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NITROBACTER AS AN INDICATOR OF TOXICITY IN WASTEWATER

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ABSTRACT

This report presents the results of a study of the use of Nitrobacter as an indicator of toxicity. Nitrobacter are strictly aerobic, autotrophic, and slow growing bacteria. Because they convert nitrite to nitrate, the effects that toxins have on them can be detected easily by monitoring changes in their nitrite consumption rate.

The bacterial cultures were obtained from two sources — the Peoria and Princeton (Illinois) wastewater treatment plants — and tests were conducted to determine the effects on the cultures of inorganic ions and organic compounds. The inorganic ions included cadmium, copper, lead, and nickel. The organic compounds were phenol, chlorophenol (three derivatives), dichlorophenol (two derivatives), and trichlorophenol.

The bioassay procedure is relatively simple and the results are reproducible . The effects of these chemical compounds on Nitrobacter were not dramatic. For example, of the compounds tested, 2,4,6-trichlorophenol was the most toxic to Nitrobacter. The average median effective concentrations were 4.7 and 2.6 mg/l for the Peoria and Princeton samples, respectively. These values are very close to 3.5 mg/l, a value reported by Williamson and Johnson (1981) for tests with a single species culture of Nitrobacter. Among the inorganic compounds used, the cadmium ion was the most toxic to Nitrobacter. The median effective concentration of cadmium was generally 40-50 mg/l.

In comparison with other aquatic indicators of toxicity, the tolerance of Nitrobacter is very high. In other words, the lack of toxic response of Nitrobacter makes these microorganisms an unsuitable candidate for monitoring environmental pollution. Among nitrifying bacteria, Nitrosomonas may be a more promising microorganism for monitoring aquatic toxicity.

INTRODUCTION

The standard test specimens for aquatic bioassays have been greatly expanded in recent years (Standard Methods, 1980). Of these specimens, the majority are fishes of marine or freshwater origin. Others include phytoplankton, zooplankton, mollusks, insects, and crustaceans. In a recent paper, Williamson and Johnson (1981) proposed the use of Nitrobacter as a test organism. They described the method for a Nitrobacter bioassay as simple and the results as sensitive.

The special appeal of autotrophic bacteria, like Nitrobacter, as bioassay probes is that this group of microorganisms is an essential part of the nitrogen removal process in many wastewater treatment plants. After ammonification, nitrogen is converted to nitrite with the aid of Nitrosomonas, and subsequently nitrite is oxidized to nitrate by Nitrobacter. By monitoring the rate of nitrogen transformation, it is possible to determine the efficiency of the nitrogen removal process. The presence of toxic substances in wastewater, however, can cause problems in a wastewater treatment plant dependent on biological processes (Picard and Faup, 1980).

The cause of failure in biological wastewater treatment operations is difficult to determine because of the complexity of the entire process (Williamson and Johnson, 1981). Possible causes include shock hydraulic or organic loads, mechanical failure, improper design, operator errors, and toxicity. The problem of toxicity is perhaps the most difficult to identify. Chemical identification is hampered by the complexity of the waste being treated and the effects of interactions of a wide variety of inhibitors. Consequently, there is no simple or easy method for a plant operator to employ.

Bioassay tests may be effective in determining the extent of a toxicity problem. While they are not entirely conclusive, bioassays can give a strong indication of the presence of toxic compounds. Negative test results indicate to the wastewater treatment plant operator that remedial measures should be concentrated on the remaining possible causes of failure other than toxicity. However, if the tests prove positive, extensive sampling of all point sources should be undertaken to determine the toxic discharge. Likewise, a detailed chemical analysis of the wastewater should be conducted to identify potential toxic compounds.

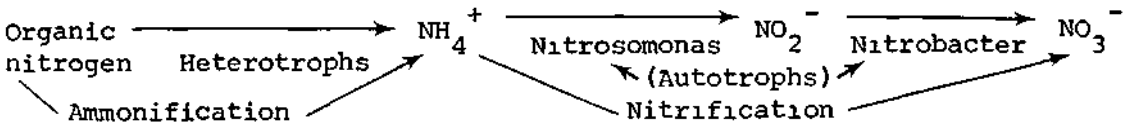
Other methods are available to measure a toxic response to biological treatment systems, i.e., continuously fed reactors and the batch-fed technique. Unfortunately, both tests are expensive, time consuming, and require more sophisticated instrumentation than is available at sewage treatment plants.

