A Bioassay to Assess Wastewater Toxicity to Aerobic Biological Treatment

by

Kenneth J. Williamson

Water Resources Research Institute
Oregon State University
Corvallis, Oregon

WRRI-49

September 1976

A BIOASSAY TO ASSESS WASTEWATER TOXICITY TO AEROBIC BIOLOGICAL TREATMENT

- Project Completion Report -

OWRR Project No. A-036-ORE-W56

July 1, 1975 - June 30, 1976

Kenneth J. Williamson
Principal Investigator

Assistant Professor
Department of Civil Engineering
Oregon State University

September, 1976

ACKNOWLEDGEMENTS

The work upon which this report is based was supported by funds provided by the United States Department of the Interior, Office of Water Research and Technology, as authorized under the Water Resources Research Act of 1964.

ABSTRACT

The objective of this research was to develop a bioassay for wastewater toxicity that could be used at sewage treatment plants. Nitrobacter was used as the test organism due to the simplicity of quantifying substrate removal rates by measuring nitrite removal, a sensitivity for most toxicants greater than that of heterotrophic organisms, and a slow growth rate which enables the use of batch-fed tests.

A method was developed for preserving the Nitrobacter organisms by freeze-drying. Substrate removal rates were measured for both freeze-dried and non-freeze-dried Nitrobacter in the presence of a heavy metal and an organic toxicant. It was found that zinc and 2, 4, 5-trichlorophenol exhibited toxic effects to the Nitrobacter at concentrations comparable to those toxic to activated sludge.

KEY WORDS

Toxicity*, Bioassay*, Biological Waste Treatment, Nitrobacter, Freeze-drying, Zinc, Trichlorophenol.

INTRODUCTION

Activated sludge has rapidly become the most common method of biological waste treatment in the United States. Unfortunately, the process is subject to many possible operational problems which can result in reduced treatment efficiency; the most common are sludge bulking, shock hydraulic or organic loads, mechanical failure, operator mistakes, temperature changes, and toxicity. It is important that the cause of process failure may be rapidly and routinely determined by the sewage treatment plant personnel. This research was orientated towards that goal for the problem of toxic wastewaters.

One method to determine if a real toxicity problem exists is through the use of a bioassay test. Such a test would not be entirely conclusive, but it would give a strong indication of the presence of toxic compounds. If the test proved negative, then remedial measures could be concentrated on examining for sludge bulking, mechanical failures, large temperature changes, operator error, or organic or hydraulic loadings beyond design capacities.

If the test proved positive, then extensive sampling of all point sources could be undertaken to determine the toxic discharge. All sanitary codes strictly forbid the discharge of toxic materials to sanitary sewers. In addition, highly-detailed chemical analyses of the wastewater could be contracted in hopes of identifying the specific toxic compounds.

Such a bioassay test would prove to be a valuable tool to increase the reliability of waste treatment plants. This goal is clearly in agreement with recent Environmental Protection Agency (EPA) policy to improve reliability through better physical designs (EPA, 1974). Improved reliability would result in less discharges of inadequately treated wastewaters to receiving streams which can be especially critical during summer periods of low flows and high contact-sport use by the public.

In this research, the possibility of using nitrifying bacteria (Nitrobacter) as a bioassay organism to measure wastewater toxicity has been examined. These organisms would have the advantage of: 1. simple quantification of removal rates by measurement of nitrite removal, 2. use of batch-fed tests because of the slow growth rates, 3. increased sensitivities for most toxicants as compared to heterotrophic organisms, and 4. independence of past toxic loads by use of freeze-dried organisms.

LITERATURE REVIEW

Kinetic Theory of Toxicity

Hartmann and Laubengerger (1968) have given a detailed summary of a biochemical theory for bacterial toxicity. The theory is based on enzyme inhibition by the toxic material; the kinetics of substrate utilization are assumed to follow Michaelis-Menten relationships as:

$$\frac{dS}{dt} = -k\frac{S}{S+K_s} X \qquad (1)$$

where: dS/dt = rate of substrate utilization,

k = maximum rate of substrate utilization,

X = organism concentration,

S = substrate concentration, and

K_s = "half-velocity" coefficient.

Two main types of enzyme inhibition are identified: competitive and non-competitive. In the case of competitive inhibition, the inhibitor, I, can react with the enzyme and the enzyme-substrate complex which results in an enzyme-inhibitor complex in both cases. The resulting Michaelis-Menten relationship is:

$$\frac{dS}{dt} = -k\frac{S}{S+K_c} X \qquad (2)$$

where: K_c = "half-velocity" coefficient with competitive inhibition.

K_c will always be larger than K_s.

For the case of non-competitive inhibitor, the inhibitor can react with the enzyme to form an inhibitor-enzyme complex and with the enzyme substrate complex to form an enzyme-substrate-inhibitor complex. The appropriate Michaelis-Menten relationship is:

$$\frac{dS}{dt} = -k_n\frac{S}{S+K_s} X \qquad (3)$$

where: k_n = maximum velocity with non-competitive inhibition.

k_n will always be less than k.

In both cases of inhibition, the inhibitor will cause a reduction in the substrate removal rate. Hartmann and Laubenberger (1968) showed that dichromate and H^+ ions exhibited non-competitive inhibition as compared to lead and copper which exhibited a competitive inhibition. Most toxicants demonstrated a mixed case where both non-competitive and competitive inhibition were present. The importance of this distinction to the measurement of toxicity with Nitrobacter will be presented in a later section of this report.

Mechanism of Toxicity for Various Compounds

Chemical agents which are toxic to microbial populations act by a variety of mechanisms which cause the inhibition or destruction of cellular components vital to cell function. Several categories of these agents are known for their anti-microbial activities which were first investigated because of their importance as disinfectants.

Halogen compounds have long been recognized for their germicidal qualities. Chlorine, which is usually present in an aqueous environment as hypochlorous acid, attacks the sulfhydryl (-SH) groups of enzymes involved in essential metabolic pathways (Hedgecock, 1967). Complete termination of glucose oxidation occurs at lethal levels, indicating the sensitivity of the particular enzymes in that pathway. Hypochlorite ion

also attacks proteins to form derivatives containing chlorine linked to nitrogen. The oxidative character of derivatives produced in the chlorine reactions also allow them to act directly in the destruction of cell constituants by oxidation. A second halogen, iodine, exhibits its most toxic character in the I_2 form. Reaction of elemental iodine with organics or surface active agents produces iodophors which exhibit germicidal qualities according to the availability of the complexed iodine. These compounds attack proteins by direct iodination or oxidation of their constituent groups, rendering them inactive. Bromine, another halogen compound, acts in much the same manner as those mentioned above. Existing primarily as hypobromous acid as neutral pH, the chemical is a strong oxidizing agent which alters protein structure.

A second major category of anti-microbial agents consists of the heavy metals. Exposure to inhibitory metals can result in the occurrence of a variety of abnormalities. Conversion of bacterial rods into spherical forms by copper suggests interference with the normal process of cell wall synthesis (Sadler, 1967). Decreased activity of certain oxidative enzymes occurring in the cytochromes has taken place in some organisms grown in the presence of cobalt or copper. Whether these are the direct result of action by the metal or secondary symptoms due to some other primary effect is not known. This uncertainty arises because no thorough investigation of all the effects produced in microorganisms due to exposure to a heavy metal has been performed.

The supposed mechanisms of toxicity for some of the heavy metals have been presented (Hedgecock, 1967). The most lethal of the compounds, mercury, occurs as an organic ion and also in the form of organic mercurial cations. These chemicals react specifically with available sulfhydryl groups of the enzyme constituents of the cells, causing decreased enzymatic activity and, ultimately, cell death. Reversal of the anti-bacterial properties of the mercury compounds can be brought about by the addition of sulfides. The anti-microbial action of another metal, silver, is exhibited when it assumes the free ion configuration. The mode of action of this agent involves the formation of metal complexes with polynucleoxides, DNA and RNA, causing their inactivation. Copper is also thought to bind DNA and thereby bring about interference of its function. In addition to this action, the germicidal effects of copper are also due to the coagulation of bacterial colloids.

In some cases, metal toxicity is also known to mimic the symptoms of essential metal deficiency. As an example, zinc toxicity in the fungus Aspergillus niger exhibits symptoms very similar to those of magnesium deficiency. In cases such as this, it appears that the inhibitory metal is somehow interfering with the normal activity of the corresponding essential metal (Sadler, 1967).

