Toxicity Testing Using Microorganisms

Volume II

Editors

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Chapter 1

INTRODUCTION AND REVIEW OF MICROBIAL AND BIOCHEMICAL TOXICITY SCREENING PROCEDURES

G. Bitton and B. J. Dutka

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I. INTRODUCTION

With the increased world-wide industrialization over the past 25 years, and with the concomitant higher demand for chemicals, both the developed and developing nations face increasing ecological and toxicological problems from the release of toxic contaminants to the environment. In response to these expanding stresses on the environment and in the belief that there is no single criterion by which to adequately judge the potential hazard (either to the environment or man) of a given substance,[1] a multitude of biological assay procedures have been developed, proposed, and used to assess toxicant impacts.[2,3] Due to our newly acquired awareness of the long-term effects of chemicals discharged into receiving waters, research efforts are being directed at short-term bioassay tests in an attempt to alert monitoring agencies as well as dischargers of toxic conditions.[4-8]

As industrial pollutants and toxicants such as herbicides, insecticides, fertilizers, and car exhaust fumes affect aquatic biota systems at different levels and in many ways, it is acknowledged that the battery approach utilizing several different short-term biological tests would be preferred in any monitoring scheme. In some studies, investigators[9] have employed a battery of ecological and health effect tests to estimate the toxicity and mutagenicity of industrial effluents.

In general there are two main groups of toxicity screening tests: in vitro "health effect" tests and "ecological effect" tests.

"Health effect" toxicity tests are based on the use of subcellular components (e.g., enzymes, DNA, RNA), isolated cells (e.g., cell cultures, red blood cells), tissue sections, or isolated whole organs.[10-12] These tests consist of determining cell viability (vital staining-dye inclusion test, plating efficiency, colony formation), cell reproduction, or macromolecular biosynthesis.[10,12]

"Ecological effect" tests are conducted to measure mainly the acute toxicity of chemicals to aquatic organisms representing various trophic levels of the food chain. These tests help in the estimation of chemical toxicity in natural and man-modified ecosystems. Bacteria, algae, zooplankton, benthic invertebrates, and fish have been used in these tests.[13-15]

Bacteria and enzymes may be exposed to a wide range of toxic, organic, and inorganic compounds in natural waters, soil, and in sewage treatment processes. The toxicity of the compounds depends on environmental parameters as well as on the microorganism or enzyme systems being tested. The compounds may be metabolically altered to nontoxic metabolites or may exert a direct toxic action on microbial populations. Bacteria also may be subjected to synergistic or antagonistic effects between components of toxicant mixtures. In sewage treatment plants, toxicants may cause shifts in microbial populations, and this may adversely affect the operation of the plant.[16] The effect of toxicants on waste treatment processes will be reviewed in Chapter 5 by Koopman and Bitton.

Toxicant action is concentration dependent. For example, phenol can be metabolized at low concentrations but becomes toxic at higher concentrations. Toxicant action also depends on the presence of other chemicals in solution.[17]

The purpose of this chapter and book is to survey the literature on microbial and enzymatic tests which are used to screen for chemical toxicity in the research laboratory or in the aquatic system, and to present in detail some of the more commonly used microbial toxicity screening procedures.

II. EFFECTS OF TOXICANTS ON MICROORGANISMS

There are many proposed mechanisms by which toxicants inhibit and eventually kill bacteria.[18] Toxicants may cause damage to the genetic material or may lead to protein denaturation, e.g., halogens. They may also disrupt bacterial cell membranes (e.g., phenol

and quaternary ammonium compounds), the result of which is the leakage of DNA, RNA, proteins, and other organic materials. Certain toxic chemicals may displace cations (e.g., Na^+, Ca^+) from adsorption sites on the bacterial cell, e.g., acids and alkalis.

A more subtle action of toxic pollutants is their ability to block bacterial chemoreceptors[19] which may lead to the inhibition of organic decomposition and self-purification processes in sewage treatment plants and in waters receiving fecal material.[20] It is believed that one of the most important effects of the toxic action of chemicals on bacteria is on enzyme activity.[21] However, in any toxicity study, one must also take into account the physicochemical factors (presence of other cations, pH, oxidation-reduction potential, temperature, organic matter, clay minerals, etc.) that control the toxic action towards microorganisms.[22,23] Chapter 2 (Babich and Stotzky) focuses on the effect of abiotic factors on toxicant impact.

The impact of toxicants on bacterial cells may be measured via biochemical tests which include measurement of enzyme activity, ATP content, and bioluminescence. Some biochemical indicators (e.g., ATP, lipopolysaccharides, muramic acid) have been used for the determination of microbial biomass in environmental samples.[24] We will now briefly review the major categories of tests which are used or could potentially be used in toxicity assays.

