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MICROBIOLOGICAL ASSAYS: SCREENING TECHNIQUES FOR NATIONAL WATER QUALITY MONITORING

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MANAGEMENT PERSPECTIVE

One of the goals of the National Water Research Institute's Biomonitoring Project is to provide the Water Quality Branch with leadership and expertise in the establishment of a national biomonitoring program to complement the existing chemical monitoring program.

Bacteria and enzymes are sensitive sensors of chemical toxicity since they respond quickly to changes in their environment. This report briefly reviews many of the enzyme and bacterial growth tests which have been developed for monitoring or screening toxicants in water and effluents. Compared to fish bioassays, these tests are rapid, inexpensive, relatively reproducible.

The document provides a succinct summary of available technology suitable for the development of a biomonitoring component of the national water quality monitoring program.

PERSPECTIVE-GESTION

L'un des objectifs du projet de bio-surveillance de l'Institut national de recherche sur les eaux est de bien informer la Direction générale de la qualité des eaux pour lui permettre de mettre au point un programme national de bio-surveillance en vue d'assurer un complément aux programmes de surveillance chimique.

Les bactéries et les enzymes sont des capteurs sensibles de la toxicité chimique étant donné qu'ils répondent rapidement à toute modification de l'environnement. Le présent rapport passe brièvement en revue de nombreux essais de prolifération d'enzymes et de bactéries qui ont été mis au point pour la surveillance et la détection de substances toxiques dans les eaux et les effluents. Comparés aux dosages biologiques pratiqués sur les poissons, ces tests sont rapides, peu coûteux et relativement reproductibles.

Le présent document fournit un résumé des technologies disponibles permettant de mettre sur pied un volet de bio-surveillance dans le cadre du programme national de surveillance de la qualité des eaux.

INTRODUCTION

With the increased world-wide industrialization over the past 25 years, and with the concomitant higher demand for chemicals, Canada faces 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, a multitude of biological assay procedures have been proposed, developed and used to assess toxicant impacts. In response to recent public concern for the long-term effects of chemicals discharged into receiving waters, NWRI microbiological methods research has been directed at short-term bioassay tests which can be used to screen for the presence of adverse toxic conditions. Such tests would be a valuable compliment to the Water Quality Branch monitoring programme.

As industrial pollutants and toxicants such as herbicides, insecticides, fertilizers, and car exhaust fumes affect aquatic biota systems in many ways and to different degrees, it is acknowledged that the battery approach utilizing several different short-term biological tests is to be preferred in any toxicity screening scheme. Commonly, investigators 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. These tests consist of determining cell viability (vital staining-dye inclusion test, plating efficiency, colony formation), cell reproduction, or macromolecular biosynthesis.

Ecological effect tests, which are stressed in NWRI research, are conducted to measure mainly the acute toxicity of mixture 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.

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 the compound mix, environmental matrix, as well as the microorganism or enzyme systems being stressed. 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.

Toxicant action is concentration dependent. For example, phenol can be metabolized at low concentrations but becomes toxic at higher concentrations (1). Toxicant action also depends on the presence of other chemicals in solution. For example, Ca^{++} and Pb^{++} can ameliorate the toxicity of Hg^{++}, Zn^{++} can ameliorate the toxicity of Ni^{++} while Cu^{++} and Zn^{++} can enhance the toxicity of Hg^{++} (2).

There is an urgent need within the Water Quality Branch for rapid biological assessment tests. A battery of such tests would make the national monitoring programme more useful in the management of aquatic ecosystems and, at the same time, more economical by enabling areas of little concern to be quickly identified. The information provided by toxicant screening tests can help set reclamation priorities, monitor reclamation efforts and denote present uncontaminated areas.

The role and goals of Microbiology Laboratories Section, NWRI, are to develop a battery of tests which can be used in water quality management to screen environmental samples for toxicant and mutagen activity. To develop this battery, tests are developed, evaluated, and modified, if necessary, and then the proposed battery of tests is intensively field evaluated with samples collected from various parts of Canada. After each field evaluation the data are examined and tests which fail to live up to expectations are discarded from the battery and new tests are added.

The goal of these studies is to develop a battery of 3 or 4 toxicant and mutagen screening tests which could be applied with confidence and low cost to water, effluent and sediment extracts

collected from all parts of Canada (target date, December 1987). Data from such tests can be used to produce a priority index of areas of concern for more intensive studies by conventional chemical analyses. This optimized battery of tests should form an integral part of the national water quality monitoring network.

