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# A Simple, Rapid Bioassay for Detecting Effects of Pollutants on Bacteria

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Soil and aquatic environments have long been a respository for agricultural chemicals, discarded materials from manufacturing, and household refuse. Approximately 80% of the 344 million metric tons of wastes generated in this country are disposed of into nonsecure ponds, lagoons and landfills (COUNCIL ON ENVIRONMENTAL QUALITY 1980). The U.S. Congress has provided legislation to monitor the proper release and disposal of these hazardous materials. It is estimated that 43,000 compounds are subject to regulation by these acts. Both acts call for the establishment of criteria for identifying hazardous materials and determining levels of toxicity.

Since approximately 90% of hazardous wastes reach soil and water for permanent disposal, it is logical that microflora (bacteria) contained in these environments be used to establish initial toxicity levels. The microflora are especially important in the metabolic biodegradation and activation of various chemical compounds (ALEXANDER 1980). Bacteria can be suitable bioassay tools because they are inexpensive to cultivate, grow rapidly and contain physiological and enzymatic processes also found in higher organisms.

Bioassays have been described that utilize manometric methods and bacteria as indicators of toxicity (DAWSON & JENKINS 1950, MALANEY ET AL. 1959). The primary goal of these and other similar assays was to furnish information regarding metal ion toxicity on the biological oxidation processes which occur at municipal wastewater treatment plants. Other assays involving the toxicity of pollutants on bacteria have been

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proposed which involve measuring loss of viability (ANDERSON & ABDELGHANI 1980) changes in growth rates (NARKIS & ZUR 1979), and changes in biochemical oxygen demand (MOWAT 1976).

A screening bioassay needs to be accurate, inexpensive, rapid, and sensitive. The bioassay described in this study meets these criteria and utilizes bacteria as the toxicity predictor. The basis of the test involves measuring the kinetics of dissolved oxygen depletion by a mixed microbial population following exposure to a pollutant and allows results to be obtained in as little as 40 min.

## MATERIALS AND METHODS

Organisms and Growth: Samples of activated sludge or trickle filter effluent were collected for each experiment from a municipal wastewater treatment plant. A 0.1% volume/volume inoculum of sewage effluent was made into each of two 100 ml volumes of nutrient broth (Difco). Cultures were incubated in a gyrotory incubator shaker at 200 rpm for 24 h at 25°C.

Experimental procedure: One-hundred ml portions of nutrient broth cultures were added to two 2.8 L flasks each containing 900 ml of pH 7.2 standard methods buffer (AMERICAN PUBLIC HEALTH ASSOCIATION 1975) equilibrated at 25°C. The pollutant was dissolved in the experimental flask of buffer prior to the addition of the cell suspension. The 10-fold diluted cell suspensions typically gave an optical density of 0.18-0.20 at 600 nm. Three-hundred ml portions were removed from the experimental and control flasks, shaken vigorously for 1 min in sterile 1L plastic bottles, poured into 300 ml biological oxygen demand (B.O.D.) bottles and immersed into a 25°C waterbath.

Depletion of dissolved oxygen (D.O.) was monitored every 2 min with two oxygen meters (YSI models 51B and 54, Yellow Springs, Ohio) containing self-stirring probes (model 5720A) which fitted securely into the B.O.D. bottles. Meters were calibrated for each run according to manufacturer's instructions.

Initial D.O. concentrations ranged from 8-9 ppm. Readings were continued until the D.O. concentration was reduced to at least 3-4 ppm in the control bottle and until at least 10 readings were recorded from the experimental bottle. The time which corresponded to the point of 50% D.O. depletion was designated $T_{50}$. With pollutants which severely inhibited the D.O. depletion, it was necessary to determine the $T_{50}$ by linear regression analysis of the experimental recordings. This protocol was designated the brief exposure assay. An extended exposure assay was achieved by incubating control and experimental cell suspensions at room temperature (21°C) for 22h in the plastic 1L bottles. The contents were reshaken, and D.O. depletion rates were measured again.