The phenols form a group of anti-microbial compounds consisting of a variety of derivatives of phenol. The unsubstituted compound displays a high degree of surface activity and acts by disrupting cell

membranes and inhibiting oxidase enzymes associated with the surfaces. As the substance enters the cell, damage may be done by the precipitation of proteins. The compound 2, 4-dinitrophenol is thought to uncouple oxidation from phosphorylation so that the energy obtained by the oxidation of the substrate ceases to be stored as ATP.

Alcohols act in much the same way as the phenols, causing inhibition of respiration and phosphorylation, possibly due to severe damage to the membrane. Other agents responsible for membrane disruption include the quaternary ammonium compounds. They also do damage by inhibiting bacterial oxidase and dehydrogenase systems as a result of protein denaturation and enzyme suppression.

Acids and alkalies affect the pH of the bacterial media causing an over-abundance of either H^+ or OH^- ions. Hydrogen ions are known to displace essential ionic species such as Na^+ and Ca^{++} from their absorption sites on the cell. This displacement may result in a deficiency of the ions required for bacterial growth. Hydrolytic reactions involving the hydrogen or hydroxyl ions could also produce lethal damage to the cell surface.

Toxicity can result in several different ways for bacteria. All of the methods reviewed here would be common for both heterotrophic organisms (activated sludge) and autotrophic organisms (Nitrobacter). Thus, there appears to be no theoretical reason why Nitrobacter could not be used as a bioassay organism for toxicity to activated sludge.

Methods of Measuring Toxicity to Activated Sludge

Many different methods have been used to measure a toxic response in activated sludge. In this section several of these methods will be described and their advantages and disadvantages summarized.

The most common method is to use continuously-fed chemostats. Duplicate reactors are fed with and without the toxicant and the organic removal rate monitored. This technique is expensive to operate and extremely time consuming. Equipment requirements include reactors and constant-feed pumps, and a large source of fresh sewage is necessary. This method does approximate the conditions in activate sludge tanks, although the hydraulic and solids retention times of the activated sludge treatment are difficult to maintain in the chemostats. Aeration rates and solids separation methods are usually radically different than the field conditions. Unfortunately, the bioassay organisms are typically taken from the treatment plants which has already experienced the toxic discharge. Short-term studies often never approach assumed steady-states and long-term studies do not provide information rapidly enough.

Unfortunately, chemostats are notoriously difficult to run. Pumping the small amounts of influent at a controlled rate is difficult. Several weeks may be necessary for stabilization of growth to occur. Often the effects of toxicity are masked by the normal variability in COD or BOD removal. For these reasons, chemostats cannot be used as a standard

analysis procedure. Examples of toxicity test performed by this method are reported by Moulton and Shumate (1963), Ayers, Shurrate and Hanna (1965), and Barth, et al., (1964).

Another common toxicity test could be termed the BOD bottle technique. There are many variations, but the test essentially consists of measuring the BOD of a known organic with or without the toxic waste. The method is extremely simple and can be accomplished in standard sewage treatment plant laboratories. However, the dilution of the waste for the test may reduce its concentration below its threshold level. The small range of only 2 to 7 mg BOD/l greatly limits the accuracy. In addition, the method is slow since it requires 5 days to complete. The toxic waste will undoubtably add to the measured BOD which is difficult to account for in the calculations. Mowat (1976) used this method to determine the toxicity of various metals to mixed heterotrophic growth from sewage.

Most of the difficulties of the BOD bottle technique can be alleviated by using a batch-fed technique. Basically the test involves measurement of the substrate utilization rate and the cell mass. The most common method of determining the substrate utilization rate is manometrically as described by Hartmann and Laubenberger (1968), although the decrease in concentration of the organics can also be used as described by Bunch and Chambers (1967). The cell mass can be measured as total suspended solids, volatile suspended solids (Neufeld and Hermann, 1975), organic

nitrogen (Hartmann and Laubenberger, 1965), or ATP (Patterson, Brezonik, and Putnam, 1969).

The batch-fed techniques typically require more advanced instrumentation than available at sewage treatment plants. Respirometers are expensive and difficult to operate even for highly-trained personnel. Specific measures of organics will require sophisticated instruments or difficult chemical extractions. The length of the tests using batch-fed techniques are usually long compared to the generation times of the heterotrophs, so the number and types of organisms can vary significantly between the reactors with toxicants and the control reactor. The activity will vary accordingly, which makes the results difficult to interpret.

None of the three methods (chemostat, BOD bottle, batch-fed) are directly applicable for routine monitoring of toxicity in sewage treatment plants. These tests primarily require too much effort or advanced instrumentation. Any test adopted for routine testing at sewage treatment plants must be both rapid and simple, and the results must be easy to interpret.

The Test Organisms

For this research, Nitrobacter bacteria have been chosen as the test organisms. These organisms are strict aerobes which obtain their energy from the oxidation of nitrite to nitrate. Carbonate is their sole

carbon source. The cells can be grown in a media of nitrite, oxygen, carbonates, phosphorous and basal salts and, therefore, the growth medium does not contribute organics to the bioassay test.

The substrate utilization rate for Nitrobacter can be accurately expressed by the Monod model:

$$\frac{dS}{dt} = -\frac{kSX}{K_s + S} = -kX. \quad (4)$$

Williamson (1973) measured the half-velocity coefficient as 0.07 mg NO_2^--N/l and the maximum utilization rate as between 1 to 4 mg N/mg TSS-day. The substrate concentration in the bioassay test would always be above several mg/l, so the substrate removal rate (dS/dt) will be constant and equal to -kX.

The growth rate of these organisms is very slow due to the small free energy captured from the oxidation of nitrite and because carbon dioxide is used for cellular carbon. The thermodynamically calculated cell yield value is 0.084 mg TVSS/mg NO_2^--N oxidized. As a result of such low growth rates, the bioassay test can be conducted in batch-fed reactors over detention times of several hours without significantly changing the organism concentrations or the linear decrease in concentration versus time. By avoiding continuously-fed reactors (chemostats), the bioassay test is greatly simplified and can be conducted without ordinary glass beakers.

Nitrifying bacteria have been found to be especially sensitive to toxic compounds which makes them an ideal test organism. Barth, et al. (1965) found that in activated sludge systems nitrification ceased at concentrations of copper, chromium, nickel and zinc which only slightly inhibited the chemical oxygen demand reduction efficiency of the system. Downing, Tomlinson, and Truesdale (1964) also found nitrification inhibition in activated sludge from many toxic organic compounds. However, nitrification readily proceeds in activated sludge without toxic inputs so these autotrophic organisms are not adversely affected by normal organics found in sewage.

Since the substrate concentration will always greatly exceed the half-velocity coefficient, most competitive inhibition will probably not be measured in the toxicity test. The half-velocity coefficient with competitive inhibition would have to be increased by at least a factor of twenty before it would affect this test. This does not appear to be a serious shortcoming, however, because almost all toxicants show some non-competitive inhibition which will decrease k. A decrease in k can be readily measured in batch-fed tests as proposed for the bioassay test.

Compounds Specifically Toxic to Nitrobacter

Studies of nitrification have typically emphasized inhibitory compounds which directly affected the energy reaction. In one such study, Lees and Simpson (1955a) proposed a very specific mode of action by

which chlorate inhibited the oxidation of nitrite to nitrate. Their results indicated that chlorate when present at a concentration of 10^{-3}M displaced nitrate from, and combined irreversibly with, a nitrite oxidizing enzyme. This replacement apparently occurred at the stage of nitrite oxidation when the enzyme was carrying a nitrate ion. Later work by the same authors (1955b) indicated that cyanate also specifically inhibited nitrite oxidation, but at an earlier stage in the process than the chlorate inhibition. Stafford (1974) reported results of inhibition studies on nitrifiers in activated sludge using phenols and pyridine compounds. He found inhibition by phenol occurred at 4-10 mg/ℓ for the conversion of ammonia to nitrite; however, the Nitrobacter induced reaction was not affected even at 100 mg/ℓ. Pyridine completely inhibited the nitrite reaction at 100 mg/ℓ, while ammonia oxidation was only partially inhibited.

Cyanide, an inhibitor of respiratory enzymes, has been found to reduce the activity of Nitrobacter in activated sludge by 50 percent at 2×10^{-5}M and 79 percent at 1×10^{-4}M (Tomlinson, 1966). Work in the same laboratory has indicated a 75 percent reduction of nitrite oxidation in activated sludge if 2, 4-dinitrophenol is added at a concentration of 370 mg/ℓ. Concentrations of 2×10^{-3}M potassium chlorate, 5.7×10^{-5}M sodium cyanide, 1.5×10^{-3}M hydrozine sulfate, and 2.2×10^{-4} sodium azide were also reported to inhibit nitrite oxidation by 75 percent.

