Methods for Assessing the Effects of Chemicals on Reproductive Functions
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Methods for Assessing Effects of Chemicals on Algal Reproduction

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1 INTRODUCTION

Reproduction in lower plants, including algae, is often quite complex, involving a series of stages of very different shape, size and function. In many cases these stages, which constitute the life cycle of the plant, have not all been identified, and their special requirements are unknown.

To illustrate the complexity of the problem, the life cycle of a macroscopic, diplobiontic, red alga is schematically depicted in Figure 1. There is ample opportunity for chemicals to interfere anywhere in this complicated cycle, and it is quite understandable that toxicologists have primarily assessed effects in terms of the simpler process of vegetative proliferation.

Relatively less complex reproductive systems are found among the microscopic algae. These species are very important since they supply the larger portion of primary production in lakes, rivers and especially in the oceans. However, the

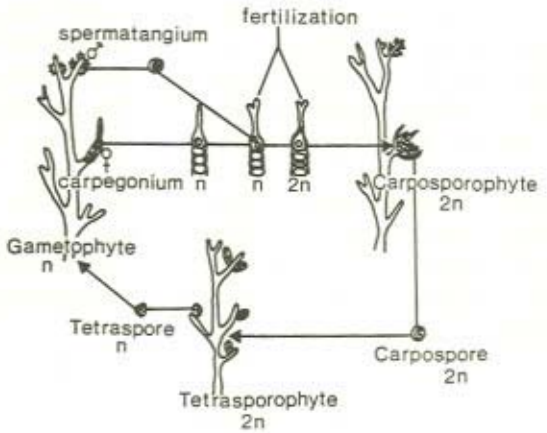

Figure 1 Schematic presentation of the life cycle of a macroscopic, diplobiontic red algae

complete life cycle of many microscopic algae are still quite complex. Reproduction in these plants can remain on the simple vegetative level for very many generations before it suddenly and for no apparent reason switches a sexual stage. Since the vegetative proliferation of many microscopic algae is easily maintained in culture, toxicological and other studies, including biochemical and physiological investigations, have concentrated on this phase of algal reproduction. Current and conventional toxicological methods for the predictive assessment of reproductive injury to algae caused by chemicals are therefore normally restricted to analysis of vegetative proliferation of cells in culture.

These methods assess the influence of chemicals on the growth of algal populations, as characterized by the change in the number of cells in the populations per unit time, or by evaluating other parameters proportional to algal biomass. The results are interpreted in terms of concentrations response curve and IC 50 value, and further information on the influence of the chemical agent on the final cell density.

The major test requirements are:

(1) that desired levels of the toxicant can be prepared, maintained in the active form and measured during the test;
(2) that the cell proliferation can be followed accurately; and
(3) that the test organisms are not stressed unreasonably by the normal test conditions.

Several methods have been developed for this type of toxicological testing, and a few have been adopted nationally and even internationally as standard algal toxicity tests. These tests seem to provide satisfactory data for the ranking of chemicals according to their relative toxicity to test species; however, their virtue as a means of evaluating the real hazard of chemicals in the environment is less obvious. The rather artificial conditions prevailing in these laboratory tests are very different from those encountered in the natural environment and do not generally account for the interference of effects of multiple toxic agents interacting with all the natural factors operating simultaneously in natural habitats. This fact has led to the development of *in situ* test systems, and one such method using marine microalgae in cage cultures has been adopted internationally.

These and other current methods will be reviewed in the following presentation. The characteristic features, as well as the inadequacies of the methods, will be discussed. Problems related to interpretation of results and application of test findings to the evaluation of the effect of chemicals in the environment will be given special attention.

2 CURRENT METHODS

Bioassays based on the proliferation of microscopic algae are in routine use in a number of countries for the testing of toxic substances and for the assessment of

cultural eutrophication. It is sufficient review to list chronologically the development of major algal tests: the Algal Assay Procedure Bottle Test of the National Eutrophication Research Program (US Environmental Protection Agency, 1971); the Marine Algal Assay Procedure Bottle Test of the Eutrophication and Lake Restoration Branch (US Environmental Protection Agency, 1974); the Algal Assay Procedure Batch Technique introduced in Denmark (Gargas and Søndergård Pedersen, 1974); the Dutch Water-Determination of Toxicity with Algae proposed as a standard test in 1979 (NEN 6506, 1979); and the Test with a Unicellular Alga for Determining the IC 50 Concentration suggested for inclusion in the OECD Guideline for Testing of Chemicals in 1980.

