG. 6
ARTIFICIAL STREAM CONSTRUCTED OF CEMENT CANALS, ZURICH, SWITZERLAND



of embryos has been developed to render this approach highly productive for future workers.

It is perhaps debatable whether regenerative change should be included with embryological change, with growth change, or should more properly be assigned a separate category. I have placed it here because of the many similarities it has with ontogenetic mechanisms, even though it is realized that the regenerative processes normally require a base of intact, fully developed tissues. The patterns of cell migration and organization, although quite distinct in the two processes, have nevertheless specific schedules, sequences, and end results.

Unfortunately, I have not found sufficient evidence of the use of regeneration as a bio-assay to evaluate its potential properly. The available evidence does suggest that it has promise. For example, Quaglio et al. (1957) employed the regeneration technique, exposing planarians to holothurin, a steroid saponin toxicant produced in the sea cucumber. They found the technique very useful in characterizing the nature of the toxicity of the compound. I found (Warner, unpublished data, 1962) that toxaphene had a decidedly suppressive effect on regeneration in planaria at 0.32 μg/l, with the severity of impairment directly related to the toxicant concentration down to that level. No histological studies were made of the patterns of regeneration, which may prove even more sensitive to microchemical toxication than the over-all rate of regeneration. Patricia Hudelson has informed me (personal communication) that she has found the rate of regeneration in planaria a very sensitive basis for the bio-assay of certain heavy metals.

Certainly, sufficient base-line work has been done on normal regeneration patterns to render the approach highly productive for bio-assays. Brønsted & Brønsted (1954) have studied the fragment size in relation to the rate of regeneration in planaria; Singer (1952), Kamrin & Singer (1955) and Singer et al. (1955) have carried out important investigations on regeneration in vertebrates, and Balamuth (1939) and others (e.g., Tartar, 1961) have considered in detail " regeneration " in protozoa. A variety of other studies on regeneration are also available.

It now remains for interested workers to pool the available information on normal regenerative processes, possibly by construction of " flow-charts " of the cell migrations, accretions, and subsequent differentiations that occur on regenerating surfaces. It would also be useful to establish criteria for the various " stages " in the regenerative process, for use when histological examination is impracticable or undesirable.

Bio-assays using growth change

The dilemma of assignment which we found with " regeneration " is upon us again with " growth ". Taking a very wide view of the question, it can be successfully argued that embryology represents the processes of early ontogeny; growth, those of later ontogeny; and regeneration, the processes of ontogenetic repair (and in some cases a process associated with reproduction). Hence they all fall comfortably into one biological category.

It is worth pointing out here that this assignment to various categories is not simply an exercise in semantics: the different categories reflect the various areas of specialization in which these processes have been most thoroughly investigated, and give a certain posture to the bio-assay methods using the biological mechanisms encompassed in the category.

From a heuristic point of view, the separation of growth as a category has distinct advantages. For example, the use of organ-weight : body-weight ratios has proved extremely useful. Krumholz (1956) found in a study of radioactive waste contamination of a lake that the ratios of liver and kidney weights to body weight provided highly significant information on the condition of the exposed fish. Matsue et al. (1957) found a retardation in the growth of aquatic organisms due to sublethal toxication with parathion. In this case the retardation was associated with a decrease in feeding behaviour.

Crandall & Goodnight (1963), Frieders (1954), Dilling et al. (1926), Mount (1962), Allison et al. (1964) and others have employed direct measurements of growth parameters (weight, length, etc.) and have found them to be useful indicators of fish response to chronic sublethal toxication. Collier (1953) and Butler & Springer (1963) have successfully applied the measure to invertebrates undergoing long-term toxication. The method of Butler & Springer is simple, clever, and quite sensitive. The outer edges of the shells of molluscs of known age are ground off flat. Growth increments can then be quickly and quantitatively determined using the ground surface as the zero point.

Growth, like most forms of quantified behavioural parameters, is essentially a composite measure, governed as it is by a host of biochemical and physiological processes. Consequently, little can be said from growth data about internal functions, except that they are disturbed in some way. The collective

nature of the measure can, none the less, be used to good advantage, especially where long-term, low-level disturbances are suspected.