In summary, several compounds such as chlorate and sodium azide will exhibit toxicity to Nitrobacter which would not be seriously toxic to activated sludge. However, these compounds would not typically be present in wastewaters. Other compounds such as pesticides and heavy metals will account for most toxicity problems in aerobic treatment and should be toxic to both activated sludge and Nitrobacter.

Compounds Toxic to Activated Sludge

Many studies have been undertaken to establish the levels of heavy metals which decrease the efficiency of activated sludge organisms. Probably the most extensive such study was carried out at the Robert A. Taft Sanitary Engineering Center (Moore, et al., 1961) (McDermott, et al., 1962) (McDermott, et al., 1963) (McDermott, et al., 1965) (Barth, et al., 1964). Several pilot scale activated sludge units were assembled and run under careful supervision. Tests were run in which a particular metal or combination of metals was fed as a shock load with no acclimation, or fed continuously with samples taken two weeks following initial metal input. In either case, treatment efficiency was determined by comparison of the COD, BOD, and turbidity in the effluent of a control unit to the values obtained for the test unit.

The threshold copper concentrations in the feed which produced decreased efficiency were 1 mg/ℓ if fed continuously as $CuSO_4$, 75 mg/ℓ for a 4-hour slug dose, and between 5 and 10 mg/ℓ for a slug dose of

cyanide complexed copper (McDermott, et al., 1963). The increased effectiveness of the cyanide copper complex at a smaller slug dose was indicated to be the result of a reduced ability of that agent to bind to the sludge. Although it caused a greater immediate effect, the duration of its influence was much shorter than the sulfate salt. Large quantities of copper appeared in the effluent shortly after dosing of the cyanide copper began, which indicated that the bulk of the dose passed through the sludge quickly. Much of the sulfate copper was adsorbed by the sludge, which allowed the metal to remain and prolong its effects.

Nickel concentrations which resulted in decreased efficiency were reported as 1 to 2.5 mg/ℓ for continuous feed, and between 50 and 200 mg/ℓ for a 4-hour slug dose (McDermott, et al., 1965). The authors noted that after the initial reduction in efficiency, the concentration of metal could be increased greatly with very little further decrease in treatment efficiency.

Zinc was found to inhibit sludge organisms at between 5 and 10 mg/ℓ for continuous feed and at 160 mg/ℓ if introduced in a 4-hour slug dose (McDermott, et al., 1962). Chromium was reported to be the least inhibitory of the metals tested (Moore, et al., 1961). A concentration of 10 mg/ℓ if fed continuously and greater than 500 mg/ℓ if introduced as a slug was required to reduce efficiency. Although chromium was found to seriously inhibit nitrifying organisms in non-acclimated sludge, the authors also indicated that the bacteria could acclimatize to concentrations as high as 50 mg/ℓ.

Results of experimentation employing metal mixtures in sewage fed to the activated sludge pilot unit indicated no synergistic action in three combinations used (Barth, et al., 1964). Concern was expressed over the inhibition of nitrification because nitrification failed to occur over the 65-day duration of the test.

Moulten and Shumate (1963) studied the effects of copper on activated sludge and found a continuous dose of 45 mg/ℓ was insufficient to stop biological activity. Removal of 60 percent of the influent COD was the least efficient reduction recorded over the test period.

Ayers, et al. (1965) also experimented with copper in attempting to establish the mechanism by which the metal inhibits sludge microbial populations. Basing conclusions on his own work as well as previous studies, the author advanced several theories on the subject. They concluded that under a given set of conditions, a relatively constant proportion of the copper forms stable inactive complexes with respiratory enzymes. Effects due to the formation of such metabolically inactive complexes was observed immediately, as evidenced by their slug load data. Ayers, et al., further concluded that the degree of toxic effect depends on three factors: copper dosage, mixed liquor suspended solids, and influent sewage strength. They also suggested that reversal of the inhibition occurred by release of the copper from its attachment site, an event triggered by either dilution or addition of the uncomplexed ligands.

Neufeld and Hermann (1975) reported data on the toxicity of various heavy metals to acclimated activated sludge. A batch-fed reactor was dosed with various metals up to 1000 mg/ℓ and the effect on kinetics measured. They found a toxic response at high concentrations for all metals. Specifically, zinc was inhibitory at several hundred mg/ℓ. Similar results were found by Cheng, et al. (1975).

Jackson and Brown (1970) authored an extensive review of toxic compounds to aerobic and anaerobic treatment processes. They gave typical values of toxicity for various metals, but did not list any organics which have been reported as toxic to activated sludge. Ferguson, et al., (1976) used trichlorophenol at concentrations of about 20 mg/ℓ to initiate toxicity in their experiments with activated sludge.

In summary, the toxicity of metals to activated sludge has been reported extensively and the toxic concentration varies from several mg/ℓ to several thousand mg/ℓ. Few organic toxicants have been reported. The results on metals suggest that Nitrobacter may be more sensitive than the heterotrophic organisms.

CULTURING PROCEDURES

The enriched culture of Nitrobacter was grown in a down-flow column packed with polyethylene beads (Figure 1) similar to the procedure used by Williamson (1973). The column was 9.0 in. in diameter and 25 in. high. The feed solution contained approximately 30 mg NO_2^--N/ℓ,

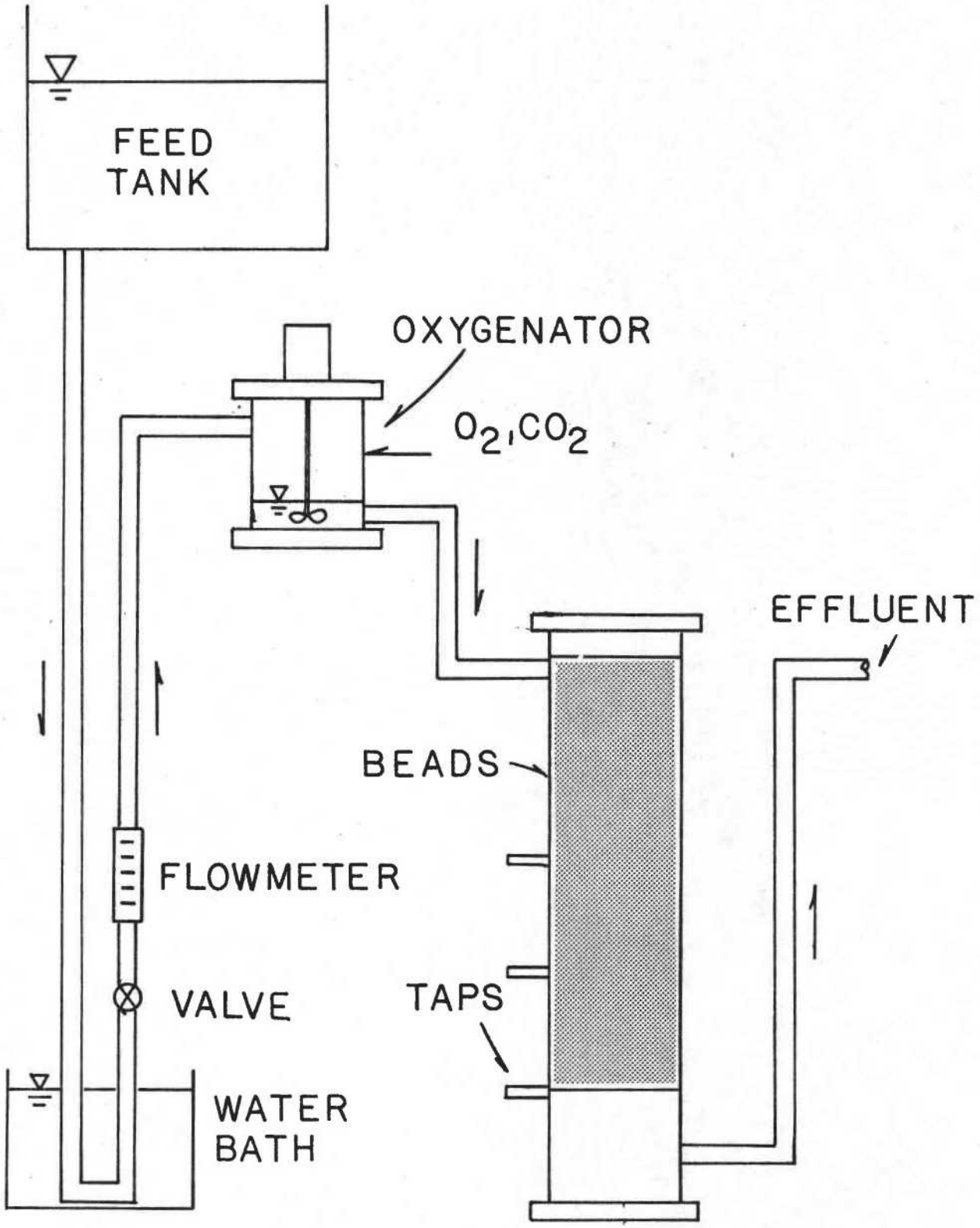

Figure 1. Apparatus for Culturing Nitrobacter.