Microbial toxicity tests have several characteristics which make them ideal for such studies. For example, the bacteria are easily handled and require relatively little space for culturing and/or testing. In addition, their short life cycle leads to fast experimental results, thus enabling the laboratory to process more samples (Liu and Kwasniewska, 1981). Consequently, bacteria are well suited for use as a primary biological monitoring system for detecting chemical toxicity. Nitrobacter are a logical choice since they are indigenous to the wastewater environment.

Bacterial bioassays are important because of the need to expand the diversity of reliable indicators for evaluating the validity of water quality standards for lakes and streams. Fishes have been employed for this purpose for quite some time, but recently the list of bioassay test organisms has been greatly expanded. The idea of using bacteria as a biological probe for aquatic toxicity is relatively recent.

Certain heterotrophic organisms, such as Arthrobacter, fungi, and actinomycetes can also nitrify. The contribution of the heterotrophs to nitrification in nature is probably rather small (M. Long, Illinois Environmental Protection Agency, personal communication, 1983).

Overall, aerobic microbial activity governing ammonification and nitrification may be simplified by the following diagram of the sequence of changes (Wong-Chong and Loehr, 1975) :



Because the activities of Nitrosomonas and Nitrobacter may be inhibited by certain substances, a brief discussion of the general biochemistry of nitrifying bacteria is presented here to explain the potential modes of toxic action. Both organisms are chemoautotrophs and depend on oxidative electron transport chains from which chemical energy is derived (Wood et al., 1981). Nitrosomonas oxidizes ammonia to nitrite via hydroxylamine (NH OH), nitroxyl (NOH), and nitric oxide intermediates. This involves a six electron change. Little is known about the oxidation of ammonia to hydroxylamine, but the oxidation of the latter to nitrite has been studied carefully in cells and cell-free extracts. It has been demonstrated that a copper protein is essential for the oxidation of both ammonia and hydroxylamine. The one step, two electron, oxidation of nitrite to nitrate by Nitrobacter, which is considerably simpler, also involves a copper-containing protein.

Although the metabolic activities of these two nitrifiers may be suppressed by certain substances, the extent of such suppression may be different for each species. The more complicated Nitrosomonas enzyme system is more susceptible to inhibition than the simpler enzyme system of Nitrobacter. For this reason and because Nitrobacter would have no substrate without Nitrosomonas producing it first, Nitrosomonas is considered to be the more important link in the oxidation chain.

Williamson and Johnson (1981) present a concise description of chemical agents which are toxic to microbial populations. For example, the halogen compounds have long been recognized for their germicidal qualities and represent an important group of toxic compounds. A second major category of disinfectants consists of the heavy metals. Exposure to inhibitory metals can result in the occurrence of a variety of abnormalities such as interference with cell wall synthesis, decreased enzyme activity, and deactivation of DNA and RNA. The phenolic derivatives represent another group of anti-microbial compounds. These chemicals display a high degree of surface activity, act by disrupting cell membranes, and inhibit oxidase enzymes associated with surface membranes. Alcohols act in a manner similar to the phenols, causing inhibition of respiration and phosphorylation, possibly due to severe damage to the membrane. And finally acids and alkalies affect the pH of the bacterial media, causing an overabundance of either H^+ or OH^- ions. This might result in a reduction of bacterial growth and/or lethal

damage to the cell surface. It is apparent then that toxicity can affect bacteria in several different ways. All of the aforementioned mechanisms can occur in the bioassay organism Nitrobacter.

According to Stensel et al. (1976), the effects of toxic materials on nitrification can vary, depending on the nature and strength of the material. The possible effects are the death of the nitrifying bacteria, temporary loss of nitrification with resumption of normal nitrification rates after removal of the inhibition, or a decrease in the growth rate of the nitrifying organisms .

Influence of Toxicants on Nitrification

Hockenbury and Grady (1977) undertook an extensive investigation that had three objectives: 1) to identify industrially significant nitrogen-containing organic compounds that inhibit the activity of autotrophic bacteria, 2) to determine the effects of concentrations of key organic compounds on the severity of inhibition, and 3) to characterize the type of inhibition exhibited. Their results indicated that more compounds were found to be inhibitory to ammonia oxidation by Nitrosomonas species than to nitrite oxidation by Nitrobacter species. Thus it would appear that Nitrosomonas species represent the weak link in the nitrification chain.

