Methods for Assessing the Effects of Chemicals on Reproductive Functions
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6 *Higher Plants, Algae and Microorganisms**

A superficial review may reveal some features common to higher plants, algae and microorganisms, such as photosynthesis, but fundamentally there are so many major differences between these groups that this section of the report had to be divided into two separate parts:

(1) higher plants, and
(2) microorganisms, including algae.

As a consequence little discussion is devoted to macroscopic algae, attention being directed principally towards the unicellular members of this large group of organisms.

6.1 HIGHER PLANTS

The term 'higher plants' includes more than 150,000 species in the division Spermatophyta which constitute a major part of the earth's visible vegetation in the form of trees, shrubs and herbs, and contains a major portion of the species used for human food, fibre and forestry products. Reproduction in higher plants, defined in an evolutionary or ecological sense, is the number of reproducing descendants generated by a species either sexually or asexually. This broad definition is necessary because higher plant species differ greatly in the length of life cycle, and chemicals introduced at any stage of their life cycle can ultimately alter reproduction.

The selection and development of accurate and meaningful tests require an understanding of the diversity of higher plant species, the diverse biological effects known to be produced by a large number of chemicals, the species–chemicals interactions, and the effects of the environment on both the species and the chemical. Differences between and within species in morphological, physiological and genetic characteristics and the environmental influences on the expression of these characteristics alter the response to a chemical. A wide range of organic and inorganic chemicals is known to change plant growth, development and reproduction, and their effects are modified by numerous environmental factors. At this degree of complexity, the development of such multitiered

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assays as are used to predict the mutagenicity and carcinogenicity of chemical compounds to human subjects is not feasible for plants (Epler *et al.*, 1978; Heath, 1978). These rapid assays are based on the apparently correct assumption that any chemical that will induce mutations and/or chromosomal damage in certain microbes or mice will induce similar effects in humans. In higher plants, possible effects of chemical pollutants include mutations and chromosomal damage and a wide array of morphological and physiological alterations which depend on species and environmental factors and can ultimately alter reproduction. *In vitro* tests involving isolated enzyme systems or organelles, single cell cultures and selected plant tissues—which are of particular value when a quantitative measure of a specific response such as inhibition of growth, photosynthesis or respiration is required—are severely limited in their predictive value for intact, integrated plant systems since a far too narrow definition of biological activity of chemicals is often imposed (Saggers, 1976). Little is known about the relationship between *in vitro* and *in vivo* plant responses and, as a result, the predictive value of *in vitro* tests—which may be useful for the solution of certain specific problems—is uncertain.

At such a level of complexity with respect to diversity of species, chemicals and environments, the selection of an 'ideal' test with adequate predictability (defined as the ability of test results gathered from a few species to predict accurately the effect of chemicals on most if not all higher plant species), reproducibility (defined as the ability to duplicate the results which, in the case of higher plants, requires sufficient control of species variability and of environmental conditions), and sensitivity (defined as the ability to distinguish small differences in response between species and chemicals) is indeed difficult. In essence, the 'ideal' test would have to incorporate an impractically large number of species grown in a very large number of 'normal' environments and exposed to an indeterminate number of complex chemicals at all stages of their life cycle. The *in vivo* tests presented here do not attain or even closely approach this high standard but are the best available at this time. Further development and refinement are necessary to attain more desirable levels of predictability, reproducibility and sensitivity.

6.1.1 Test Methods

6.1.1.1 *Stage 1: From Seed Germination to the Reproductive Phase*

In vivo standardized tests which involve a broad range of annual herbaceous species of interest to agriculture and forestry are available for routine screening of a large number of highly purified chemicals for potential use as herbicides and/or plant growth regulators (Saggers, 1976). These tests are essentially lethality tests, but a graded quantitative response can be obtained by measuring the size of progeny produced by the species. The tests at the early stages of the life cycle are conducted under controlled environmental conditions to increase

reproducibility. The tests at later stages of the life cycle must generally be conducted under field conditions, which decrease reproducibility. In general, the species for these tests are selected for their adaptability to these types of test, so that they can be propagated and grown under normal laboratory and field conditions without highly specialized professional personnel. These tests are routinely used for agricultural chemicals and can be accepted at the present time. Their predictability ranges from excellent to good depending on the number and diversity of species selected; their reproducibility is excellent under controlled environmental conditions at the early stages of the life cycle and good under field conditions; their sensitivity is unknown.