20-30 mg O_2/ℓ, and 0.2 mg/ℓ phosphorous as KH_2PO_4. The pH of the feed solution was adjusted to 7.0 using NaOH. Feed was mixed in a 300 gallon holding tank, from which it was pumped to the oxygenator. This consisted of a Plexiglas cylinder, 6 in. in diameter and 8 in. high with a motor-driven turbine propeller to mix the oxygen with the feed solution. Oxygen was supplied from compressed oxygen cylinders. The solution was then gravity-fed into the columns. A clear zone was maintained at the bottom of the column from which the effluent was withdrawn. To remove organisms, the pinch clamp on a tap near the bottom of the column was opened and a slurry of organisms and polyethylene beads flowed from the column. This slurry separated readily, the beads floating to the top and the organisms settling to the bottom of a beaker. Cells were removed at a concentration of approximately 1000 mg/ℓ. The beads were screened from the top of the beaker and returned to the column, and the bacterial mass was then used in toxicity measurements or further processed and freeze-dried.

FREEZE-DRYING

The objective of this research included the constraint that the bioassay could be used at sewage treatment plants. To enable the shipment of organisms, we investigated the feasibility of freeze-drying the organisms and rehydration for the bioassay test.

Freeze-drying provides a means to preserve organisms indefinitely so that they may be stored and shipped without special handling such as refrigeration. Successful freeze-drying of a microorganism depends first on the proper selection of a cryogenic protective agent. Fry (1966) reviewed the effectiveness of many different agents that have been utilized for different organisms. The importance of the suspending medium lies in the survival of the bacteria during the freezing and drying as well as the storage of the cells. Redway and Lapage (1974) studied the effects of carbohydrates in the suspending media for five different microbial strains, and Sinha, et al., (1974) studied the effects of the individual ingredients of skim milk as protective agents. Sourek (1974) discussed findings from the Czechoslovak National Collection of Type Cultures, which has been responsible for much study in the area of preserving pathogenic bacteria. In general, it has been found that for a given organism there may be any number of different suspending media that allow preservation by freeze-drying, but no specific medium seems to be satisfactory for all types of bacteria.

Rehydration of freeze-dried cells can be a critical step in the process of preservation by freeze-drying because certain organisms suffer high mortality at this stage. Leach and Scott (1959) found that varying the amount of solution used to rehydrate the bacteria and the rate at which it is added can affect the survival of the organisms. Sourek (1974) and Seastone (1971) have both shown that rehydration with

the growth-media broth proved more successful than using distilled water or physiological saline solution, even though the latter two dissolved the dried suspension more readily.

Procedure

Although much work has been done on freeze-drying bacteria, no work has been reported for Nitrobacter. Consequently, a large amount of trial and error has been involved in developing a satisfactory process. The most successful method found was to freeze the organisms rapidly in a dry ice-acetone bath. Slower methods apparently lysed the bacteria with large ice crystals, and consequently no survival was obtained.

Suspending media tested included various combinations of skim milk, ascorbic acid, thiourea, ammonium chloride, starch, gelatin, sucrose, dextrin, and lactose. The most successful medium was found to be a solution of 10 percent sucrose and 1 percent gelatin.

Nitrobacter were removed from the column and washed in glass-distilled water and centrifuged to remove excess water. They were then suspended in an approximately equal volume of the cryogenic protective agent consisting of a solution of 10 percent sucrose and 1 percent gelatin. Two mℓ of this suspension was then pipetted into each of several 20 mℓ glass ampules. The Nitrobacter was then frozen in an acetone-dry ice and attached to the freeze-dryer which removed the water from the frozen suspension. The ampules were then sealed and stored.

Rehydration was done with a solution of approximately 5 mg NO_2-N/ℓ. Each ampule was broken open and 2 mℓ of nitrite solution was added dropwise. After the suspension was dissolved, it was then washed twice and resuspended in this solution to remove as much of the protective agent as possible. The cells were then ready to be utilized in the toxicity testing.

TOXICITY MEASUREMENT

The bioassay tests were done in completely-mixed, temperature-controlled flasks. Since Nitrobacter neither consumes or produces acids (except for the small amounts of bicarbonate for cell growth), no pH buffer was required. One vessel served as a control and to the other, the toxic wastewater was added. The rehydrated cells were added in equal concentrations to all vessels and the NO_2^--N concentrations were measured versus time.

The measured nitrite concentrations were plotted versus time as shown in Figure 2. The slope of these plots is numerically equal to -kX. Any decrease in the slope for the vessel with the wastewater added in relation to the control vessel represented some form of toxicity. The greater the toxic effects, the greater the differences of the slopes. Under massive toxicity, the -kX value for the wastewater vessel was equal to zero.

Experiments were undertaken to determine the sensitivity of the

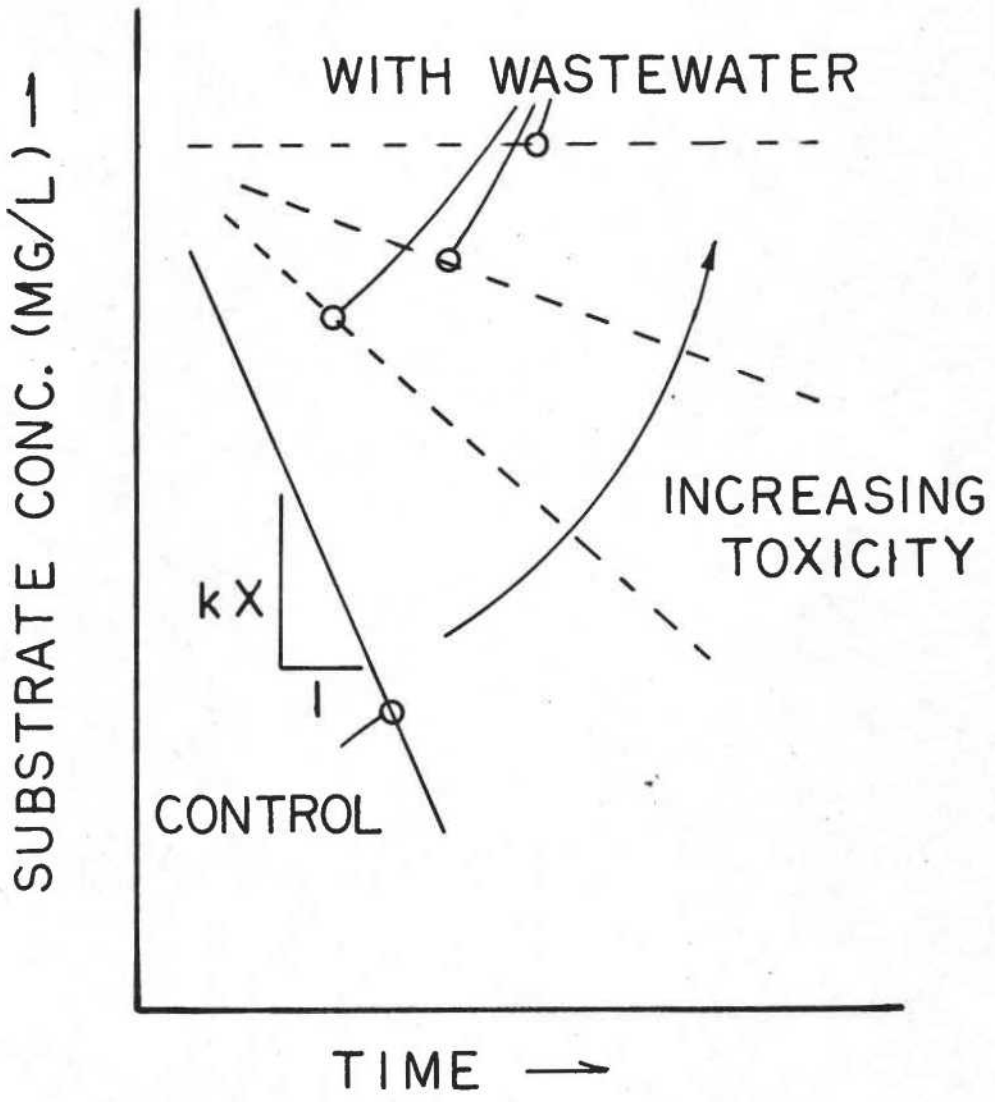

Figure 2. Substrate Concentration vs. Time for Various Toxicity Levels.