All of these tests and several other unmentioned procedure involve batch techniques and represent the conventional algal toxicity testing methods. They have been adopted as standard procedures nationally in many cases, and the EPA Bottle Tests and the OECD procedure are also in the process of being adopted on an international basis.

Continuous culture techniques, in the form of a chemostat or a turbidostat, are well established for studies of algal growth as a function of nutrients and physical factors (light, temperature, etc.), but they remain largely unevaluated as methods for toxicity testing. Cage or dialysis culture of microscopic algae seems to be the only technique which is applicable for bioassays *in situ*. The cage method is one of the few techniques available today for pollution monitoring, and its use in the marine environment has been recommended by an IOC bioassay panel (Stebbing *et al.*, 1980).

The above methods are all based on non-synchronized algal cultures, and the observed effects are related to the statistical mean of the populations. In synchronous cultures all cells divide within a very limited time range and show parallel development between the divisions. This method is in current use in biochemical and physiological studies and has been successfully applied to toxicity testing.

2.1 Batch Methods

In the batch technique several identical cultures of the test alga are exposed to systematic dilutions of the chemical to be tested. The increase in cell number is followed for each culture, and the reduction in division rate in relation to an unexposed culture (control or blank) is regarded as a measure of the toxicity. It is necessary to insure that neither nutrients nor light become limiting during the test, and that the total procedure is rigidly standardized to provide reproducible results. Accuracy corresponding to $\pm 20\%$ can then be obtained.

The test is applicable to all compounds that do not interfere directly with the counting of algal cells and do not colour the solution at test concentrations.

A fairly large number of microscopic test algae, both freshwater and marine species, are available from algal culture collections. Only a few, however, are free

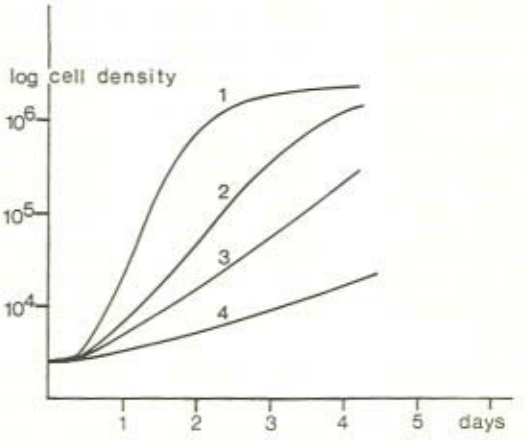

Figure 2 Expected development of a toxicity test with algae

from bacterial contamination (axenic). Axenic cultures are highly recommended for toxicological testing.

In principle the algal population is expected to grow exponentially after a short lag phase and to go gradually into a stationary state. Several techniques have been used to determine the growth rate. Counting cells under the microscope in a special chamber is the most direct method. Electronic particle counting is convenient and rapid, but it requires single cells and does not resolve algal chains or aggregates. Less direct parameters, such as turbidity, light absorption or *in vivo* fluorescence of the culture may be used to estimate growth rate in highly standardized procedures.

When cell density (number of cells per ml of culture) is plotted on a semi-logarithmic scale versus time, a graph of the type depicted in Figure 2 is expected. The curves 1, 2, 3 and 4 represent the control and test cultures receiving increasing concentrations of the toxicant.

The growth rates of the cultures are calculated from the observations, and in a new graph the reduction in growth rate relative to the control is plotted versus the logarithm of the concentration of the toxicant. The concentration estimating 50% inhibition of growth (IC 50) is read from the graph, together with the highest level demonstrating no-observed-effect.

Any one of the following detailed descriptions of this procedure is fully acceptable: the EPA Bottle Tests, the OECD Test with Unicellular Alga, or the Algal Assay Procedure used in Denmark.

2.2 Continuous Cultures

There are two types of continuous cultures, the chemostat and the turbidostat. In the former the growth rate is the independent variable, while in the latter the cell

density is varied independently. Both systems are supposed to operate at steady state.