There are two other rather well developed areas of growth and quasi-growth bio-assays which should be mentioned. These are the use of (*a*) plants and (*b*) micro-organisms as assay organisms where growth phenomena are used as parameters. For example, Ready & Grant (1947) developed a very sensitive method for detecting low concentrations of the herbicide 2,4-D using germinating seeds. They reported their procedure as having a sensitivity of 0 005 mg/l and a working range of 0.005 mg/l to 5.0 mg/l. Funderburk & Lawrence (1963) used an aquatic plant, duckweed (presumably *Lemna minor* L.), in a bio-assay procedure for diquat and paraquat. They reported a sensitivity of 0.5 μg/l for diquat and 0.75 μg/l for paraquat. They found the method successful in measuring residues not only in water collected from treated streams and ponds, but also in the expressed cell sap of submerged aquatic weeds.

O'Kelly (1965) recently reported on the culture of the micro-organism *Protosiphon botryoides* under conditions of controlled media and atmospheric environment. Bhagavan & Eiler (1961) quantitatively evaluated the inhibitory effects of phenylurethane and low oxygen levels on respiration, cell growth, and cell division of *Tetrahymena pyriformis*.

These reports typify the kind of quantitative measures of growth capable with micro-organism cultures. Precise control renders the systems highly suitable for microchemical toxicant bio-assays. Many standard and widely used assay procedures, such as the BOD (biochemical oxygen demand) test, use these same principles. There are so many developed and potential assays in this field that I am forced to choose between a highly superficial view of the subject and exhaustive treatment of the voluminous literature that has developed in this very productive and useful area. I shall therefore limit myself to emphasizing the possibilities in the use of plant and micro-organism bio-assays and call the attention of the reader to the report by Bick (1963), which discusses in greater detail some of the bio-assays developed using plants and micro-organisms.

Bio-assays using histological change

The use of cytoarchitectural or histological changes in tissues has become one of the classic forms of biological diagnosis. Histopathology is essentially the distinguishing of aberrant cellular and tissue structures from normal ones. A well-trained histopathologist can not only determine changes in morphology, but in some instances make a good guess at the causative mechanism of the change. Chronic *toxic* nephritis can, for example, often be distinguished from chronic *infectious* nephritis. And since the affected tissues are identified, predictions can be made relative to effect of the dysfunction upon the organism.

Despite these features, the method must be employed with caution, and with the knowledge that great skill is required to make confident diagnoses. Mann & Schmid (1965) were able to demonstrate severe liver damage to *Lebistes reticulatus* after exposure to a synthetic detergent. The same group (Schmid & Mann, 1961), studied the changes in gill structures of trout due to another benzenesulfonate detergent. Marchetti (1962) exposed goldfish to ammonia for a maximum of 90 hours, and then held them in fresh water for up to 110 days. He found significant histological and anatomical changes, which led him to stress the importance of long-term studies of toxicity. Palamos (1962), in a study of imipramine, found histological pathology following chronic exposure which included diffuse brain lesions and degeneration of Nissl substance, accompanied by sclerosis. No clinical symptoms of toxication were found, although he did not use any sensitive behavioural measures. Had he done so, I am confident he would have found behavioural pathology as well. Crandall & Goodnight (1963) found that sublethal concentrations of lead nitrate, zinc sulfate and sodium pentachlorophenate produced the following histological changes in the guppy (*Lebistes reticulatus*): lack of mesenteric fat, reduction of renal peritubular lymphoid tissue, apparent dilation of renal tubules with degeneration of tubular epithelial cells, retarded and aberrant gonadal development, liver damage and blood-cell destruction.

Wood (1960) has taken a very strong position regarding the use of histopathology in macro- and microchemical toxication. He also discusses the use of preservatives and other procedures. King (1962) found that chronic exposure to DDT caused degeneration of inter-renal (adrenal) tissues and severe liver and intestinal disturbances in two species of freshwater fish. Because of these changes, the accompanying modification of kidney tubules, and reported degeneration of the zona fasciculata of mammalian adrenals, she was led to suggest that DDT has a direct effect upon the glucocorticoid-secreting tissues of the inter-renal glands. Kuhn & Koecke (1956)

have also successfully employed histological and cytological techniques in analysing microchemical contaminant effects on fish.