Hockenbury et al. (1977) conducted laboratory studies on the effect on nitrifiers of domestic wastes, industrial wastes, and heterotrophic bacteria. The results indicated no effects by viable heterotrophic bacteria upon nitrate production by autotrophic nitrifiers. In fact, heterotrophic microorganisms released growth factors which slightly enhanced nitrification rates. No adverse effects from domestic or industrial wastes were noted as long as suitable pH and dissolved oxygen levels were maintained.

Neufeld and his associates (1980) conducted experiments to quantify the influence of phenol and un-ionized (free) ammonia on the rate of ammonia oxidation to nitrite by an autotrophic culture of strict nitrifiers. Concentrations of 0 to 75 mg/l phenol, as used in this investigation, caused progressive inhibition of ammonia oxidation by Nitrosomonas. In their paper, they cautioned that the influence on nitrification of toxic inhibition, such as by phenolic compounds or excursion in pH (and therefore un-ionized ammonia level), may be compensated for by rational design and operation procedures.

In a similar experiment Stafford (1974) examined toxic inhibition of phenol on nitrification in a single stage laboratory activated sludge system. Concentrations of 4 to 10 mg/l of phenol caused a progressive inhibition of respiration of nitrifiers. Nitrite oxidation was not influenced by phenol at 100 mq/l. Again, the results seem to indicate that of the two important genera of nitrifying bacteria, Nitrosomonas is more sensitive to toxic compounds than Nitrobacter.

A recent investigation by Liu (1981) utilized the resazurin reduction method to test the toxicity of certain compounds on the dehydrogenase acti-

vity of a mixed bacterial culture. Phenol; 2-chlorophenol (MCP); 2,6-dichlorophenol (DCP); 2,4,6-trichlorophenol (TCP); and pentachlorophenol (PCP) were assessed for their toxicity at the concentration level of 10 mg/l. The results (figure 1) vividly illustrate that the toxicity of chlorophenols to microorganisms is a function of the degree of chlorination in the phenol nucleus.

Three monochlorophenols (ortho-, meta-, and para-) were assessed by Liu (1981) for their toxicity to the mixed culture at concentrations of 5, 10, and 50 mg/l. Their effect on inhibition is shown in figure 2. At the 5 mg/1 level, o-MCP was found to be half as toxic to the culture as p-MCP and one third as toxic as m-MCP. Since stearic, electronic, and hydrophobic effects of the substituents could all affect the toxicity of a molecule, it is possible to speculate why m-MCP is more toxic than other isomers.

In another study by Strom et al. (1976) , nitrifying bacteria in samples from four activated sludge plants treating mainly domestic sewage were exposed to 2 mg/1 chlorine for 0-60 minutes. The findings indicate that nitrifiers are more resistant to chlorination than fecal streptococci and, more importantly, that the nitrite oxidizers, i.e., Nitrobacter, showed the greatest resistance of all.

The presence of heavy metals in wastewater is of concern not only because heavy metal discharges into a receiving body of water may be detrimental to the environment, but also because a reduced efficiency in biological wastewater treatment can occur. Although the mechanisms by which heavy metals affect biological treatment processes are not well defined, the general response of processes to varying metal concentrations is well documented. Figure 3 shows the relative response of microorganisms to increasing concentrations of heavy metals. The observations of Bagby and Sherrard (1981) regarding this are as follows: 1) relatively low concentrations of heavy metals may serve to stimulate biological systems, as indicated by the increased rate of biological reaction; 2) increased concentrations of toxic cations, however, will cause a decreased stimulation and will eventually result in the system's becoming adversely affected or inhibited; and 3) at some high concentration the system will finally fail completely as biological activity approaches zero.

The most widely held theory used to explain the effects of inhibitory metal cations on microorganisms is that the cations damage or inactivate one or more critical enzymes through the formation of an inactive complex between the metal cations and active enzymes. Detoxification can occur if the metal is chelated or complexed, or if the organism alters its metabolic process.

The response of aerobic treatment systems to increasing soluble concentrations of heavy metals is graphically depicted in figure 4. Small concentrations of soluble heavy metals generally cause a small decrease in organic removal efficiency, whereas substantially larger doses greatly decrease the efficiency.