Nitrobacter to zinc and 2, 4, 5-trichlorophenol. These particular compounds were chosen because of the known toxicity to activated sludge.

Procedure

The toxicity tests for zinc and trichlorophenol were performed by first adding 35 mℓ of primary effluent sewage, which had been allowed to settle for approximately 30 min, to 50 mℓ volumetric flasks. Three mℓ of a 100 mg/ℓ NO_2^--N solution were then added, followed by the addition of the toxic substance in an aqueous solution. Zinc was added as a solution of the sulfate salt. Each experiment included one control flask to which no toxic substance was added. The contents of each flask was then diluted to 50 mℓ with distilled water, mixed thoroughly, and emptied into 250 mℓ erlenmeyer flasks. These flasks were stoppered with cotton and placed in a shaker bath maintained at a temperature of 25°C. The solutions were then mixed on the shaker for about an hour to reach equilibrium.

While the sewage solutions were mixing, cell slurries of either rehydrated cells or cells gathered from the growth column were prepared for addition to the flasks. The freeze-dried organisms were rehydrated and prepared as described previously. The non-freeze-dried cells were removed from the column and allowed to settle. Approximately 20 mℓ of settled Nitrobacter were washed and diluted to 100 mℓ with glass-distilled water. Preparation of freeze-dried cells was done so as to

achieve approximately this same cell concentration. Five mℓ of the resulting cell slurry were added to each of the incubation flasks containing the sewage solutions. The shaker was activated to begin the run.

Nitrite concentrations in the flasks were measured at various time intervals using Method 134 (Standard Methods, 1971) in which a reddish purple azo dye is formed by the coupling of diazotized sulfanilic acid with napthylamine hydrochloride. Measurement of the absorbance of the dye solution at 520 mμ was compared with a standard curve to obtain the concentration of nitrite. Sampling was continued for 2.5 to 5.5 hours to establish the rate of nitrite utilization.

Immediately following the incubations, portions of each sample were vacuum filtered through pre-dried and weighed 0.45 micron Millipore filters. Suspended solids concentrations were determined for the runs by drying and reweighing the filters, and the filtrates were analyzed to determine the concentration of the toxic material.

Analysis of the zinc samples was performed using an atomic absorption spectrophotometer. The concentration of trichlorophenol was determined by the following procedure. Ten mℓ of sample were placed in a screw top test tube and acidified by the addition of 3 drops of concentrated phosphoric acid. Five mℓ of iso-octane was added to the tubes which were then covered with aluminum foil and tightly capped. The tubes were shaken vigorously for one minute after which the caps were loosened and the samples placed in a boiling water bath for ten minutes.

The tubes were removed from the water, the caps re-tightened, and again the samples were shaken for one minute. After standing overnight, the absorbance of the iso-octane at 298 mμ was determined and compared to a standard curve.

The proposed procedure for the bioassay test with a toxic wastewater is described in detail in Appendix 1.

Results

The activity of the bacterial cells subjected to the various concentrations of toxicant was determined by plotting the concentration of nitrite-nitrogen versus time. Typical graphs of the data appear in Figures 3, 4, 5 and 6. By comparing the rate of nitrite utilization for an incubation containing no toxic agent to those with toxicant present, the percent of activity remaining was determined. Plots of the percent of control activity versus concentration of toxic material are presented in Figures 7 and 8.

A linear removal of nitrite was observed for all controls and for the tests with trichlorophenol. A slight non-linear response was noted for those tests with zinc. For those instances, the removal rate was measured as the slope of the concentration versus time curve from 60 to 300 min. The near linearity in all tests confirmed that the substrate concentration was much larger than the half-velocity coefficient (K_s or K_c) during the test.

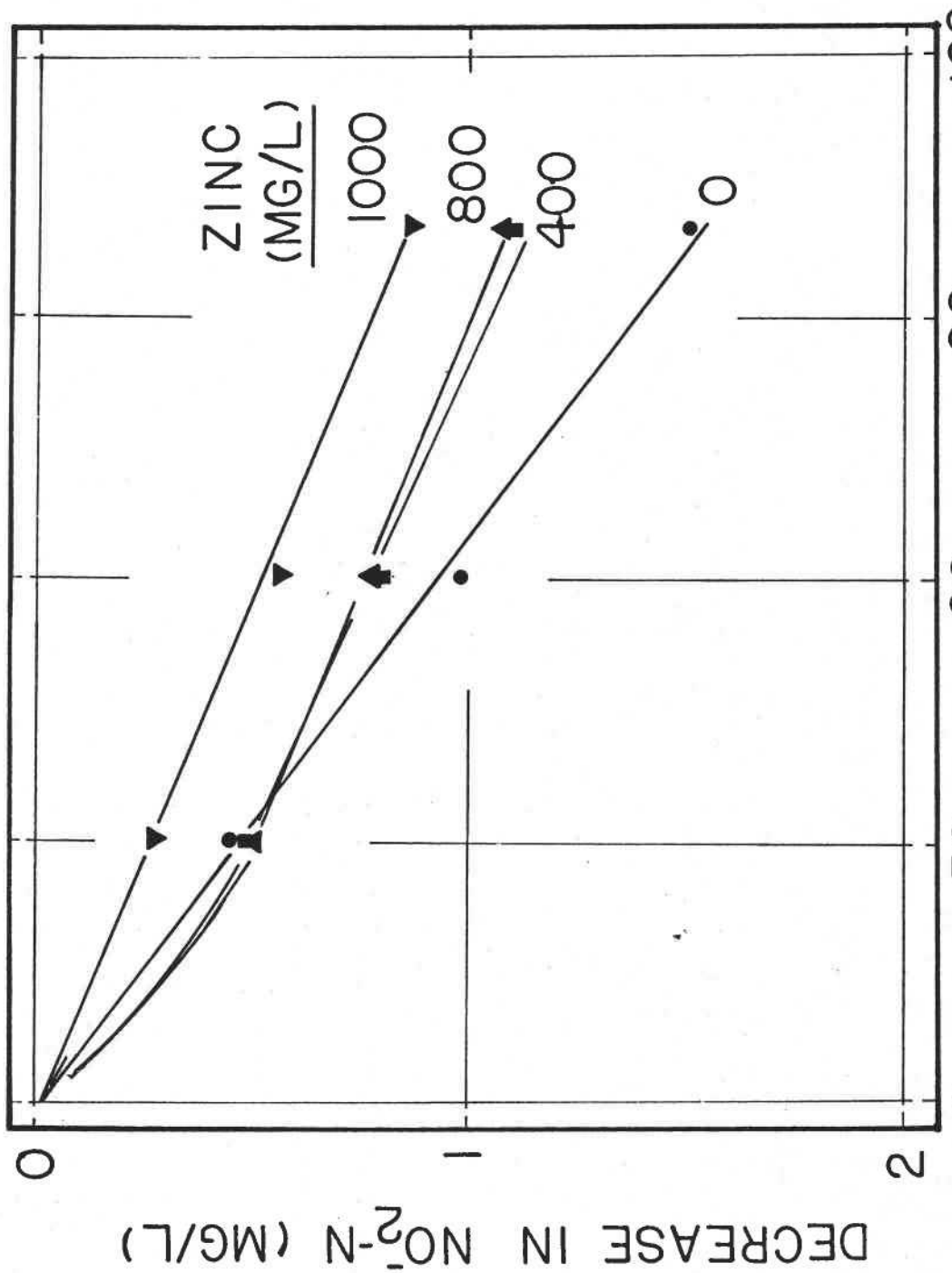

Figure 3. Incubations of Non-Freeze-Dried Cells in the Presence of Various Zinc Concentrations