It is clear that histological change can be a very useful bio-assay tool. Its application demands, however, a high degree of competence in histopathology, which relatively few workers possess, and therefore requires a specialist in its execution. The data which can be so obtained can be very meaningful, although the negative evidence obtained from its use in sublethal toxication (Warner, 1967 and unpublished data; Mount, 1962; Allison et al., 1964) has clearly shown that aquatic organisms can experience and demonstrate other pathologies due to sublethal toxication without showing any appreciable evidence of histological change. This last should be regarded as a note of caution to the over-expectant.

Bio-assays using perception by humans and by aquatic organisms

There are instances where perception of the toxicant *per se* is important. The palatability of food fishes caught inshore, the potential aversive influences of industrial effluents on the behaviour of migrating fish and the quality of domestic water supplies are examples of questions involving perception either by man or an aquatic organism of interest. It is important in this respect to distinguish responses consequent to perception of a contaminant from those resulting from uptake of the contaminant by the organism and the subsequent internal aberrations. In the former case the compound has not (necessarily) penetrated into the organism, the responses resulting instead from perception *via* the sensory modalities of the organism. In the latter (true toxication), the organism's response is changed because some internal process has been modified. Thus, conclusions derived from bio-assays involving perception may be grossly different from those involving true toxication.

This general category of perception can be divided into two natural subgroups:

(*a*) perception of a water contaminant by an aquatic organism;

(*b*) perception of a contaminant either in the water, or concentrated in an aquatic organism, by man (organolepsis).

The important work of Marcström (1959), Lindahl & Marcström (1958) and Höglund (1961) in Sweden, and of Jones et al. (1956) in the United States of America, using a fluviarium technique, well illustrates the first subgroup. Use of an apparatus (fluviarium or " water organ ") which creates a stepwise concentration gradient across a test tank allows the response of the test organism upon perceiving the compound being tested to be ascertained.

The principle of toxicant gradients has also been successfully applied to other toxicating conditions such as anoxia. For example, Whitmore et al. (1960) and Shelford & Allee (1913) have examined the avoidance reactions of fishes to low-oxygen conditions using the fluviarium technique.

A quite different approach has been used by Bull (1928, 1930), Hasler & Wisby (1949), Neurath (1949) and others. These workers have employed conditioned responses to perceived chemical stimuli in exploring the thresholds of sensory (olfactory in this case) perception of the chemicals. Thus, Neurath (1949) found he could condition *Phoxinus* to eugenol at a concentration of 0.017 μg/l, and to phenylethyl alcohol at a concentration of 0.023 μg/l. At these concentrations, the fish could distinguish one compound from the other. Hasler & Wisby (1949) found that *Hyborhynchus notatus* could detect phenol and *p*-chlorophenol at a concentration of 0.5 μg/l. In later experiments using a different procedure, they found that salmon fry (*Oncorhynchus* sp.) could detect the chemical morpholine at concentrations as low as 1×10^{-11} μg/l (Hasler, 1957). They also used a similar procedure to test the discrimination ability for stream odours, and examined the possible role of this olfactory ability in the parent stream behaviour of migrating salmon (Hasler & Wisby, 1951).

This employment of the sensory modalities of aquatic organisms has considerable potential as a bio-assay approach. Keeping in mind the limitation that the animal is responding not because its physiological state has been altered, but because the perceived stimulus has taken on meaning to it (usually through conditioning procedures), then sensing devices of incredible sensitivity become available for use with microchemical contaminants. A discussion of the general properties of the two most important senses of fishes (gustation and olfaction) which would be employed in such detection assays has recently been provided by Hasler (1957). The use of conditioned responses in fish studies has been ably reviewed by Bull (1957).

Use of human sensory mechanisms in detecting microchemical contaminants is still in the early stages of development. Its application will be limited largely by two factors: (*a*) the inherent limitations of human sensory perception; and (*b*) the lack of

necessary properties (such as being aromatic) in many microchemical toxicants.