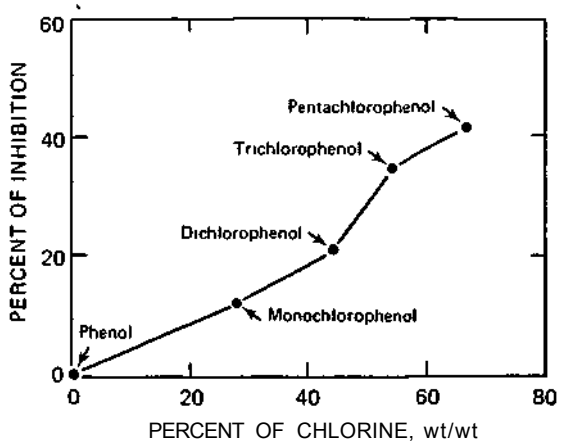


Figure 1. Percent of inhibition as a function of extent of phenol chlorination (from Liu, 1981)

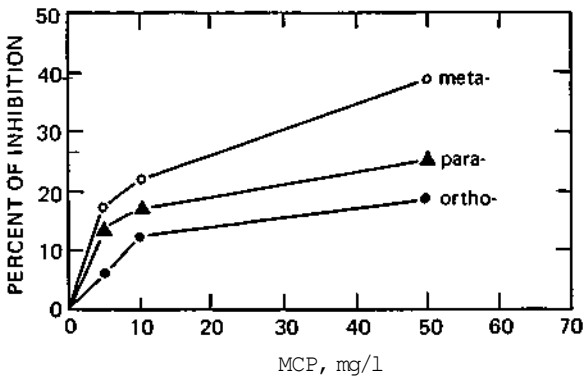


Figure 2. Percent of inhibition as a function of monochlorophenol concentration (from Liu, 1981)

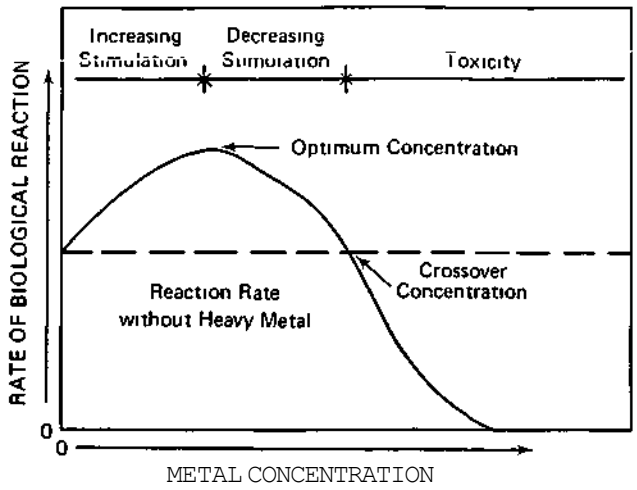


Figure 3. General effect of heavy metals on biological reactions (from Bagby and Sherrard, 1981)

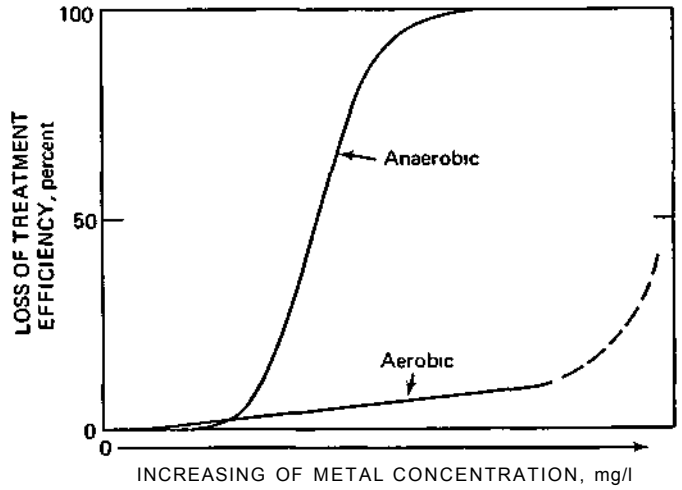


Figure 4. Effect of increasing metal concentrations on loss of treatment efficiency (from Bagby and Sherrard, 1981)

The toxicity of zinc and 2,4,6-trichlorophenol to Nitrobacter was noted by Williamson (1976) while working on the development of a bioassay for wastewater toxicity that could be used at sewage treatment plants. Nitrobacter were selected as the test organisms because they exhibited a sensitivity for most toxicants greater than that of heterotrophic organisms.