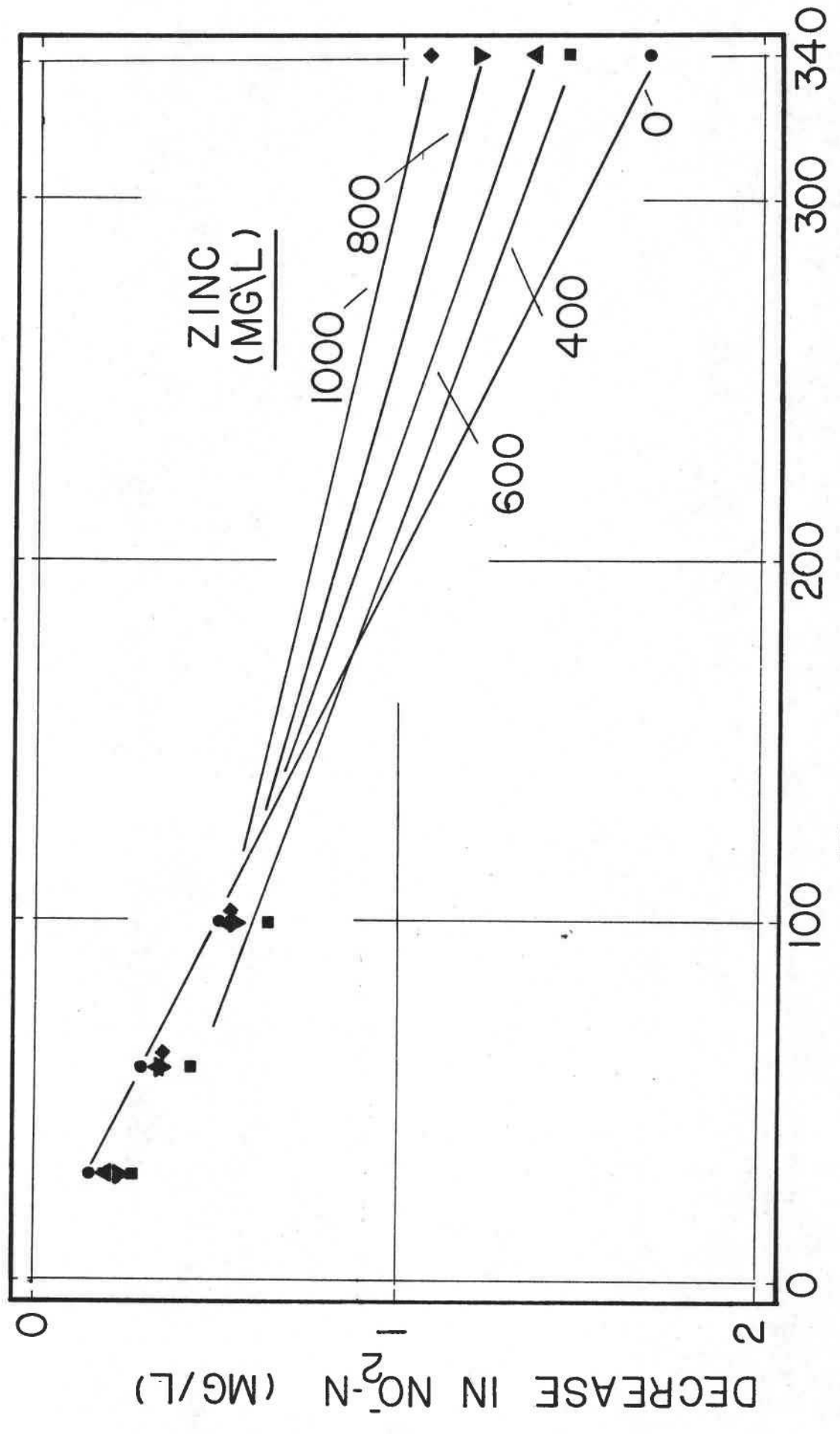

Figure 4. Incubations of Freeze-Dried Cells in the Presence of Various Concentrations of Zinc

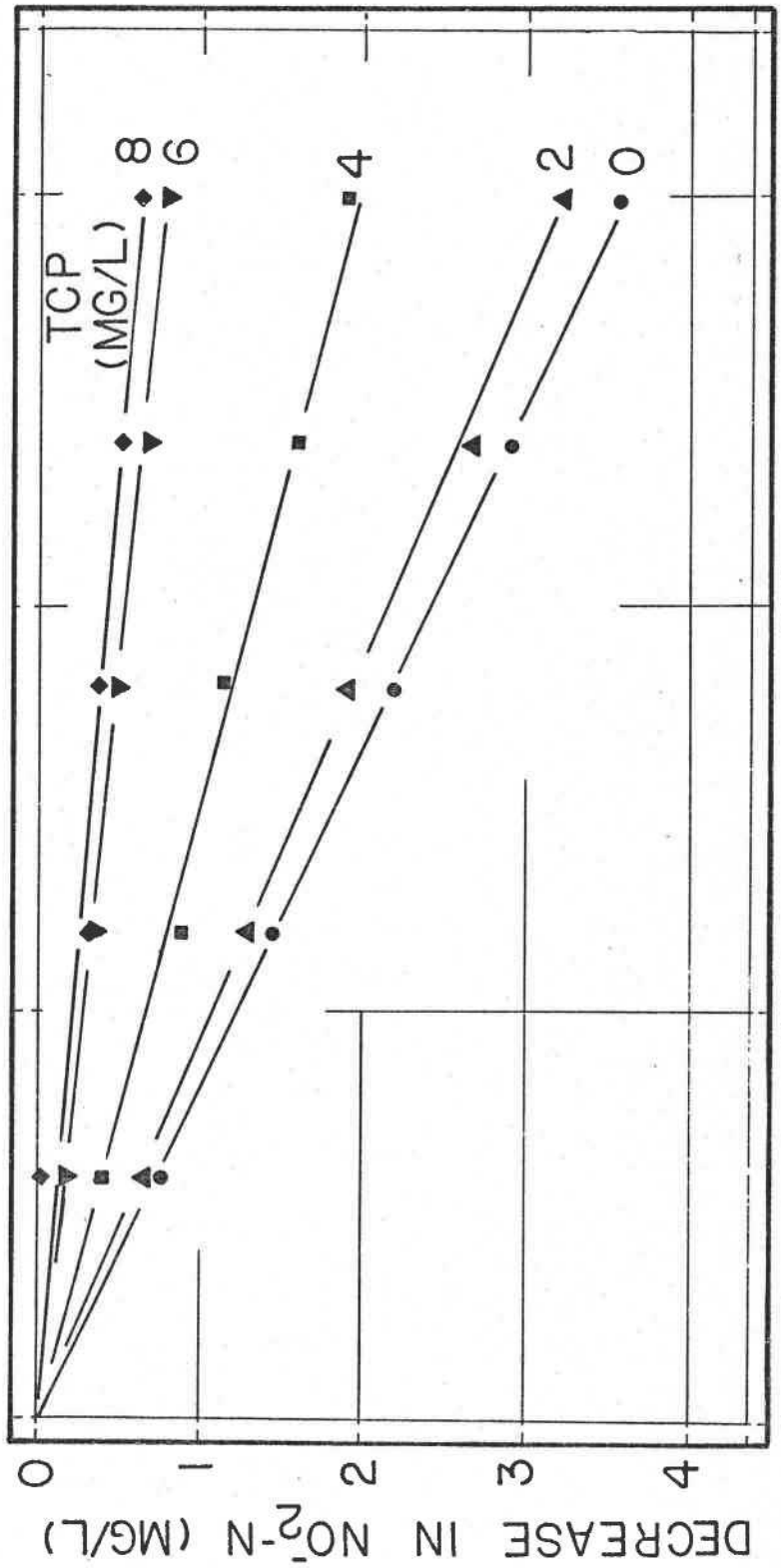

Figure 5. Incubations of Non-Freeze-Dried Cells in the Presence of Various Trichlorophenol Concentrations