Results were expressed as the ratio of the test $T_{50}$ to control $T_{50}$. This ratio is termed the "Activity Quotient" (AQ). When a compound had no toxic effect, the A.Q. = 1.00. If a compound is stimulatory (test $T_{50}$ < control $T_{50}$) the A.Q. <1.00. Toxicity was classified as slight (A.Q. = 0.8-0.94), moderate (A.Q. = 0.50-0.79), or extreme (A.Q. = <0.50).

Pollutants Tested: Pollutants tested were all reagent grade salts of heavy metals, organic pesticides, herbicides, and an organic solvent. Copper sulfate, nickel sulfate, sulfuric acid and Dimethylsulfide (DMF) were obtained from Baker. Cadmium chloride and Trichloroacetic acid (TCA) were obtained from Mallinckrodt. Diazinon and atrazine were obtained from CIBA-Geigy, Greensboro, N.C. Pentachlorophenol was obtained from Aldrich Chemical Company, Milwuakee, WI.

Statistical Treatment of Experimental Results: The D.O. depletion studies were conducted as simple paired comparisons. The pair consisted of two flasks generated from the same microbial population which differed only by the addition of the pollutant to one member. Differences between members of a pair were attributed to the treatment applied, in this case the added pollutant (SNEDECOR and COCHRAN 1967). By an analysis of variance, a difference in $T_{50}$ of 5% or less could be attributed to inherent meter variation. Any difference greater than 5% was attributed to the pollutant. All compounds were tested by this paired comparison 5 times.

## RESULTS AND DISCUSSION

Numerous chemicals must be cataloged as to amounts and potential for biological toxicity. A sequence of bioassays is envisioned to operate in a graded tier system of increasing cost and complexity. The initial screening assays should be inexpensive, rapid and sensitive enough to detect toxicity from a broad range of different chemical pollutants. The methodology described in this report has the potential of fulfilling these criteria.

Copper was tested first because of its known toxic effects on biological systems (Fig. 1). The responses were graded showing toxicity down to 0.5 ppm, detected by decreases in the D.O. depletion rate. The depletion of dissolved oxygen at 0.25 ppm occurred at a faster rate than the control indicating a possible stimulatory effect. The control suspension of the mixed microbial flora would consistantly deplete half of the D.O. in 20-30 min. The kinetics of D.O. depletion were linear during the observed time span and generally exhibited a graded response dependent upon the concentration and the time of exposure to the pollutant.

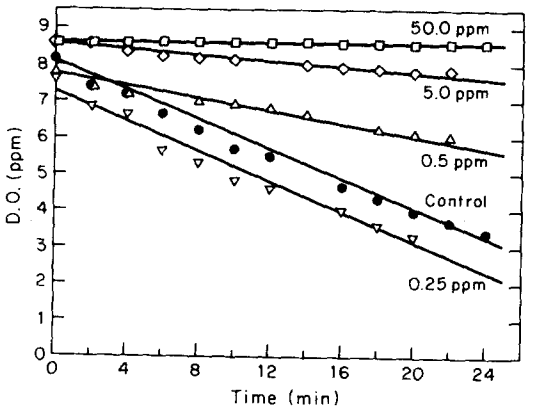


Figure 1. Kinetics of D.O. depletion by cell suspensions exposed to copper for 22 h (extended exposure assay).

Kinetics of D.O. depletion in cultures exposed to copper for a short time were compared to results found in the 22 h extended exposure. The results (Fig. 2) were expressed relative to the control $T_{50}$ and experimental $T_{50}$ rates (A.Q.). A 0.25 ppm concentration caused stimulation in D.O. depletion (A.Q. >1.00) in both the brief and extended exposures. The 0.5 ppm copper did not inhibit the D.O. depletion rate after brief exposure (A.Q. = 1.00) but was inhibitory during the 22 h exposure (A.Q. = 0.4). It was found that the $A.Q._{.5}$ for copper was 4 ppm in the briefly exposed cultures while the $A.Q._{.5}$ in cultures exposed to copper for 22 h was reduced to 0.4 ppm.

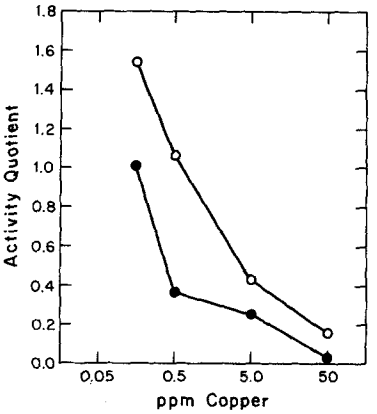


Figure 2. Activity quotients for brief exposure (open circles) and extended exposure (closed circles) to various concentrations of copper.