Toxicity screening test evaluation studies have already been undertaken in Lac Saint Louis (3) and Lake Ontario (4) by NWRI scientists and in the Ottawa River (6) as part of a joint NWRI/WQB study. Results to date have been very encouraging. It is recommended that WQB regional and headquarters personnel familiarize themselves with microbial screening tests, apply them in selected areas and integrate the results with corresponding conventional chemical data. The Analytical Methods Division is willing to assist the WQB with this introductory phase of the implementation.

An overview of microbiological assays for toxicant screening, including recent methods development research at NWRI is provided in the appendix.

APPENDIX

The purpose of this Appendix is to familizarize the reader with the scope of microbial and enzymatic tests which are used to screen for chemical toxicity in the research laboratory or in aquatic and sediment procedures developed at NWRI. It must be remembered that toxicant screening tests performed on water, effluent or sediment samples only measure effects, e.g. the response in the testing procedure is due to the total concentration of all the organic and inorganic constituents of the sample. At present, with the possible exception of the radioimmunoassay procedure, there is no biological or biochemical tests which can with certainty indicate the presence and concentration of any specific chemical or compound.

EFFECTS OF TOXICANTS OR MICROORGANISMS

There are many proposed mechanisms by which toxicants inhibit and eventually kill bacteria. 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, which may lead to the inhibition of organic decomposition and self-purification processes in sewage treatment plants and in waters receiving fecal material. It is believed that one of the most important effects of the toxic action of chemicals on bacteria is on enzyme activity. However, in any toxicity study, one must also take into account the physico-chemical factors (presence of other cations, pH, oxidation-reduction potential, temperature, organic matter, clay minerals, etc.) that control the toxic action towards microorganisms.

The impact of toxicants on bacterial cells may be measured via biochemical tests which include measurement of enzyme activity, ATP content,and bioluminescence (7). Some biochemical indicators (e.g., ATP, lipopolysaccharides, muramic acid) have been used for the determination of microbial biomass in environmental samples (7). Now briefly reviewed are the major categories of tests which are used or could potentially be used in toxicity assays.

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 (8). 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 (flavorproteins, cytochromes, etc.) which transport electrons from the nutrient source to O_2, the final electron acceptor.

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)(9).

B. ATP Assays

Adenosine triphosphate is a product of catabolic reactions, common to all protists, animal cells, 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(9). This reaction is catalyzed by luciferase and Mg.

A new toxicity test, ATP-TOX, developed at NWRI and adapted to environmental applications, is based on the inhibition of bacterial

growth and luciferase activity by toxicants (10). In the ATP-TOX System, chemical toxicity was found to be time dependent and increased with increasing exposure time up to approximately 5 hours, thus a chronic effect as well as an acute effect can be measured.

The ATP-TOX System is equally or more sensitive than the Micotox test, as easy to perform and is much less expensive (11). The ATP-TOX System applied to environmental samples can use predetermined baterial species or organisms (pure or naturally mixed cultures) indigenous to the environmental samples being tested. Organism resistance patterns to specific toxicants can also be studied to clarify and understand natural resistance patterns in the environment (11).

C. Radioimmunoassays

Immunoassays are relatively simple, powerful, and adaptable techniques that can be used to both detect and determine trace concentrations of organic compounds. Historically, Yalow and Berson (30) were the first to use radiolabelled antigens in the determination of trace antigen concentrations by means of the classical antigen-antibody reaction. Their observations provided the scientific community with a sensitive, precise and practical method for the micro-determination of proteins; the methodology has found particular application in the determination of hormonal polypeptides. Subsequently, radioimmunoassay methods were developed for the

detection and determination of non-proteinaceous compounds, such as steroidal hormones (31) and other low molecular weight organic compounds (32, 33).

Two key reagents must be prepared for an immunoassay for low molecular weight organic compounds: antibodies and labelled compounds capable of being bound by antibodies (hapten). To prepare antibodies a linkage group containing a side chain is attached to the compound of interest. The side chain separates the compound of interest from the protein carrier to which it is conjugated; this conjugate is an antigen. The antigen is injected into a rabbit, whose immune system responds by producing a family of antibodies, some of which are specific to the compound of interest. A derivative of the compound of interest is then labelled with ^{125}I or another appropriate marker. This labelled hapten is used in a competitive binding reaction with the antibody preparation to detect the compound of interest in samples.

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 and relatively large numbers of cells

can be exposed to the toxicant under study. These characteristics make bacteria suitable for rapid screening of toxicants in natural waters (7). 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 and assays to determine specific groups of bacteria and "ecological effects". Bacterial mutagen screening tests have also been evaluated.