Moulton and Schumate (1963) attempted to explain the effects of copper toxicity on aerobic biological systems. A continuous copper dosage up to 45 mg/l was not sufficient to stop biological activity in the aerobic biological waste treatment units. Copper, however, was retained by the units in varying amounts. It was hypothesized that copper in the mixed liquor was eventually complexed or chelated in a form which is largely inert to the bacteria in the sludge.

Beg et al. (1982) reported on the feasibility of removing nitrogen in a fixed-film biological reactor from fertilizer plant wastes by microbial nitrification processes in the presence of inorganic arsenic, fluoride, and chromium. For the normal operations of a fertilizer plant, the reported concentrations of fluoride, arsenic, and chromium in the plant effluents are in the range of 0 to 200, 0 to 20, and 0 to 10 mg/1, respectively. The ammonia oxidation rates were inhibited by chromium at 50 mg/1, by arsenic at 292 mg/1, and by fluoride at 1218 mg/1.

A considerable amount of knowledge now exists about the effects of various environmental factors on the kinetics of nitrification. A study was undertaken by Charley et al. (1980) to investigate the kinetics of nitrification under various temperatures and different levels of dissolved oxygen in activated sludge from a domestic sewage works. The reaction was found to be in agreement with the Michaelis-Menten model in the temperature range of 10-35°C, with a temperature optimum at 15°C. After acclimation, high amounts of dissolved oxygen (38 mg/1 O_2 at 30°C) had little effect on nitrification. However, the reaction was inhibited by nitrite levels in excess of 20 mg/1. Such nitrite concentrations would not normally occur under natural conditions because of the rapid oxidation of nitrite by Nitrobacter. The oxidation of nitrite to nitrate is not the rate limiting step. Instead, it is those factors which specifically influence the kinetics of ammonia oxidation that affect the overall rate of the nitrification process.

Srinath et al. (1976) developed a technique to estimate the concentration of active nitrifiers in wastewater mixed liquor. The oxidation rates for ammonium and nitrite in the mixed liquors were compared with those obtained with pure cultures of Nitrosomonas and Nitrobacter at known concentrations. It seemed that the substrate concentrations did not affect the oxidation rates. The oxidation rates of nitrite and ammonium at concentrations above 10 mg/1 nitrogen had zero-order kinetics, with pH optima in the range of 7.4 to 7.9. The population of nitrifiers in the system influenced the rate of oxidation. Jenkins and his associates (1980) took that conclusion one step further by stating that the nitrification process is more dependent upon the activity levels of the nitrifying bacteria than on their

absolute densities. Similarly, Mattern (1973) attempted to explain the growth parameters of the organisms which control nitrification. He concluded that the Nitrosomonas bacteria, which convert ammonia to nitrite, control the nitrification process since Nitrobacter bacteria, which convert nitrite to nitrate, have a higher maximum specific growth rate.

A technical analysis of the joint influence of substrate concentration and temperature upon the rates of microbially mediated ammonia oxidation and nitrite oxidation was prepared by Quinlan (1980) . This analysis stated that: 1) a family of substrate isoconcentrates is required to describe the thermal sensitivity of each oxidation step; 2) the optimum temperature of each step increases as the logarithm of its substrate concentration; and 3) the thermal rate maximum of each step increases as its substrate concentration is raised to a fractional power. The thermal sensitivity of ammonia oxidation differs substantially from that of nitrite oxidation.

Finally, no discussion of nitrification inhibition would be complete without mention of the Michaelis-Menten biochemical theory for bacterial toxicity. The theory is based on enzyme inhibition by the toxic material, and the kinetics of substrate utilization are assumed to be as follows:

$$R = -k \frac{S}{S + k_s} X$$

where

R = rate of substrate utilization
k = maximum rate of substrate utilization
S = substrate concentration
k_s = "half velocity" coefficient
X = total organism concentration

Enzyme inhibition can be identified as competitive or noncompetitive (Williamson and Johnson, 1981) and each may be rationalized by reference to theoretical pure culture "enzyme-substrate" (Michaelis-Menten) biochemical equation counterparts. Typically, a toxic agent will produce both competitive and non-competitive inhibition. The Nitrobacter bioassay will detect either one as a decrease in R although the test is most sensitive to non-competitive inhibition.