III. BIOCHEMICAL TESTS

A. Enzymes

Since enzymes drive numerous key metabolic reactions in microbial, plant, and animal cells, their inhibition could be the underlying cause of toxicity to the cells. Thus, numerous studies have been carried out to test the effect of toxic pollutants upon enzyme activity, although most of them dealt with dehydrogenase enzymes. The latter catalyze the oxidation of substrates by transfer of electrons through the electron transport system (ETS), which consists of a complex chain of intermediates (flavoproteins, cytochromes, etc.) which transport electrons from the nutrient source to O_2, the final electron acceptor.[25]

Specific dyes can be used as indicators of ETS activity. They act as artificial hydrogen acceptors and they change color upon reduction. Thus, the activity may easily be measured with the aid of a spectrophotometer. The most widely used indicator dyes are methylene blue, triphenyltetrazolium chloride (TTC), tetrazolium blue, rasazurin, and 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT). Toxicity tests based on the reduction of these indicator dyes, as well as on other enzymatic assays (e.g., ATPases, esterases, ureases), are described in Chapter 3 of Volume I.

B. ATP Assays

Adenosine triphosphate (ATP) is a product of catabolic reactions, common to all protists and animal and plant cells. Since ATP is rapidly destroyed after cell death, one then has an ideal means of distinguishing between live and dead cells. The basic assay consists of measuring the light emitted following the reaction of firefly luciferin with ATP. This reaction is catalyzed[26] by luciferase and Mg^{2+}. Brezonik and Patterson[27] first proposed the use of ATP in toxicity testing in activated sludges. This was further explored by other investigators (see Chapter 3 of Volume I for more details).

IV. BACTERIAL TESTS

Bacteria are involved primarily in the mineralization of organic substrates and in the recycling of mineral nutrients. Their activities are essential to self-purification processes in aquatic environments. They have relatively short life cycles and respond rather quickly to changes in the environment. They are stable and easily maintained at low cost. Relatively large numbers of cells are exposed to the toxicant under study. These characteristics make bacteria suitable for rapid screening of toxicants in natural waters. The various bacterial

toxicity screening tests can be divided into three main categories: assays based on bacterial luminescence, assays based on the measurement of viability or growth of specific bacteria or specific groups of bacteria, and "ecological effect" assays.

A. Assays Based on Bacterial Luminescence

Bioluminescent or luminous bacteria are mostly marine microorganisms which live freely in ocean water or in association with higher marine organisms. The three major luminous bacteria are *Photobacterium* (vibrio) *fisheri, phosphoreum,* and *Beneckea harveyi.*[28] From a biochemical standpoint, bioluminescent systems are considered as a branch of the electron transport system where the enzyme luciferase catalyzes the oxidation of $FMNH_2$ (reduced flavin mononucleotide) and an aldehyde, resulting in the production of FMN, acid, and light. Some early reports[29,30] have suggested the use of bioluminescent bacteria in toxicity testing. More recently, a Microtox® assay based on measurement of bacterial bioluminescence was developed by scientists at Beckman Instruments. Inc. (Carlsbad, Calif.) to screen aquatic pollutants for their toxicity. The numerous applications of this assay are explored in Chapter 4 of Volume 1 of this book.

B. Assays Based on the Measurement of Growth Inhibition, Respiration, and Viability of Bacterial Cells

Bacterial assays for chemical toxicity in aquatic environments are based on measurement of growth inhibition, respiration, or viability of the cells. Sewage microorganisms as well as bacteria belonging to the genera *Pseudomonas, Klebsiella, Aeromonas,* or *Citrobacter* have been suggested for these assays. Some representative methods used in these bacterial bioassays are described in detail by Trevors in Chapter 2 of Volume I.

One particular bioassay is based on the nitrifying ability of *Nitrobacter* in sewage treatment plants. These bacteria have been proposed as bioassay microorganisms to measure the toxicity of heavy metals and industrial wastes. Nitrite disappearance or nitrate formation are monitored in these tests. The toxicant concentration (ED_{50}) that causes 50% inhibition of nitrite conversion to nitrate can be obtained from plots of the relative metabolic rate of *Nitrobacter* as a function of toxicant concentration.[18]

Another particular bioassay is the *Spirillum volutans* test which is based on loss of coordination and subsequent loss of mobility in the presence of toxicants.[4,31] (see also Chapter 2 of this Volume).