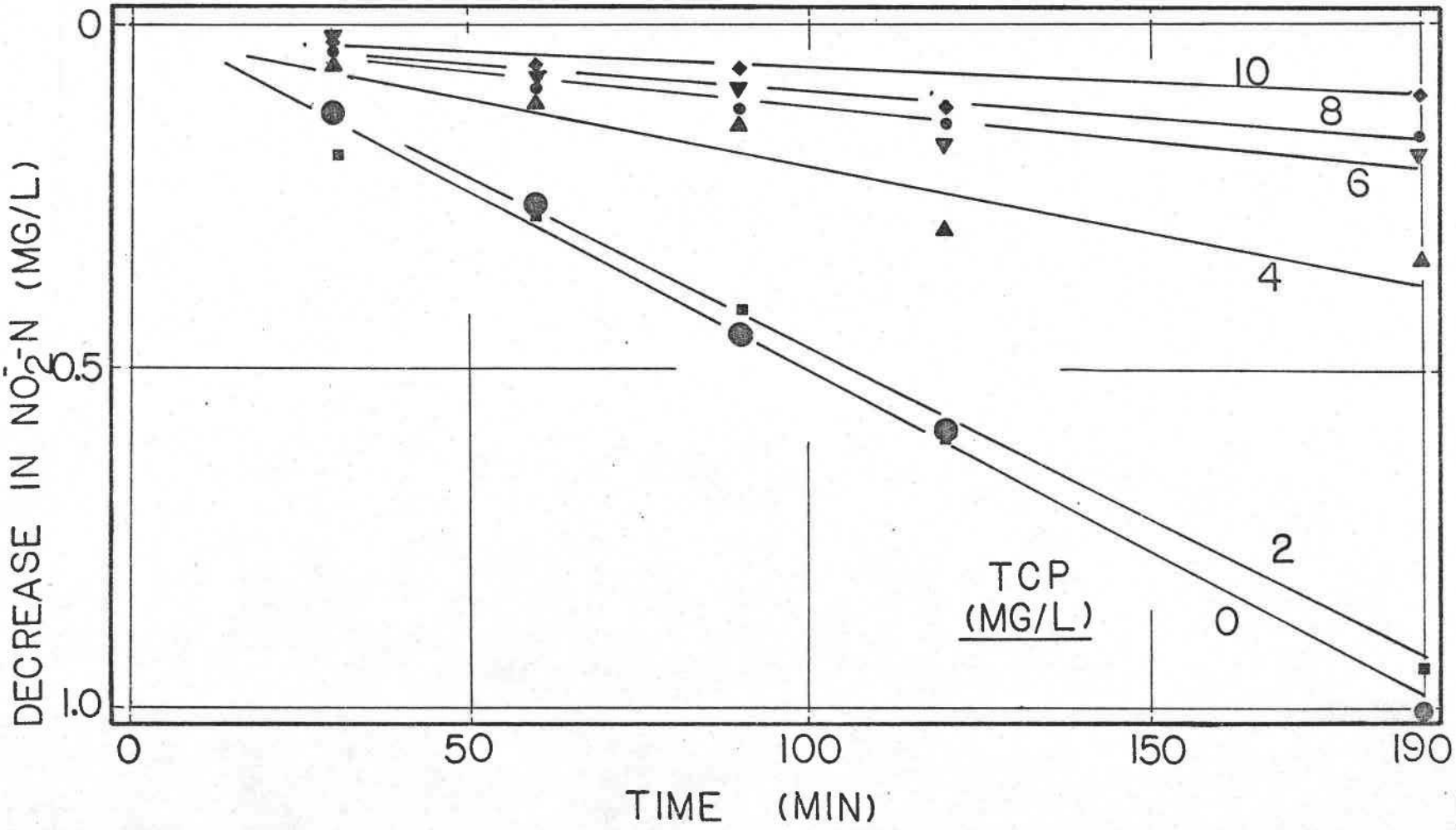

Figure 6. Incubations of Freeze-Dried Cells in the Presence of Various Trichlorophenol Concentrations