Fortunately, recent work is demonstrating that human senses are far more acute than had been appreciated in the past. The work that is currently being done in the area of organolepsis indicates the substantial possibilities of this approach. For example, Mann (1964) used olfaction to detect the quantity of phenols and oils in fish tissues, and found that the presence of synthetic detergents in the water materially enhanced uptake in the exposed organisms. Althaus & de Jong (1965), Popp & Bahr (1954) and Cohen et al. (1961) all found that toxaphene had a quite low threshold odour concentration (5 μg/l. The first two groups used olfactory (organoleptic) means of tracing the compound in natural streams. Popp & Bahr (1954) were able to trace lindane contamination of a stream, by olfactory means, to its point of entry (the effluent of a chemical manufacturer) 15 km upstream.

Ivanov (1960) found the odour threshold of chloroprene in water to be 0.25-0.50 mg/l, and since this concentration was below that causing detectable effects in his experiments he recommended that the threshold concentration in waste waters be the same as the odour threshold concentration.

The use of organoleptic measures is being vigorously pursued in Sweden, under the leadership of Dr Sölve Widell of the National Swedish Institute of Public Health (Hasselrot, 1964). This group uses a classification for odour and taste tests on polluted waters and exposed organisms based on the following scale:

5 — no noticeable effect;

4 — very weak effect;

3 — weak effect;

2 — distinct effect;

1 — pronounced effect.

Using this scale, they can assign values to the quantity of contaminant (for example, kraft pulp mill wastes) that has been discharged into a lake or river system.

An intriguing possibility is suggested by these various points. Should " olfactory indicators " be added to pesticides, herbicides and other synthetically produced potential microchemical contaminants, their presence and distribution in the environment, if they were present in any quantity, could be quickly determined. That this is feasible is indicated by the studies of Cohen et al. (1961), who found that while rotenone normally has a threshold odour concentration of 13.8 mg/l, this threshold could be reduced to 0.007 mg/l by use of a different solvent. Olfactory labelling of toxic compounds would make them far more detectable than they now are, vastly reducing problems of secondary contamination, inadvertent toxication through drift, leakage, or other unpredicted movements of the toxicants. While " olfactory indicators " would not supplant the need for the other detection methods now required when toxicants have been absorbed by organisms or otherwise altered from their original form, they would perform an important and useful function before, during, and for a variable period following release into the environment.

DISCUSSION AND CONCLUSIONS

Selection of appropriate tests

The variety of bio-assays applicable to the study of microchemical contaminants is obviously very great. I believe that the material presented herein fully establishes this point, although I also realize that the presentation does not adequately indicate the relative amount of work being done in the various areas, nor in many cases specify useful aspects such as relative thresholds of sensitivity. Such data would be best compiled in separate reviews on the current status of, and potentials for, each of the bio-assay categories described. Such reviews would ideally include:

(*a*) cursory descriptions of the procedures;

(*b*) descriptions of the organisms employed and/or employable;

(*c*) classes of toxicants testable;

(*d*) thresholds of sensitivity for toxicants tested; and

(*e*) time, cost and number of data produced per test.

I have not done so in the present instance because I wished to give a more general view of the subject.

I am, however, confident that within each of the bio-assay categories a multitude of highly functional response parameters, in addition to those listed herein, will be found. The limiting factors restricting their immediate use as microchemical toxicant bio-assays will be: (*a*) unresolved problems of quantitation; (*b*) extrapolation from the test situation to the field or ecological situation.

This problem of extrapolation is a knotty one, for while biological changes of any sort due to a toxicant are of significance and interest to the

scientist, laymen involved with application of the law to biological evidence have often demonstrated great reluctance to accept bio-assay findings utilizing life processes more than a few steps removed from the intuitively obvious indicators of debility. Consequently, measures of death, growth, reproduction, and change in number of organisms present often inspire more confidence than equally or more meaningful data based on physiological or behavioural pathology. This condition persists, despite the recognition by biologists that virtually all toxicant-induced deviations from normal in healthy organisms have been found to be pathological and deleterious to the organism's well-being. I have discussed this point in greater detail elsewhere (Warner, 1967; Warner et al., 1966). It is to be hoped that this naïveté will diminish with time, for it has proved to be a major stumbling-block to effective pollution control in many parts of North America.