The biological activity of wastewater is usually determined via respirometric methods.[32] Oxygen uptake may be determined using a wide variety of techniques described by King and Dutka in Chapter 5 in Volume I. A toxicity test based on respirometry consists of measuring the effect of a toxicant (e.g., percent inhibition) on the oxygen uptake rate of a wastewater sample. This approach has been used to measure the toxicity of heavy metals and organic chemicals in wastewater treatment plants.[33]

C. "Ecological Effect" Assays

"Ecological effect" tests provide information on the adverse effects of toxicants upon natural and man-modified ecosystems. Some of these tests have been published in the *U.S. Federal Register*[34] and consist of evaluating the effects of pollutants on nutrient cycling, and include organic matter decomposition, nitrogen transformations (ammonification, nitrification), and sulfate reduction.

V. ALGAL TESTS

Algae are primary producers widely used for assessing the impact of nutrient and toxic input to aquatic environments. Algal bioassays are relatively simple and inexpensive as

compared to fish or invertebrate bioassays. These tests may be carried out under laboratory conditions using batch or continuous cultures of algae. Among the most widely known "batch culture" tests is the "Algal Assay Procedure Bottle Test" developed by the U.S. Environmental Protection Agency[35] to assess limiting nutrients in aquatic environments.[36] However, these laboratory methods have been criticized since they may not adequately simulate the natural environment. Hence, some investigators propose the use of mixed natural algal populations in toxicity assays.

Algal bioassays for toxicity testing are based on a wide range of parameters which include cell counts, in vivo fluorescence, ^{14}C assimilation, nitrogenase activity, or adenylate energy charge. These method and others are extensively described by Wong and Couture in Chapter 4.

VI. FUNGI AND YEAST BIOASSAYS

Along with bacteria, fungi and yeasts play an important role in the decomposition of organic matter in solid and aquatic environments and in industrial processes. Some species are, however, pathogenic to plants and animals and others may colonize and deteriorate various surfaces. Bioassays using fungi and yeasts are based on a myriad of methods such as measurement of radial growth rates on solid media and growth inhibition in broth, spore germination tests, agar diffusion methods, respirometry, ETS activity, or measurement of K^+ release following exposure to a toxicant. These methods and others are discussed by Gadd in Chapter 3.

Although these bioassays have not been widely used in the water pollution field, their further development remains nonetheless essential, especially with regard to the control of biodeterioration of natural and synthetic surfaces as well as applications in phytopathology and medicine.

VII. OTHER APPROACHES

Recently, two procedures, which are not in themselves new, have started to attract researchers interested in toxicity screening tests. These procedures are the use of microcosms to study toxicant effects and the use of microcalorimetric techniques. Both of these procedures show promise and were the subject of several papers at the First International Symposium on Toxicity Testing Using Bacteria, May 17 to 19, 1983, Burlington Ont. Canada.

A. Microcosms

Microbial degradation of a potential toxicant or pollutant in the natural environment depends upon the relative concentration and availability to the indigenous microbial community. One of the ways of monitoring this degradation in the aquatic environment is through the use of microcosms.

Microcosm approaches using natural waters, solid, or sediments as microbial seed are now being used to develop a correlated interpretative analysis of the fate and effect of a variety of xenobiotics in aquatic environments. However, quantitative estimates for environmental fate can still only be achieved by the extrapolation of laboratory estimates to an *in situ* ecosystem, and we suspect laboratory conditions may overestimate degradation rates or toxicity effects.

Portier[37] and Portier and Myers[38] have pursued the use of microcosms and have much experience with both the batch-type and continuous-flow microcosms. They have used the microcosm procedure to analyze the effects of three major classes of toxicants: organophosphates, organochlorine, and phenol. A summary of their techniques and results has recently been published.[38]

B. Microcalorimetric Techniques

The use of microcalorimetry to study the effect of potential toxicants on microorganisms is a new, exciting, and developing concept. Basically there are two main responses in heterotrophic microorganisms when they are subjected to stress. One response is to effect changes in biomass or community structure and the other response is based on changes in total or specific activities, e.g., motility and heat production. Heat changes which accompany all biological activity reflect the total activity in a community and could be a useful parameter for studies on the integrated effect of ecocontaminants under aerobic as well as anaerobic conditions.[39]

In principle, the measurement of the heat flux in the presence of inhibitors can provide a basis for evaluating inhibitory effects and the "microtoxicity" of contaminants. The main limitations in the past for the use of this procedure have been related to instrumental requirements, namely, sensitivity, response time, ease of operation, and automation.[40]

One of the major reasons for using microcalorimetric techniques is that a community effect is measured rather than the effect of pure or slightly diversified cultures, which could lead to the misinterpretation of toxicity effects. With the recent developments in flow microcalorimeters, it has been found that microcalorimetric techniques are sensitive to $\sim 10^4$ cells per cubic centimeter, exhibit a response time of ~ 1 min, and may be used virtually for any type of microorganisms, substrate, and toxic contaminants.[40] The ease of operation is comparable to standard chromatographic techniques and hence, measurement systems could readily be automated for dedicated analysis in continuous monitoring or control operations.[40] This topic is further discussed in Chapter 11.