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, P. phosphoreum, and Beneckea harveyi (12). 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. Recently, a Microtox assay (13), based on measurement of bacterial bioluminescence, was developed by scientists at Beckman Instruments, Inc. (Carlsbad, Calif.) to screen aquatic pollutants for their toxicity. NWRI has modified this test and has been applying it to the screening of environmental samples for toxic activity since 1978.

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 (14, 15). Some representative methods used in these bacterial bioassays are described in papers by Dutka and Kwan.

One particular bioassay is based on the nitrifying ability of Nitrobacter in sewage treatment plants (16). These bacteria have been proposed as bioassay microorganisms to measure the toxicity of heavy metals and industrial wastes. Nitrite disappearance or nitrate formation is monitored in these tests. The toxicant concentration (EC_{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.

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 (14). Pseudomonas fluorescens and Aeromonas hydrophila density inhibition tests and synthetic activated sludge tests have also been developed and assessed for environmental applications (14, 15).

The biological activity of wastewater is usually determined via respirometric methods (17). Oxygen uptake may be determined using a wide variety of techniques which have been described by King and Dutka (17). 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.

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 and consist of evaluating the effects of pollutants on nutrient cycling, and include organic matter decomposition, nitrogen transformations (ammonification, nitrification), and sulfate reduction (18).

D. Mutagen Assays

Many of the microbiological tests used for assessing mutagenicity/carcinogenicity are based on the the bacteria's attempts to repair damages caused by the stressing chemicals (19). Based on this knowledge, a new test has been developed (the SOS Chromotest)in Israel, which directly measures the damage to bacterial DNA through

the actions of the SOS DNA repair system (20). This test has been put into kit form for the testing of medical samples and pure chemicals. An adaptation and modification of this test has been made by Microbiology Laboratories Section and applied to environmental samples (21).

In testing for mutagenic or genotoxic activity in environmental samples, the SOS Chromotest appears to have advantages over the traditional Ames test (21). The use of the microplate with the 96 cells makes the test easy to perform, especially when a large number of samples are to be screened for genotoxicity. In doing the test, two end points can be reached, the SOSIP and toxicity. In addition, the procedure uses only one sample strain which reduces the number of tests required and the results can be easily visualized by two simple colorimetric enzyme assays. Last but not least, the test can be easily performed with results obtained in hours at a fraction of the cost of the Ames test.

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" test is the "Algal Assay

Procedure Bottle Test" developed by the U.S. Environmental Protection Agency to assess limiting nutrients in aquatic environments (22). 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 (23).

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 (23).

FUNGI AND YEAST BIOASSAYS

Along with bacteria, fungi and yeasts play an important role in the decomposition of organic matter in soil 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 (24).

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.

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 and Second International Symposia on Toxicity Testing Using Bacteria, 1983, Burlington, Ontario and 1985, Banff, Alberta.

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, soils, 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 (25). However, quantitative estimates for enviornmental 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 (25, 26).

Portier and Portier and Meyers (25) 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 (25).

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 co-contaminants under aerobic as well as anaerobic conditions.

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 to the use of this procedure have been related to instrumental requirements, namely sensitivity, response time, ease of operation, and automation.

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 ~10^4 cells per cubic centimeter, exhibit a response time of ~1 min and may be used virtually for any type of microorganism, substrate, and toxic contaminant (27, 28). 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. Dr. Jolicoeur, Sherbrooke University is one of the world leaders in this area (29).

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. Most of these tests are rapid, relatively reproducible and inexpensive, and require little space and time compared to fish bioassays. Microbiological screening techniques provide a useful and rapid screening tool for aquatic toxicologists, sanitary and environmental engineers, and microbial ecologists. Bacteria appear to be sensitive sensors of chemical toxicity since they respond relataively quickly to changes in their environment.

More information is now becoming available on comparative studies of short-term bacterial assays for estimating the impact of toxicants on the aquatic environment. These studies will provide valuable information about reproducibility, sensitivity, cost, and rapidity of the various tests. With the recent initiation of the International Symposia on Toxicity Testing Using Microbial Systems by NWRI staff, it is hoped that these biennial symposia will provide the forum for obtaining more of 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 Microbial Systems may be the vehicle for the above.

There is a strong need to standardize microbial toxicity screening tests, and efforts are being made towards that goal under the sponsorship of the American Society for Testing and Materials (ASTM) and the International Standards Organization (ISO). The use of 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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