6.1.1.2 *Stage 2: Sporogenesis*

No standardized tests for sampling a broad range of species and environments are available for studying either megasporogenesis or microsporogenesis. *In vivo* tests involving a limited range of species and environments have been developed to study the effects of chemicals on microsporogenesis (Nelson and Rossman, 1958; Kaul and Singh, 1967; Hanna 1977). These *in vivo* tests are conducted in controlled environments or under field conditions and are quantitative using the seed number as the variable measured. In general, the species selection for these studies is limited to self-pollinated or cross-pollinated plants expressing hybrid vigour which have agricultural or forestry significance. These species can, therefore, be grown under normal laboratory and field conditions without highly specialized professional personnel. Predictability of such tests is severely limited because of the narrow range of species involved, but the reproducibility is excellent under controlled environmental conditions and good under field conditions. The sensitivity is unknown.

6.1.1.3 *Stage 3: From Mature Gametes to Mature Seeds*

No standardized tests applicable to a broad spectrum of species and environments are available. A small number of *in vivo* tests involving a limited range of species and environments are available to examine the pollination process in general, pollination control, pollen germination and tube growth, fertilization and seed development (Balatkova and Tupy, 1972; Schwartz and Osterman, 1976; Church and Williams, 1977; Kroh *et al.*, 1979). Test-tube fertilization (Balatkova and Tupy, 1972) has great potential if the predictability could be improved by increasing the number and diversity of species studied. For these *in vivo* tests, the species have been selected on the basis of their particular adaptability to the study rather than their economic significance. Obviously, most of the species selected could be grown under normal laboratory and field conditions without highly specialized professional personnel. The predictability is severely limited because

of the narrow range of species involved. The reproducibility is excellent under controlled environmental conditions, and good under field conditions. The sensitivity is unknown.

6.2 ALGAE AND MICROORGANISMS

Before discussing the methods for assessing the effects of chemicals on the reproductive capabilities of these groups of organisms, it is necessary to state clearly two major considerations which affect the approaches that can be adopted.

First, it is important to recognize that the Protista and the algae represent a complete spectrum of nutritional and metabolic types found in the biosphere. Thus, among the members of these groups are heterotrophs (chemo-organotrophs), phototrophs, including photoautotrophs and photo-organotrophs, chemoautotrophs, including chemolithotrophs and chemo-organotrophs, obligate aerobes, obligate anaerobes, facultative aerobes and other specialized groups with respect to their carbon and/or energy metabolism. Furthermore, within these groups there may be vast differences in the capacity to metabolize known natural organic compounds. These groups represent a plethora of different, and in many cases unique, metabolic mechanisms with substantial differences in their potential susceptibility to the same chemical.

Accordingly, in considering the effects of chemicals on the reproductive processes of microorganisms and algae, care has to be taken to ensure that an appropriate organism is tested for a given compound. To quote the example used by Slater (1983, this volume), the assessment of a compound known specifically to inhibit nitrogen fixation can be tested only with an organism able to fix nitrogen. Conversely, if the target of the chemical is unknown there are serious difficulties in selecting a test organism. Rationalizations will have to be made by a judicious selection of putatively appropriate organisms, but care must be taken not to underestimate possible effects on organisms not yet tested.

Second, it is necessary to define the reproductive function of microorganisms and algae, since in terms of mechanism this function is substantially different from most other groups of organisms considered in this workshop. Many algae and 'higher' microorganisms, especially those with cells of the eukaryotic type, frequently exhibit life cycles with sexual reproductive stages (e.g. microscopic red algae) and simple vegetative growth cycles (see Jensen, 1983, this volume). Much less is known about complex life-cycle modifications, particularly with respect to establishing and maintaining these modified life-cycle stages in the laboratory. Thus, most toxicity studies have been undertaken on vegetative cells growing by a mechanism of cell size increase followed by division into daughter cells. Many microorganisms, particularly those with a prokaryotic cell form, do not exhibit any morphological differentiation and reproduce simply by division of a parent cell into two or more daughter cells. Thus, cell growth and division, i.e. the cell

cycle, represent the reproductive sequence, which transmits genetic information from one generation to the next. By and large the growth of a microbial population is a measure of its reproductive potential. Thus, within the terms of reference of this workshop, the assessment of the effects of a chemical on reproductive function may be measured in terms of its effect on the growth of a microbial or algal population. This is in fact one of the few ways in which it is possible to treat in a meaningful manner these vastly diverse organisms as a unified group.