VIII: CONCLUSIONS

Many of the enzyme and bacterial growth tests which have been developed for monitoring or screening of toxicants in water or effluent discharges have been touched on or reviewed. Most of these tests are rapid, relatively reproducible and inexpensive, and require little space and time as compared to fish bioassays. Microbiological screening techniques provide a useful and rapid screening tool to aquatic toxicologists, sanitary and environmental engineers, and microbial ecologists. Bacteria appear to be sensitive sensors of chemical toxicity since they respond relatively quickly to changes in their environment.

However, little information is available on comparative studies of short-term bacterial assays for estimating the impact of toxicants on the aquatic environment. Such studies could give information about reproducibility, sensitivity, cost, and rapidity of the various tests. With the recent initiation of the International Symposia on Toxicity Testing Using Bacteria (First, May 17 to 19, 1983), it is hoped that these biannual symposia will provide the forum for obtaining this type of information. Also, as in the case for mutagenicity testing, the use of a battery of short-term tests to screen for toxicity of aquatic pollutants should be entertained.

There are, however, still some problems as scientists and engineers still attempt to associate the relationship of bacterial and enzyme assays with animal toxicity tests. Other problems concern the attitude of government agencies and engineers toward enzyme and bacterial assays. This attitude can be changed through further research on bacterial toxicity tests and better education of the potential users. Again, the International Symposia on Toxicity Testing Using Bacteria may be the vehicle for the above.

There is a strong need to standardize bacterial tests, and efforts are being made towards that goal under the sponsorship of the American Society for Testing and Material (ASTM) and the International Standards Organization (ISO). The use of the battery approach must be emphasized as there are no absolute techniques.

The field of microbial toxicology is in its infancy and we believe microbial toxicity screening is the future for toxicological screening tests.

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Chapter 2

ENVIRONMENTAL FACTORS AFFECTING THE UTILITY OF MICROBIAL ASSAYS FOR THE TOXICITY AND MUTAGENICITY OF CHEMICAL POLLUTANTS *

H. Babich and G. Stotzky

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* The literature search and manuscript for this chapter were completed in 1983.

I. INTRODUCTION

The 1970s have been termed the "environmental decade", as during this period a variety of federal statutes in the U.S. were promulgated that strengthened many pre-existing laws and set new legislation for the regulation of chemical toxicants with respect to their adverse effects on human health, the environment, and the indigenous biota. For example, the Marine Pollution, Research, and Sanctuaries Act (MPRSA) of 1972, the Federal Water Pollution Control Act (FWPCA) of 1972, and the Resource Conversation and Recovery Act (RCRA) of 1976 charged the U.S. Environmental Protection Agency (EPA) with protecting various components of the biosphere from chemical contamination and stress.[1] Of particular importance were the 1970 Amendments to the Clean Air Act (CAA), which required the EPA to formulate National Ambient Air Quality Standards and included both primary standards to protect human health and secondary standards to protect the environment and its biota. Standards were set for ambient levels of sulfur dioxide, particulates, nitrogen oxides, carbon monoxide, hydrocarbons, photochemical oxidants,[2-4] and lead.[5] The Toxic Substances Control Act (TSCA) of 1976 mandated the EPA to regulate the manufacture, processing, distribution, commercial use, labeling, and disposal of substances on the basis of "unreasonable risk" of injury to the health of human beings or to aquatic and terrestrial environments. Section 5 of the TSCA required manufacturers to submit a premanufacture notice and environmental and human health data to the EPA before the manufacture of any "new chemical substance" or the manufacture or processing of any existing chemical for a "significant new use." However, the TSCA did not delineate the specific types of data that manufacturers *must* include in their premanufacture notice. Although the EPA cannot specify the scientific information that must be submitted by manufacturers, it does have the authority to require additional testing by manufacturers if the existing data are insufficient for determining whether an "unreasonable risk" to health and the environment exists. To guide manufacturers in providing sufficient information for risk assessment of these chemicals, the EPA has identified the types of data (Table 1) on the physical and chemical properties and the health and environmental effects that should be submitted by manufacturers.[6]