The toxicity of cadmium and nickel, was compared to that of copper (Table 1). Five ppm cadmium was more toxic in the bioassay than 5 ppm of copper while 5 ppm nickel was less inhibitory. Both copper and nickel were added to the bioassay as the sulfate salts. A control with 50 ppm sulfate ion (added as $H_2SO_4$) confirmed that toxic effects were caused by the metal ions alone.

Table 1. Quantitative effect of pollutants on D.O. uptake.

| Pollutant | Concentration ppm | A.Q.[a] brief | A.Q.[a] extended | Effect[b] brief | Effect[b] extended |
|---|---|---|---|---|---|
| Cadmium | 5.0 | .27 | .11 | extreme | extreme |
| Copper | 5.0 | .43 | .24 | extreme | extreme |
| Nickel | 5.0 | .51 | .54 | moderate | moderate |
| Sulfate ($H_2SO_4$) | 50.0 | 1.00 | 1.00 | none | none |
| Diuron | 40.0 | .83 | .56 | slight | moderate |
| Pentachlorophenol | 14.0 | .86 | .36 | slight | extreme |
| Atrazine | 33.0 | 1.00 | .76 | none | moderate |
| Tricholoracetic Acid | 50.0 | .96 | .88 | none | slight |
| Dimethylformamide | 50.0 | .95 | .85 | none | slight |
| Diazinon | 40.0 | 1.01 | 1.03 | none | none |

[a] $\frac{\text{Control } T_{50}}{\text{Test } T_{50}}$

[b] 1.00 = no effect; >1.05 = stimulatory 0.94-0.80 = slight; 0.79-0.50 = moderate; <0.49 = extreme.

The assay was used to determine the toxicity of several chemical classes of organic compounds which were tested at their maximum water solubility (Table 1). Only Diuron (a pre-emergence herbicide) and Pentachlorophenol (a wood preservative and pesticide) induced detectable and statistically significant inhibition of D.O. depletion in the short term exposure. However, in the 22 h exposure assay, all organic compounds, except Diazinon, were found to be toxic with effects ranging from slight to extreme. Pentachlorophenol exhibited only a slight effect (A.Q. = .86) in the brief exposure but an extreme inhibition (A.Q. = .36) was noted after the extended exposure. The lack of inhibition by Diazinon, an organophosphate, is not surprising since its primary target is the cholinesterase enzyme system which is absent in prokaryotes.

The D.O. uptake assay offers several advantages over some existing conventional animal test systems. It avoids major capital expenditures for equipment and reagents and provides data that are easily interpreted and little time is required to obtain results. The activated sludge and trickle filter samples provided equivalent results and both sources are widely available for testing in all regions throughout the world. Some

variation in bacteriological content of the samples could be expected due to seasonal influences and various industrial discharges which may enter wastewater plants. Little variation was observed in the current study in samples which were collected during a 1 year period. The variation that did occur was controlled by two procedures. First, the inoculation of a 0.1% v/v sample and its subsequent cultivation in rich medium was sufficient to eliminate any inhibitory substances which might be anticipated in the influent of the treatment plant. Second, the presentation of data as the activity quotient provided a simple means to normalize inherent sample variation. The test system is also adaptable to extended exposure responses which can increase assay sensitivity. Toxicity was detectable at 5- to 10-fold lower concentrations of compounds when cultures were preincubated with the chemical for 22 h prior to measuring D.O. depletion. The mode(s) of inhibition ellicited with the brief and extended exposures may be different but any resolution of the mechanisms observed are beyond the scope of this study. The periods of incubation are sufficiently short to avoid the emergence of pollutant resistant microbial populations. Future studies are planned to ascertain the impact of pollutants on pure cultures and to develop procedures for increasing the assay sensitivity.

Mention of products does not constitute endorsement by the U.S. Environmental Protection Agency. Technical paper #5976, Oregon Agricultural Experiment Station.

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