6.2.1 Approaches to Testing for Effects on Reproduction

There are two basic approaches to the analysis of the effects of chemicals on microbes and algae (see Jensen, 1983; Slater, 1983; this volume).

First, test procedures, whether in the laboratory or in the field, need to demonstrate a statistically valid difference in response between the treated and untreated populations in terms of their growth potential. For pure culture systems the responses may be neutral, growth stimulatory, growth depressive (i.e. reduced rates of growth; growth inhibition but maintenance of viability; formation of resting structures such as spores) or lethal (resulting in cell death). There may be different sequences of these basic processes (e.g. growth depression leading to cell death) and their timing may be useful in characterizing the types of test chemicals under consideration. For mixed-culture systems the same basic responses may be monitored. In addition, differential effects on the component species of the mixture can be observed, which provide potentially valuable information on the specific target of the chemical (i.e. one metabolic type or one particular species may be a specific target for the test chemical). Such differential effects of chemicals will result in changes in the composition and/or diversity (e.g. loss of highly susceptible species) of the community. This general approach will provide information which may be extrapolated to natural habitats. In general terms, this first category of test procedures could be adequate, at least for initial screening and ranking of chemicals to assess the desirability of using a particular compound. Again in general terms, this approach has been adopted as the basis of most regulatory guidelines and directives.

Second, where possible, test procedures should identify specific cellular targets. Major processes such as photosynthesis, nitrification, denitrification, respiratory activity, carbon dioxide evolution, methanogenesis and many others may be screened for effects. These test procedures ought to be considered within the first tier of assessment tests since they are of major importance for the function of an ecosystem. Associated with these overall tests, attempts should be made to pinpoint the effect at the biochemical level, a procedure which may lead to the development of specific assays based, for example, on particular enzymes. Such tests have been developed for dehydrogenases, nitrogenases and various catabolic enzymes, but more detailed work on test development is required.

6.2.2 Choice of Organisms

A primary requirement for developing laboratory tests is the choice of the growth system and the type of organism to be used. The preceding section has indicated the nature of the problem of choosing organisms. Standard organisms covering all the possible metabolic modes cannot be recommended since much depends on the nature of the chemical under test. Furthermore, the use of type organisms or standard organisms may induce a false sense of security in that consideration of the possible effects on other organisms may be reduced if not excluded.

Standard algal cultures and standard bacterial strains have been recommended by recourse to national and international culture collections. There is clearly a value in using standard strains in terms of data comparison between laboratories and different test programmes. However, there are dangers in using a limited number of standard organisms. The organism may not have the required characteristics and alternative strains or species from either culture collections or newly isolated organisms must be considred. There are arguments against the use of single organisms (pure cultures), and test procedures using defined or undefined natural mixed cultures must be employed (e.g. OECD 1981); activated sludge systems and river die-away tests using, in both cases, undefined mixed-culture inocula).

Whichever organism or organisms are selected for the test systems, the same experimental techniques should be used for growth and population analyses in order to facilitate comparison of the results.

6.2.3 Methods of Growth

Laboratory test procedures require populations of microorganisms and algae grown under defined conditions, so that the treated and untreated populations can be compared. Differences in growth response may be quantified in terms of various characteristics which the growing cultures may, but need not, have. Thus a primary requisite in devising test procedures is to devise an appropriate growth system.

6.2.4 Growth Media and Physicochemical Conditions

The initial consideration must be to select an appropriate growth medium. For most test procedures a complete medium which supports the organism's growth is required. However, there may be occasions (e.g. testing growth promoting substances, such as vitamins) when an incomplete medium which is supplemented with varying amounts of the growth promoting substance is required. But, in general, most test procedures are aimed at detecting deleterious effects on organisms initially requiring complete media.