In 1979, the EPA set new criteria for chemical pollutants occurring in aquatic environments, both freshwater and marine. The Water Quality Criteria for various categories of chemicals (Table 2) — which were later defined in terms of 129 specific priority pollutants — considered toxic under the Clean Water Act (CWA) of 1977 were set at distinct levels to protect human health and the aquatic biota. For example, in freshwaters, the criterion for pentachlorophenol is 6.2 ppb for "aquatic life" and 140 ppb for human health. In formulating the Water Quality Criteria, the EPA noted that the toxicity of a pollutant may be reduced in some environments, whereas in other environments with different physicochemical properties, the toxicity of an equivalent concentration of the pollutant may be potentiated. For example, as the toxicity to the biota of heavy metals appears to be directly related to the degree of hardness in freshwaters, the criteria for beryllium (Be), cadmium (Cd), chromium (Cr), copper (Cu), nickel (Ni), lead (Pb), and zinc (Zn) were formulated on a sliding scale, recognizing that as hardness increases, the levels of metals that can be tolerated by the biota also increase.[8-11] The focus by the EPA on only hardness reflected the lack of significant data to establish relations between other abiotic factors and pollutant toxicity: "Although EPA recognizes that other water characteristics such as pH, temperature, or degree of salinity (as in estuaries) may affect the toxicity of some pollutants, the data base at this time is not detailed enough for further specificity."[7] As there is no "Clean Soil Act", there are no standards or criteria for toxicants in terrestrial environments.

Protecting the environment from the adverse effects of potential chemical pollutants is a herculian task, as it has been estimated that 70,000 chemicals are currently in use in the U.S. alone and that 700 to 3000 new chemicals will be introduced each year.[12] To accomplish

Table 1
SCIENTIFIC DATA, RECOMMENDED BY THE U.S. ENVIRONMENTAL PROTECTION AGENCY, TO BE SUPPLIED BY CHEMICAL MANUFACTURERS IN THEIR PREMANUFACTURE NOTICE[6,7]

- Physical/chemical data
  - Melting point/melting range
  - Boiling point/boiling range
  - Density of liquids and solids
  - Vapor pressure
  - Water solubility
  - Partition coefficient, *n*-octanol/water
  - Hydrolysis, as a function of pH
  - Absorption spectra (ultraviolet and visible)
  - Soil adsorption/desorption
  - Dissociation constant
  - Particle size distribution
- Degradation/accumulation data
  - Ready degradability
  - Bioaccumulation (uptake from medium)
- Acute toxicity data
  - Acute oral toxicity
  - Acute dermal toxicity
  - Acute inhalation toxicity
  - Skin irritation
  - Eye irritation (for chemicals showing no skin irritation)
- Repeated dose toxicity data
  - 14 to 28 Days, repeated dose test(s), using probable route(s) of human exposure
- Mutagenicity data (screening tests)
  - Gene (point) mutation (*Salmonella typhimurium* reverse mutation assay preferred)
  - Chromosome aberrations (in vitro mammalian cytogenetics test)
- Ecotoxicity data
  - Acute toxicity, LC_{50} study, fish (96 hr)
  - *Daphnia* reproduction study (three broods)
  - Growth inhibition, unicellular algae (4 days)

the task of evaluating the risk to human health and to the environment of chemical pollutants, various bioassays, ranging in complexity from in vitro tests with single microbial species[13-18] to more complex assays using whole animals or microcosms,[19-23] have been developed.

The intent of this chapter is not to review the benefits and limitations of the various bioassays, but to emphasize the need to recognize and incorporate into the assay procedures the mediating influence on pollutant toxicity of the abiotic physicochemical characteristics of aquatic and terrestrial environments. Assays that are standardized, although important for evaluating initially the toxicity of a potential pollutant, have limited value in predicting the toxicity of that pollutant in different natural environments, inasmuch as the data obtained will reflect only the biotic response to the toxicant under one specific set of environmental variables. Merely knowing the concentration of a chemical that evokes a deleterious response in vitro is not likely to produce meaningful information for the environmental management of that pollutant.[24] As the response of the biota to chemical toxicants *in situ* (i.e., in the "real world") is dependent on numerous abiotic factors (i.e., the physicochemical characteristics of the specific recipient environment), the potential mediating influence of these factors on pollutant toxicity must be incorporated into the in vitro assays. Only then can regulatory agencies, such as EPA and state and local environmental agencies, utilize these data to formulate risk assessments of and to set criteria and standards for these chemicals that will adequately protect the biosphere. A criterion or a standard for an environmental pollutant that is based on only one set of abiotic factors may be either overprotective or underprotective for environments with different physicochemical properties.[25-28] Attention