The chemostat holds the algal culture in a reactor which receives a constant flow of the nutrient solution. This flow displaces an identical volume of the culture. The growth rate of the culture is therefore identical to the flow rate when a steady state (constant cell density) is reached. In the turbidostat the cell density is predetermined and regulates the dilution rate. For both types of continuous cultures, the steady-state kinetics of growth is similar and the theory well developed. Addition of chemicals which reduce the growth rate will lead to a lowered steady-state cell density (or washout) in the chemostat, and to a reduced dilution rate in the turbidostat. Both systems are widely used in biochemical studies of nutrient requirements and in studies of the dependence of light and temperature. They have not been generally applied to studies of the influence of toxic substances on the proliferation of algal cells. They do represent, however, very promising potential toxicity testing procedures, especially since they offer constant testing conditions in regard to nutrient concentration, light level, cell density, toxicant levels and concentration of metabolic by-products. In these respects continuous culture methods are superior to the batch method.

A review of the current and future utility of continuous algal cultures in ecological research has recently been published by Rhee (1980).

2.3 Cage Cultures

In the cage culture the test alga is grown in a cage which is open to the movement of light, nutrients and most chemicals but not to the cells themselves. The cage with the culture can be placed in a tank in the laboratory through which the test medium is flowing, or it may be operated *in situ*, i.e., immersed in the lake, river or seawater. In the laboratory, control cultures are run in tanks with the control medium, and the test cultures are maintained in test tanks supplied with selected concentrations of the toxicant dissolved in the medium. *In situ* cultures are preferably operated in series of decreasing pollutant concentrations, including a control culture in a non-polluted or practically non-polluted habitat. In both systems cell proliferation is followed by cell counting or with an alternative technique as previously described.

Besides being the only known way to operate a test culture in the natural environment, cage methods ensure, through the frequent or continuous replacement of external medium, constant external conditions and a stable level of the toxicant throughout the test. The cage culture method is described in detail by Eide *et al.* (1979), and is recommended by a Bioassay Panel of IOC for *in situ* monitoring of pollution effects in the marine environment (Stebbing *et al.*, 1980).

2.4 Synchronous Cultures

Under specific conditions all cells of an algal culture may be forced to develop simultaneously, i.e., at a certain time all cells divide. The daughter cells will then

grow in size for a fixed period, prepare for division and divide again in a synchronized pattern. This synchronous pattern of development offers a unique possibility for both rapid and detailed studies of the influence of chemicals on the life cycle and the proliferation of algal cells. Toxic effects of chemical agents can be detected and quantified rapidly (within hours) by means of synchronized cultures because the steps in the life cycle can be sharply defined in such cultures. It is also possible to measure the effect of the toxicant on specific processes, such as the synthesis of DNA, RNA, proteins (including specific enzymes), and carbohydrates. This method has recently been adopted by Norwegian authorities. Presently all dispersants to be used in oil spill clean-up require testing based on synchronous cultures of the green alga, *Chlamydomonas reinhardthi*, in addition to the brown shrimp and limpet tests. The test synchronous culture procedure has been described in detail by Nordland *et al.* (1978).

3 METHODOLOGICAL PROBLEMS

As long as the studies of the effect of chemicals on algal reproduction are restricted to the vegetative proliferation of cells, the risk that crucial problems in the complex reproductive cycle of algae will be overlooked always remains. It is rather likely that processes other than the vegetative division may be more sensitive to chemical interference.

In addition to this general problem, there are specific difficulties in establishing and maintaining constant biologically active doses of the toxic substance throughout the tests. For instance, heavy metals present special problems in algal test systems because the organisms frequently deactivate the toxic forms of the metal by exuding chelating substances. Light-sensitive chemicals also provide special difficulties in algal bioassays, since light cannot be excluded during the test.

A problem common to all algal tests is inherent in the need to determine increments in biomass of the growing population. Cell number is an obvious growth parameter, but average cell size frequently changes during the development of a culture. Other growth characteristics, such as increment in pigment content (chlorophyll *a*), ATP, protein, carbon, and in the levels of other chemical compounds are all dependent on the constant chemical composition of the cells during the test. However, it is well documented that the chemical composition of algal cultures vary with age, nutrient levels, light conditions and other factors that are not maintained at constant levels either within individual tests or between testing programmes.

The crucial question in all test systems of this type is the relevance of their results to chemical effects in environmental situations. This is a question of how well the test organisms represent the natural populations and how relevant the test conditions are to the situations in nature. Large differences in tolerance to heavy metals between algal species have been established, therefore, any test

programme should examine a set of algal test species rather than a single population.

The relevance of these tests to effects under natural conditions is difficult to evaluate. *In situ* cage cultures are likely to be more relevant than laboratory batch cultures in this respect. There is a need for experimental tests *in situ* to establish how well estimates based on concentration–response data obtained in the laboratory predict the effects found under more natural conditions.