The range of media available both commercially or constructed for specific

organisms is extensive. Where possible standard media ought to be used since this facilitates comparisons between tests and between laboratories; one should at least use recognized formulations of undefined, complex media. The selection of appropriate media must reflect the nature of the organisms to be cultured. It is, however, imperative that the media used are defined or described accurately, since the microbial and algal form, function, activity and chemical make-up are highly dependent on the growth conditions and nutrient composition. It is imperative that these are reported accurately, since in many cases comparison of the effects of chemicals cannot be undertaken between different tests and different laboratories if major variations are induced by differences in the composition of growth media. Similar restrictions must be applied to aeration, pH, temperature and other conditions used. Frequently these conditions are not adequately standardized, controlled or reported.

6.2.5 Growth Systems

6.2.5.1 *Systems Using Solidified Media*

A standard Petri dish technique is used with growth media normally solidified with agar. The technique may be adapted to include the test chemical at varying concentrations and to allow measurement of the effects in terms of concentrations that inhibit the growth and, perhaps, in terms of modifications of the colony and of individual cell morphology.

The system may be modified along the agar plate–well bioassay system producing inhibition zones in a lawn of microbes or algae. Standard calculations and statistical procedures exist to estimate variables such as potency and minimum inhibitory concentrations.

The system can be used to determine the development of the rate of resistance to a chemical, the rate of mutation and rate of survival in the presence of a compound. Similarly, the procedure can be used to select populations able to metabolize the compound, a particularly important factor in determining the rate of and/or potential for removal of a toxic chemical from the environment. Finally, this growth system is the basis for determining viable cell counts.

The technique is cheap, simple to handle, requires relatively low levels of technical competence, is reproducible and provides a powerful, quantitative assessment procedure.

6.2.5.2 *Closed (Batch) Culture Systems Using Liquid Media*

These systems include tube cultures (a known, usually small volume of a medium in a test tube); bottle cultures (especially for natural populations and natural nutrient sources); and flask cultures (usually conical flasks containing a known culture volume). The common feature of these systems is that they are closed (i.e.

there is no addition or subtraction of nutrients once the growth has started). The organisms grow at their maximum rate over a limited period and there is a characteristic sequence of transitory growth phases. Frequently, in comparison with microbial and algal growth in natural environments, these characteristics are inappropriate. Chemicals may be tested and the response monitored in a fashion similar to that for plate cultures. Methods for growth assessment are described in section 6.2.6.

This is often the method of choice despite various limitations, since it is cheap, reliable, technically simple to operate and may be replicated easily. It has the potential to provide data in screening procedures, an important consideration for any evaluation program.

6.2.5.3 *Continuous-Flow Culture Systems Using Liquid Media*

Many different continuous-flow culture systems, essentially based on chemostat or turbidostat principles, have been developed. There are some important features of these systems which are advantageous compared with closed culture procedures, including the capacity to examine growth rates at a range of submaximal values; growth under different substrate-limited conditions; growth at different population densities; and the growth of stable, interacting microbial populations. All these features represent important environmental characteristics and enable the effects of chemicals to be assayed under physiological conditions which cannot be achieved in plate or closed-culture systems.

The disadvantages are that these techniques require relatively complex equipment and more technical competence. The growth systems are more elaborate and costly. These features normally preclude the use of continuous-flow culture systems in screening programmes. However, they are strongly recommended as important methods of choice for second-tier analysis of chemicals identified as potential problems by the first-tier screening tests. Furthermore, continuous culture systems must be used to examine the effects on microbial communities grown in laboratory environments.

6.2.5.4 *Cage (Dialysis) Culture*

This technique, which was originally developed for bacteria and later adapted for algal cultures, allows the test medium to be more or less continuously renewed without losing the test organisms, thereby securing constant nutrient levels and stable doses of the chemicals to be tested. It also makes it possible to carry out the bioassay *in situ*, whether this is a host organism of reasonable size or a natural habitat. The cage technique is also one of the few systems which allows test organisms to exert extensively their ability to accumulate chemicals from dilute solution. Its relevance to environmental problems is also promising. Despite all these potential advantages, experience with this method is limited. There is still a

need for standardization of techniques and conditions. The method can be regarded as an intermediate stage between laboratory and field experiments.

6.2.6 Methods for Assessing Algal and Microbial Growth

There are numerous methods for assessing growth. In many instances the selected method is probably immaterial as long as valid comparisons between control and treated samples can be made. However, interlaboratory comparisons require a uniform approach, and attempts ought to be made to refer the assessment method to a standard measure, namely the dry weight biomass of the organism.