MATERIALS AND METHODS

Culture

Nitrobacter were selected as the test organisms for this research. These organisms are strict aerobes that obtain their energy from the oxidation of nitrite to nitrate. Their rate of metabolism can be measured easily by plotting nitrite removal or nitrate production as a function of time.

Originally, all bioassays were to be performed with the use of three species of Nitrobacter, all freeze-dried specimens commercially available from the American Type Culture Collection in Maryland. Despite a lengthy period of incubation, however, all the specimens failed to grow, so an alternate method of obtaining a viable Nitrobacter culture was sought. It was decided that the investigation into the toxicity of various wastewaters to Nitrobacter could proceed if a mixed culture was used.

Two Illinois locations were selected for collecting Nitrobacter populations : the Greater Peoria Sanitary District and the Princeton Sewage Treatment Plant. Peoria is an industrial center with heavy equipment manufacturers, breweries, etc. About 40 percent of the influent wastewater is of industrial origin. Princeton is a small town with minimal industrial activity. The culture samples were taken by scraping biomass from rotating biological contactors (RBC). To be sure of the predominance of Nitrobacter, the point of sampling was the last or next to the last unit of the RBC train. Autotrophic bacteria are more abundant in the latter stages of RBC units and appear as light-gray in color.

Approximately 500 ml of biomass was placed in a 2-liter flask. To it was added 1.5 liters of nutrient solution. The nutrient solution used for maintaining microbial cultures was that recommended by the American Type Culture Collection, Maryland, catalog 480 26C. The mixture was aerated with a small aquarium pump and stirred constantly with a magnetic stirrer. As nitrite is the only energy source for Nitrobacter, nitrite was fed into the mixture by adding 500 ml of stock solution (300 mg/l nitrite-N) every 3 days.

Methods

The measurement of a Nitrobacter population is a difficult task that precludes it from widespread application. Instead, this study relied upon the quantification of Nitrobacter activity. This method is not only much easier, but also more significant since it deals with viable, active organisms.

The determination of Nitrobacter activity can be achieved by measuring the rate of either utilization or yield. Most researchers prefer determining nitrite utilization over nitrate production simply because of the ease of nitrite determination (Standard Methods, 1980) . A calibration curve was prepared daily with at least four standards and it was linear in the range 0-0.20 mg/1. Figure 5 represents a typical calibration curve.

The experimental procedure for detecting toxic effects on Nitrobacter was quite simple. A small quantity, usually 2-5 ml, of the culture was placed in a 500 ml flask and diluted with 100 ml de-ionized water. To this was added the nitrite solution (0.2 mg/1 nitrite-nitrogen) and toxicants. The flask was placed on a shaker at 100 rpm for 4 hours . Nitrite-nitrogen was determined initially and hourly thereafter.

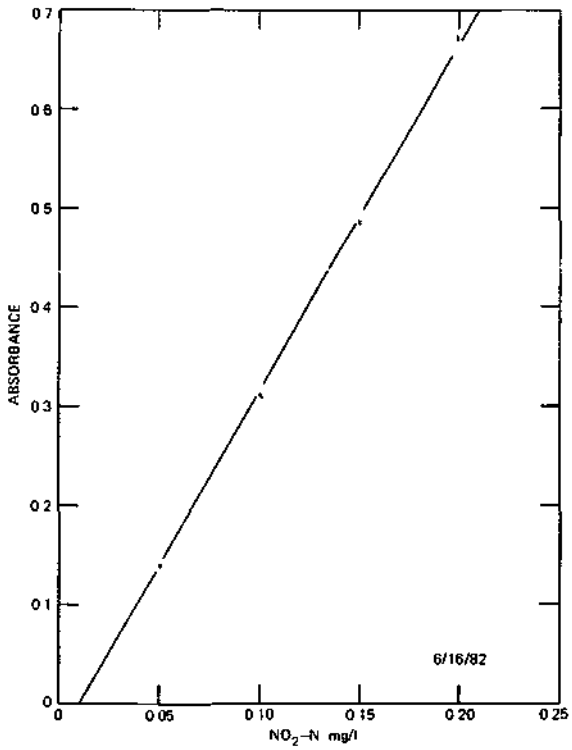


Figure 5. Typical calibration curve

A control sample was always tested in addition to the treated samples containing toxic substances. Duplicate tests were made for every experiment.