There is sufficient evidence at hand to suggest the approaches of greatest utility to specific bio-assay problems. However, since the evolution of methods is still in progress, any such guide-lines should be considered just that, with more general rules laid down only after the subject has further matured.

For monitoring of potentially toxic discharges, or where the important base-line data on sublethal acute and chronic effects have been adequately explored, *acute-response* bio-assays can be extremely useful. One must, however, keep constantly in mind the problems of toxicant loss if static tests are employed, and be prepared to wield large (sometimes disturbingly large) " application factors ".

Where extreme sensitivity is desired and the question of toxicant impact is a relatively general one, measures of *behavioural change* appear at present to offer the greatest promise. Certain physiological and biochemical tests also appear of potentially great sensitivity, and offer the additional advantage of providing evidence of the change that is nearer, if not at, the site of action. The complexity of measurement with the latter types of bio-assays will continue to be the major deterrent to their widespread use.

Growth and *histological changes* appear at present to have their greatest utility as indicators of chronic dysfunction, although some evidence, principally that of Butler (personal communication) and Butler & Springer (1963), suggests that growth may be successfully used on a short-term basis as well. As with behavioural measures, growth bio-assays have the trait, which can be used to advantage, of indicating dysfunction at any of several levels of organization, thus broadening the scope of the assay. This can be very valuable when one has no idea of the mode or site of action of the toxicant being tested.

Perception per se also offers a considerable potential, both in terms of altering the behaviour of exposed organisms, and in the detection of the presence of toxicants.

Bio-assays using *ecological change* will ultimately be widely used to link laboratory findings to environmental conditions, and to explore the energy changes in the complex and interwoven circumstances of natural habitats. Artificial streams offer great potential for integrating laboratory findings with environmental circumstances.

Sequential concentration: a major problem

It has been stated above that sequential concentration has proven to be one of the more serious aspects of microchemical contamination. Indeed, evidence is accumulating which strongly implicates this phenomenon as one of the most dangerous aspects of environmental contamination, especially by the stable or persistent synthetic biocides and the radionuclides. The dynamics and effects of this process can be best illustrated by examining the findings of Hunt & Bischoff (1960) and Hunt & Keith (1962), who documented one striking case of sequential concentration at Clear Lake, California.

Clear Lake, a fresh-water lake of approximately 46 000 acres (19 000 hectares), is located in the hills north of San Francisco. In years past the lake has maintained a breeding population of the western grebe, *Aechmophorus occidentalis*, estimated at about 1000 pairs. In 1949 the lake was treated with DDD at a rate of approximately 0.02 mg/l to control a species of non-biting but pestiferous gnat. The lake was subsequently treated in 1954 and again in 1957 as the gnats had rapidly recovered from the earlier treatments and developed a resistance to the pesticide. From 1950 until 1962 reproduction in the lake's grebe population dropped to zero. In 1962 a single young was successfully produced; in 1963, three young were fledged. Cottam (1965), in an excellent paper which included a summary of the data of Hunt, Keith and Bischoff which so clearly delineated the pattern of sequential concentration leading to the destruction of the grebe population, wrote:

" In plankton, DDD was found as high as 5.3 ppm, a 265-fold increase over the maximum applied; from the visceral fat of frogs and carp, from 5 to 40 ppm, representing a 2000-fold increase of the toxicant; in

bluegills (fish) 125 to 250 ppm, up to a 12,500-fold increase; in bullhead fish from 342 to 2,700 ppm, up to a 135,000-fold increase; in grebes, up to 1,600 ppm, an 80,000-fold increase; in largemouth bass from 1,550 to 1,700 ppm, up to an 85,000-fold increase; in whitefish, from 80 to 2,375 ppm, up to a 118,750-fold increase."

It should be noted that DDD could not be detected in the lake water after two weeks following an application.