3.1 Batch Methods

The batch method is the simplest but probably also the least predictive technique. This is because the test conditions change significant during the test. Nutrient levels go from superabundant to below concentrations essential for normal algal growth. The light intensity is considerably reduced as the culture develops. Metabolic by-products build up, and the measurable concentration of the toxicant very often decreases during the test through various losses.

The countermeasures proposed to partially alleviate these interfering factors are use of dilute cultures, analysis of only the exponential growth phase, and completion of the test in the shortest possible time.

Extended lag periods in population response often occur in toxicological studies. Lag periods, which tend to be proportional in length to the concentration of toxic substances and which are followed by relatively unaffected exponential growth of the culture, strongly indicate that some sort of detoxification process is operating in the system. Normal dose–response effects on the growth rate and the corresponding IC 50 value cannot be determined under such response conditions. The cause of the delayed growth pattern must be identified and removed before useful bioassay data can be expected. Despite its many problems, the batch technique will presently remain the primary method for rapid screening of the relative toxicity of chemicals to algae.

3.2 Continuous Cultures

Continuous cultures of microalgae require pumps, electronic devices for control and recording, and are considerably more complicated to operate than the batch type. Both the chemostat and the turbidostat need several days of growth to establish steady state before the toxicant can be introduced, and several instruments have to be run in parallel to provide satisfactory control. It is definitely advisable to run batch experiments for localization of the effective concentrations of the toxicant prior to tests in the turbidostat or the chemostat. The provision of constant conditions, including defined concentrations of the toxic chemical throughout the test, therefore involve time-consuming operations and expensive equipment.

The use of these techniques will probably be limited to special studies of those

chemicals which can be kept at constant level in batch systems only with great difficulty or for which natural detoxification processes necessitate frequent exchange of the batch medium.

3.3 Cage Cultures

Cage cultures are not commonly used for algal species, and experience with this technique is lacking in most toxicology laboratories. This method requires fairly large volumes of the test and the control media in comparison to the batch technique, and there is a need for additional space and equipment.

When the cage (bag) is composed of regenerated cellulose, toxicants with high molecular weight (> 10,000) cannot penetrate to the test organism. There is also a slight loss of light through the bag, especially toward the end of the test when the cellulose tends to become opaque.

A major problem of *in situ* tests is the growth of bacteria and foreign microalgae on the outside of the culture bag. This growth reduces the flow of nutrients and light to the test culture and may also change the concentration of the toxicant which enters the bag.

When the method is applied in the field, a power source to stir the culture is required. Problems of light and temperature shock upon transfer of the caged algae from the laboratory to the site and during sampling of the culture at the site may also arise, and it is often difficult to find suitable unpolluted sites for reference cultures. In the summer, bacteria in the water will normally break down the cellulose bags within 6–10 days; thus, at that time the test has to be interrupted. Other problems encountered are related to the high cell density developed toward the end of the growth period of many algae in cage cultures.

Several of these problems can be overcome by daily cleaning of the bags, by sampling and replacing cultures in the evening (to avoid light shock), by using other membranes instead of cellulose, and by keeping the cell density low in the test cultures.

The cage culture remains the only method applicable for *in situ* testing, and it is the simplest way to insure constant conditions during laboratory tests.

3.4 Synchronous Cultures

Synchronization of the vegetative proliferation of algal cells has been accomplished with a limited number of species. Therefore, presently only a few test organisms are available for toxicity testing utilizing this technique. The developmental synchrony may be lost quite rapidly in some cases, and when the rhythm is maintained, questions have been raised regarding the effects of stress that may result from the conditions needed to assure synchrony. The intermittent illumination/culture dilution principle used for *Chlamydomonas reinhardthi* by

Nordland *et al.* (1978) seems, however, to insure synchrony and excellent growth of healthy cells.

The technique does offer more test opportunities than can be exploited in routine toxicity testing of chemicals, for which it may appear too sophisticated. Synchrony, however, does increase the sensitivity of the test, especially when an alga which yields 12–16 new cells per cycle is used (Nordland *et al.*, 1978). The procedure developed by these authors is based on electronic counting and volume measurements of the algal cells. The cells are separated into dead and living fractions and can be used to estimate LC_{50} values when the relative lethality of the various dose levels is plotted versus the logarithm of the doses on a probit diagram. The estimated LC_{50} is not identical to the IC 50 value which is related to reduction in growth rate caused by the chemical rather than cell survival.

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