The reliability of conversion factors used must be demonstrated. The major problem which is not universally recognized is that many cellular components and, indeed, basic characteristics such as organism size are highly dependent on the growth environment (Jensen, 1983; Slater, 1983; this volume). In many instances comparison of toxicity data is not feasible because of gross discrepancies between measurements of the same variable. Similarly, comparisons between control and treated populations require that non-specific chemical effects (e.g. simple changes in growth rate) are accounted for.

The following variables have been used to measure population numbers and/or density (biomass), and have been extensively described and discussed by Jones (1979):

(1) dry weight determinations by centrifugation or membrane filtering;
(2) turbidity or absorbance measurements;
(3) biomass determinations by correlation with a species cellular component including protein, DNA, RNA, lipid, lipopolysaccharide (LPS method), chlorophyll a, ATP, and total organic carbon;
(4) total cell number estimates by microscopic haemocytometer counting chamber methods or electronic particle counters, e.g. Coulter counter; and
(5) viable cell number estimates by dilution methods and recovery on solidified medium, i.e. plate counts on slide cultures, fluorescent antibody labelling, and immunofluorescent flow cytometry in conjunction with particle counting.
(6) Under certain circumstances there may be value in estimating the effects of chemicals on overall population characteristics which are related to growth, such as oxygen consumption. These methods include biological oxygen demand (BOD) assays and carbon dioxide fixation rates for photosynthetic organisms.

6.3 CONCLUSIONS AND RECOMMENDATIONS

(1) Well established methods exist which are suitable for analysis of the effects of chemicals on algal and microbial vegetative reproduction. They are highly

quantitative and applicable to routine laboratory-based testing that enables the characterization and ranking of a wide range of chemicals. By and large, the methods are simple, inexpensive and amenable to statistical analysis. Also, they are directly relevant to the problem of the interaction between the chemical and the organisms, i.e. the test systems may be made to reflect accurately situations likely to occur in the natural environment provided the chemical reaches the cell in the active form. Standardization of test procedures for systematic evaluation and ranking of the effects of chemicals on microbial and algal reproduction recommended.

(2) Notwithstanding the success and usefulness of these procedures, further attention must be given to devising appropriate test systems, to improve their predictive value with regard to the effects of chemicals in natural ecosystems. This must include parallel studies in a number of laboratories in order to develop reproducible procedures which model the effect of interphases, species diversity, physical and chemical heterogeneity (i.e. light–dark regimens; concentration gradients; temperature profiles), diurnal rhythms and interactions between organisms.

(3) There is a limited number of test organisms available from culture collections; this facilitates comparison. For a limited number of purposes reference organisms may be applicable but the diversity of microorganisms as a group must be borne in mind. The test procedures are generally based on organisms which are readily cultivated. These organisms represent a small fraction of the existing microflora, and, since differential effects of chemical action may be expected, this is a major problem. Efforts must be encouraged to establish in the laboratory some organisms with complex life cycles in order to evaluate the effects of chemicals on the complete reproduction cycle of these species. Similarly, it would be desirable to encourage work aimed at a controlled cultivation of organisms which cannot be currently grown in the laboratory.

(4) Although laboratory test procedures are adequate for predicting the immediate or direct effects of a chemical on a given organism, the ecological effects which may arise as a result of the initial chemical/organism interaction by and large will not be observed or quantitated in simple test systems. The test systems tend to be gross simplifications of conditions usually prevailing in natural habitats. They do not, normally, reflect the heterogeneity of natural habitats, transient phenomena, natural rhythmic cycles or interphases. Thus the chemical, physical and biological interactions may modify the effect of the chemical, a consequence which cannot be detected by standard test procedures. This problem may be partially resolved by using mixed culture systems, continuous culture systems, microcosms and, particularly, field studies. Such test programmes are often not simple or easy to mount, and they are expensive.

(5) It is recommended that attention be given to providing a limited number of test chemicals which can be used to examine the validity of both the developed and proposed tests, with particular attention focused on their sensitivity and

reproducibility between laboratories. A consequence of this recommendation is that an international organization needs to take responsibility for the coordination of this activity.

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