The implications of these findings for the present discussion are clear. Studies of microchemical contamination using bio-assays, no matter how sensitive or inclusive, which do not encompass the extraordinary potential effects of sequential concentration will yield insignificant and misleading information. Thus a holistic approach must be taken, both in selecting bio-assays and designing programmes for the characterization of suspected microchemical contamination of the environment.

Toxic response syndromes

The multitude of possible aberrations which can be induced by microchemical toxicants has been amply illustrated in the previous discussion. This great heterogeneity of potential toxic responses, combined with the limited data available from any single bio-assay, render imperative the application of the concept of *toxic response syndromes*. For only by using an approach embodying a diversity of measurements on a variety of living systems can the multifaceted effects of microchemical toxication be effectively demonstrated. Several workers have begun employing either multiple measures on one or two organisms (e.g., Fujiya, 1962) or single measures on a variety of organisms (e.g., Cabejszek et al. (1964); Cabejszek & Stanislawska, 1965; Klock, 1960) in assessing the toxic properties of a microchemical contaminant. These efforts, and the work of Kaminski discussed above, constitute first approaches towards the application of the toxic response syndrome concept. My work, discussed above, is an approach to the same concept using multiple behavioural measures. We shall, without doubt, ultimately establish the toxic response syndromes for the current major contaminants. But equally important is the need to establish comprehensive toxic response syndromes for the many new and even more exotic compounds that will undoubtedly be appearing in the future. When one considers that about 500 different biocides, involving some 56 000 different pesticide formulations, are now registered in the United States of America alone (Cottam, 1965), the magnitude of the general problem becomes clearer. In the absence of comprehensive toxic response syndromes on the veritable flood of new compounds that can be confidently expected to appear as potential environmental contaminants, we shall have virtually no sound basis for predicting their effects when they are released into the environment.

Other possible approaches

Dr H. Van Genderen, of the Institute of Veterinary Pharmacology, Utrecht, Holland, has brought forward a proposal that merits serious consideration.[1] He has advanced the concept of *allowable* (or permissible) *levels* of microchemical contaminants in the environment. In practice, this would mean first the elucidation of toxic response syndromes for important organisms in an ecosystem being polluted by a microchemical contaminant. Based on the findings of an appropriately selected battery of sublethal bio-assays, the upper limit of allowable environmental contamination by the toxicant in question would be established.

Should this upper limit be exceeded, then further use of the toxicant in that specific ecosystem would be banned until the environmental level had dropped below the allowable level. Successful application of such a programme would require knowledge of the behaviour of the toxicant once it has been released into the environment. Peter Ames (personal communication) has pointed out that because of the dynamics of distribution in the ecosystem, the level of toxicant circulating in the ecosystem may continue to increase for a considerable period after its use has been discontinued.

Any such programme would, of course, require thorough and carefully planned multiple bio-assays having sufficient sensitivity and yielding applicable response data. An effective environmental monitoring programme would also be necessary, employing gas chromatography (Skrinde et al., 1962) and/or other analytical procedures. Many countries already have such monitoring programmes in effect. Its successful application would also require recognition of the great complexity of ecosystem-toxicant interactions, and of our present ignorance concerning this complexity. The proposal has the distinct merit of permitting continued use of biocides until they reach a functional danger point. Whether or not this specific proposal finds wide acceptance, it is

[1] Paper presented to the Symposium on Pesticides in the Environment and their Effects on Wildlife, Monks Wood, England, 1-14 July 1965.

quite clear that this type of thinking is desperately needed if we are ever to achieve some measure of effective control over the problem.

The question of standardization

Sufficient evidence is now on hand to permit recommendations as to standard procedures for bio-assays of microchemical contaminants. That any such efforts towards standardization should be subject to frequent modification as the sophistication of bio-assays improves is apparent. Nevertheless, the variety of sensitive, highly meaningful bio-assays now available is sufficiently great for such a move.

The bio-assays to be standardized could best be split into two general categories:

(*a*) simple, easy to perform bio-assays requiring only limited laboratory facilities; and

(*b*) more elaborate, but highly informative bio-assays requiring a greater degree of instrumentation in their employment.