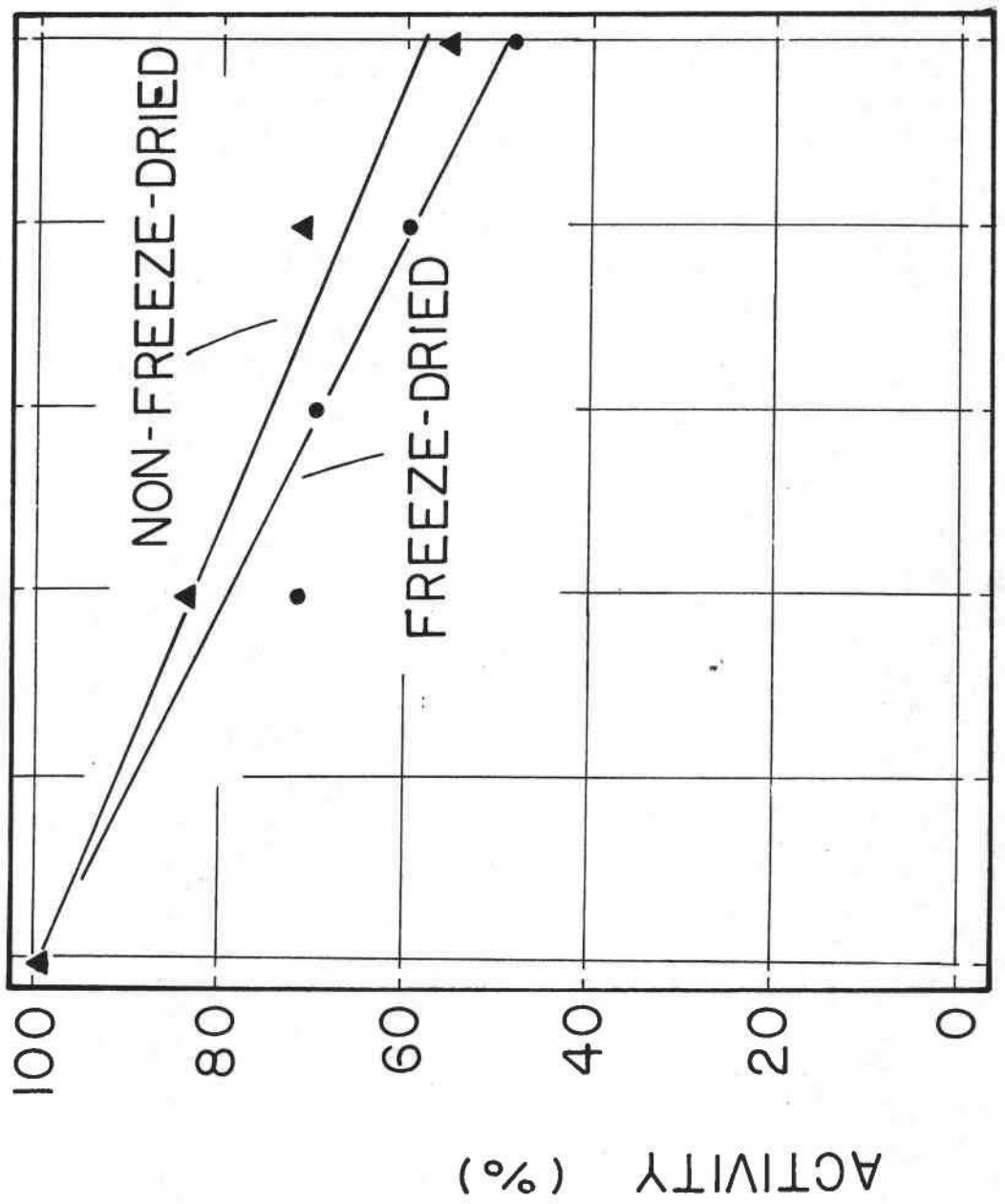

Figure 7. Loss of Activity as a Function of Zinc Dosage

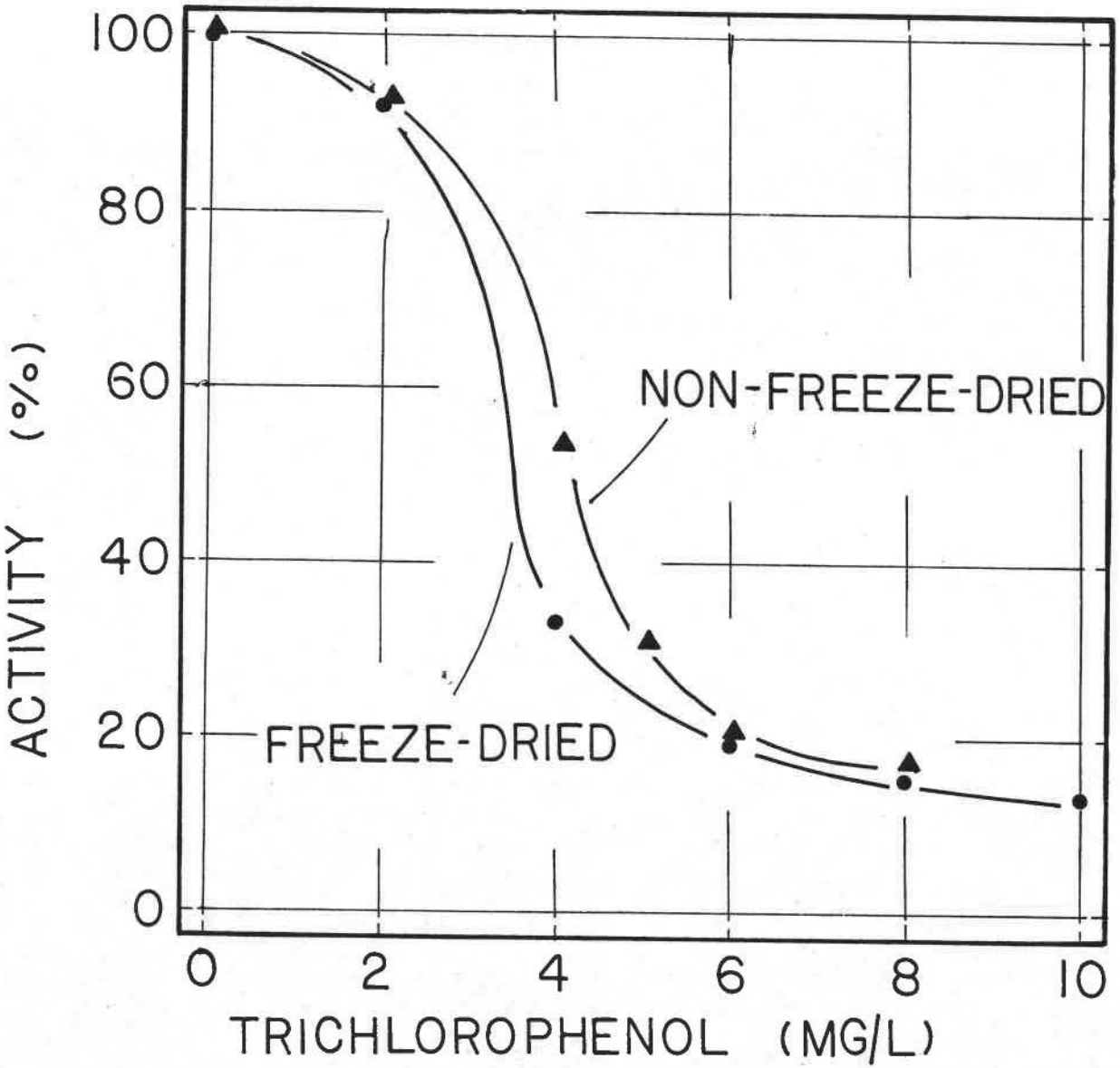

Figure 8. Loss of Activity as a Function of Trichlorophenol Concentration

The percent activity versus concentration of toxicant plots showed a near linear relationship for zinc and a non-linear relationship for trichlorophenol. The consistent decrease of activity with an increase of toxicant showed that the bioassay test would be adequate for conclusive determination of toxicity.

Zinc resulted in approximately a 50% reduction in activity at a concentration of 1000 mg/ℓ. These zinc concentrations for a toxic response were higher than the several hundred mg/ℓ reported for activated sludge by Neufeld and Hermann (1975). This discrepancy may have resulted from an interaction of the zinc with the sewage or a higher tolerance for zinc by the Nitrobacter as compared to activated sludge. However, concentrations of zinc of about 50 mg/ℓ were found to be significantly toxic to non-freeze-dried Nitrobacter during preliminary experiments which were run exactly like the results presented. This suggests that a variable interaction occurs between the zinc and the domestic sewage which altered the toxicity.

The trichlorophenol resulted in an 85% reduction at a concentration of 10 mg/ℓ. This value is below the toxic level reported for continuously-dosed activated sludge of 20 mg/ℓ (Ferguson, et al., 1976). An increased sensitivity as observed for trichlorophenol for most toxicants would make this bioassay test a useful method for toxicity control.

Comparison of the percent activity versus concentration plots for the two toxicants showed that the susceptibility of the toxicant to Nitrobacter was not lost during freeze-drying. Susceptibility could have been

lost for a number of reasons such as carry-over of the cryoprotective agent. Activity of the freeze-dried Nitrobacter was only about 30 percent of the activity of the non-freeze-dried cells. However, similar toxicity results were obtained for both the freeze-dried and non-freeze-dried cells when the activity in the presence of toxicants is compared to the activity of the respective controls. The 30 percent activity, which probably represents 30 percent viability, was considered to be adequate for the bioassay test.

The sample filtrates were analyzed for the concentration of toxicant to determine the actual amount of toxic substance remaining in solution. These values are presented in Tables 1 through 4 for the four test conditions. These data showed that most of zinc and trichlorophenol remained in solution throughout the bioassay test. The material could have been adsorbed to the particulate fraction in the primary-treated sewage which could have affected the toxic responses.

CONCLUSIONS

Based on the results of this study, it was concluded that:

1. Nitrobacter can be freeze-dried and reconstituted with a resulting activity of about 30 percent compared to the original cells.
2. Zinc and trichlorophenol showed increasing toxicity to Nitrobacter over ranges of approximately 0 to a thousand mg/ℓ,

Table 1. Soluble Zinc Concentrations for Bioassay with Non-Freeze-Dried Cells

Zinc Dosage (mg/ℓ)	Soluble Zinc After Bioassay Test (mg/ℓ)	Suspended Solids (mg/ℓ)
0	0.3	465
400	335	465
800	675	465
1000	900	465

Table 2. Soluble Zinc Concentrations for Bioassay with Freeze-Dried Cells

Zinc Dosage (mg/ℓ)	Soluble Zinc After Bioassay Test (mg/ℓ)	Suspended Solids (mg/ℓ)
0	0.3	465
400	325	465
600	500	465
800	700	465
1000	875	465

Table 3. Soluble Trichlorophenol Concentrations for Bioassay with Non-Freeze-Dried Cells

Trichlorophenol Dosage (mg/ℓ)	Soluble Trichlorophenol After Bioassay Test (mg/ℓ)	Suspended Solids (mg/ℓ)
0	0	430
2	2.0	435
4	3.8	435
5	4.9	430
6	5.6	435
8	7.7	435
10	9.6	430
15	14.6	430
20	19.2	430

Table 4. Soluble Trichlorophenol Concentrations for Bioassay with Freeze-Dried Cells

Trichlorophenol Dosage (mg/ℓ)	Soluble Trichlorophenol After Bioassay Test (mg/ℓ)	Suspended Solids (mg/ℓ)
0	0	450
2	1.7	450
4	3.6	450
6	5.0	450
8	7.0	450
10	9.0	450

and 0 to 10 mg/ℓ, respectively.

3. The toxic response of zinc and trichlorophenol to Nitrobacter was approximately the same before and after freeze-drying.
4. Toxicity of zinc and trichlorophenol occurred to freeze-dried Nitrobacter at concentrations similar to those concentrations reported for toxicity to activated sludge.
5. The bioassay test described in Appendix 1 showed potential as a routine monitoring test for wastewater toxicity to activated sludge treatment.

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APPENDIX 1

A Method for Measuring Toxicity in Wastewaters

I. Preparation of NO_2^--N Stock and Feed solutions.

A. Stock solution - 250 µg NO_2^--N/mℓ

Dissolve 1.232 g of $NaNO_2$ in 1000 mℓ distilled water.

B. Rehydration and feed solution - 5 mg NO_2-N/ℓ.

Dilute stock $NaNO_2$ solution 1:50.

II. Rehydration of Nicrobacter.

A. Break open one ampule of freeze-dried Nitrobacter per test to be run and one for the blank.

B. Add 2 mℓ of 5 mg NO_2^--N/ℓ solution to each ampule dropwise. Place contents of all ampules into a centrifuge tube and rinse ampules with same solution and pour into the centrifuge tube.

C. Centrifuge long enough to separate cells from liquid. Pour off liquid.

D. Resuspend cells in feed solution, centrifuge again. Repeat twice.

E. Resuspend cells in feed solution.

III. Toxicity Test.

A. Dilute 1 mℓ of stock $NaNO_2$ solution to 50 mℓ using the wastewater to be tested. Place in a 250 mℓ erlenmeyer flask.

B. Place 50 mℓ of feed solution in a 250 mℓ erlenmeyer flask. This will serve as the blank.

C. Pipette equal volumes of the Nitrobacter suspensions into each flask. Shake or stir at a constant temperature of about 20 to 25°C.

D. Sample each flask initially and every hour thereafter for at least four hours. To sample, remove 0.8 mℓ and dilute to 50 mℓ. Analyze for nitrite concentration according to Method 134 (Standard Methods, 1971).

E. Plot NO_2^--N removal vs time for each flask. Determine the slope of each data set.

F. Using the slope obtained in the blank as 100 percent, find the percent cell activity in each wastewater sample. A reduced cell activity in the test flasks as compared to the control confirms a toxic response from the wastewater.