All chemical compounds used in this study are reagent grade. The study was conducted by setting an upper limit concentration of 50 mg/l for metal ions and 75 mg/1 for phenolic compounds. These artificial limitations were set in order to achieve experimental results consistent with environmental realities.

RESULTS

The experimental results will be discussed in four parts: preliminary tests, metal toxicity, organic compounds toxicity, and the time effect of toxicants on Nitrobacter activity. The toxicity of the various compounds to Nitrobacter will be expressed in terms of median effective concentration (EC50), i.e., that concentration which suppresses the Nitrobacter activity to one-half that of the control sample. (In some cases the median effective concentrations could not be determined because of the low toxicity of the compounds tested.) All concentrations are given in mg/1. Pe and Pr are abbreviations for Peoria and Princeton, respectively.

Preliminary Tests

Some preliminary tests were performed to confirm the presence of an active and healthy population of Nitrobacter. First, nitrite was added to a small sample of the bacterial culture. It quickly disappeared. Second, a small sample of bacterial culture was boiled. Nitrite was added after the culture cooled to room temperature, and this time the nitrite concentration remained constant. Third, a small sample of the bacterial culture was treated with 4 percent formaldehyde and then nitrite was added. Again the nitrite concentration remained unchanged. The first test indicated the possibility of the Nitrobacter's presence. The second and third tests confirmed that only utilization of nitrite by Nitrobacter was responsible for the disappearance of nitrite during the first test.

Metal Toxicity

The toxic effects of metal ions on Nitrobacter activity are depicted in figures 6 through 12. Among cadmium, nickel, copper, and lead, cadmium appears to be the most toxic. Figures 6 and 7 show the effect of the cadmium ion on microbial metabolism as determined by nitrite consumption. At the shorter exposure time, 1-2 hours, there was some stimulation of metabolic activity with the cadmium ion in the range of 10-20 mg/l. At 3-4 hours, cadmium's toxic effect became rather pronounced. A nearly linear relation can be seen in figures 6 and 7, showing the adverse effect of cadmium on Nitrobacter activity. In six of the eight relationships shown in figures 6 and 7, the median effective concentration was in the range of approximately 40 to 50 mg/1, as shown with arrow signs in these figures. In the other two cases, the median effective concentrations could not be determined.

A nickel ion experiment was performed twice, and typical results are shown in figures 8 and 9. The nickel ion is not as toxic as the cadmium ion, as can be seen by comparing figure 6 with figure 8 and figure 7 with figure 9.

In figures 6 to 9, there appears to be a specific toxicity pattern. At a low concentration, 10 mg/l, the metal ion stimulated the microbial metabolism, as indicated by the increase of nitrite consumption over that in the control sample. This is particularly noticeable at the 1-hour exposure time. For example, the loss of nitrite-N in the Peoria sample treated with 10 mg/1 cadmium for 1 hour was 0.04 mg/1, while the loss in the control sample was 0.015 mg/1 (figure 6). By the same token, the loss for the Peoria sample treated with 10 mg/1 nickel for 1 hour was 0.03 mg/1, compared to a loss of 0.025 mg/1 for the control sample (figure 8).

The loss for the Princeton nickel-treated sample was 0.025 mg/1, compared to a loss of 0.005 mg/1 for the control sample (figure 9). As shown in figure 7, the biostimulatory effect of cadmium on the Princeton sample was not apparent until the metal concentration reached 20 mg/1; the loss of nitrite-N was 0.04 and 0.025 for the treated and control samples, respectively.