A battery of bio-assays of the first group could be set up at modest expense and used where budgets are limited and/or for the solution of short-term problems. The more elaborate bio-assays would be employed by fully equipped laboratories committed at least in part to this activity, with competent and well-trained staffs of biologists. Such groups or centres would include state, regional, and federal laboratories to which potential or actual contaminants could be submitted for a thorough appraisal.

Implementation of such a programme of bio-assay selection, with the attendant considerations of adequate description of technique, provision where necessary of standard test organisms, information on culturing techniques (see, for instance, Anderson et al., 1948; Bond, 1937; Buss, 1959; Galtsoff et al., 1937; Surber, 1949) and a suitable programme of frequent and periodic revision based on the most recent data, could be successfully undertaken by an organization such as the World Health Organization. The benefits of this move would be manifold, among the most important being the establishment of internationally accepted criteria for quantifying toxic responses. Until such a step is taken, the vast amount of data now being generated on the toxic effects of microchemical contaminants will remain scattered, ununified, and of only limited usefulness to the growing numbers of scientits, sanitary engineers, and others actively engaged in studies of environmental contamination.

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RÉSUMÉ

Dans cette importante contribution au problème de la pollution du milieu, l'auteur formule d'abord quelques considérations générales sur les polluants microchimiques et sur leurs propriétés physiques et biologiques. A l'opposé des polluants macrochimiques, relativement peu toxiques mais souvent déversés dans le milieu en grande quantité, les polluants microchimiques sont des composés biologiquement actifs, capables d'affecter les organismes

vivants à des concentrations de l'ordre de 1 partie par million ou moins, et qui témoignent d'un haut degré de toxicité sélective. Leur importance croît à mesure que s'intensifie l'emploi des pesticides et des herbicides.

De très nombreuses épreuves biologiques ont été élaborées afin d'évaluer quantitativement et qualitativement l'action toxique de la pollution sur un matériel vivant, qu'il s'agisse d'une culture microbienne, de protozoaires, etc., ou d'une fraction définie d'un système écologique. Le procédé employé, quel qu'il soit, utilise essentiellement la notion de « déviation par rapport à la norme », indice de la réponse au toxique. Cette réponse ne sera correctement mesurée qu'après de multiples épreuves biologiques.

L'auteur passe en revue les épreuves biologiques généralement utilisées pour évaluer la contamination du milieu hydrique, qui peuvent être aisément adaptées à l'étude de la pollution en général. Elles sont de plusieurs types, différant par les paramètres de réaction recherchés: réaction aiguë, modification du comportement, perturbation des normes physiologiques, altérations biochimiques et histochimiques du milieu intérieur, changement des caractéristiques écologiques, répercussions de nature embryologique et troubles de la régénération, action sur la croissance des plantes et des microorganismes, altération de la morphologie et des structures cellulaires, perception du polluant par l'homme ou les organismes aquatiques. Les avantages et les limites des différentes méthodes, sous le rapport de l'interprétation et des possibilités d'extrapolation des résultats, sont examinés à la lumière de la documentation existante.

Le choix des méthodes d'épreuve qui permettent de caractériser les toxiques microchimiques est examiné en détail. Il faut éviter le recours systématique aux épreuves biologiques dont les résultats sont les plus directement mesurables en raison des risques de distorsion qu'elles impliquent. Le phénomène de la concentration croissante des toxiques microchimiques dans une série d'organismes vivants pose d'importants problèmes qui influencent le choix et l'application des procédés d'épreuve à mettre en œuvre.

Les épreuves biologiques sont actuellement suffisamment développées pour que l'on puisse formuler des recommandations en vue de leur normalisation sur le plan international. Un tel programme de normalisation, soumis à révision périodique en fonction des données les plus récentes, devrait notamment comporter le choix de méthodes d'épreuve spécifiques; l'établissement de souches d'expérience et la diffusion d'informations sur les méthodes de culture; l'adoption de critères internationaux pour l'enregistrement des résultats. Une telle action permettrait de déceler, de mesurer et de combattre plus efficacement la pollution microchimique du milieu. Elle contribuerait à unifier et à coordonner les recherches, encore très dispersées, actuellement en cours et faciliterait l'examen du problème dans son ensemble.

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