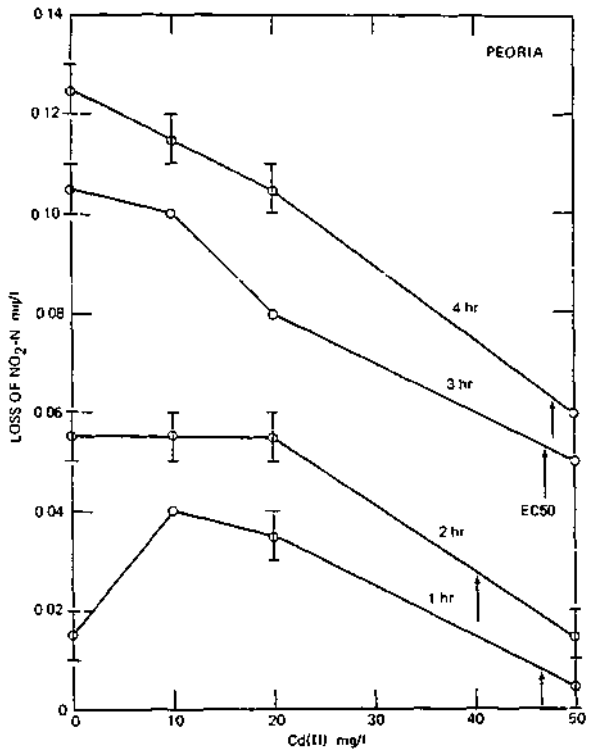


Figure 6. The effect of cadmium on the Peoria sample (7/7/82)

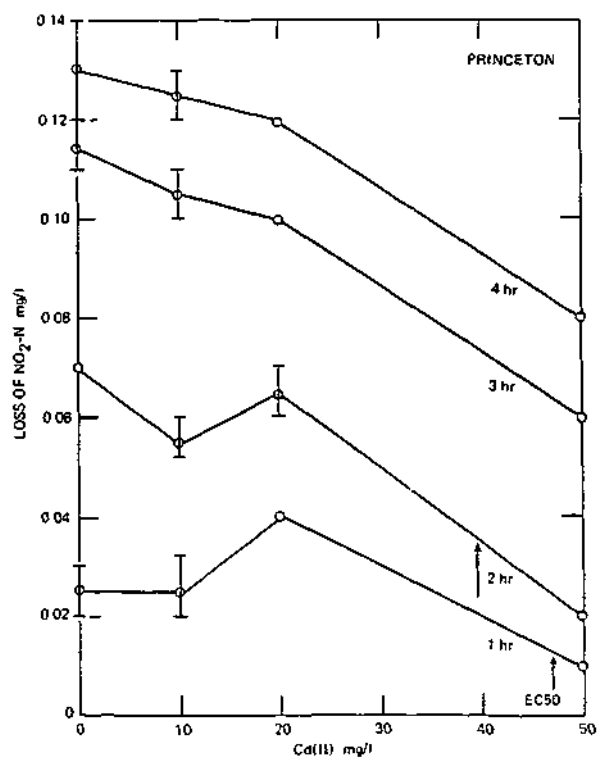


Figure 7. The effect of cadmium on the Princeton sample (7/7/82)

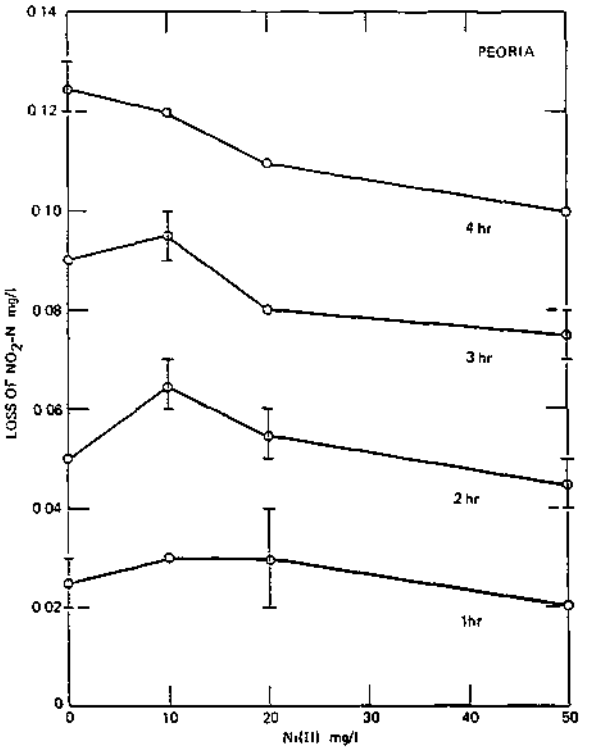


Figure 8. The effect of nickel on the Peoria sample (7/9/82)

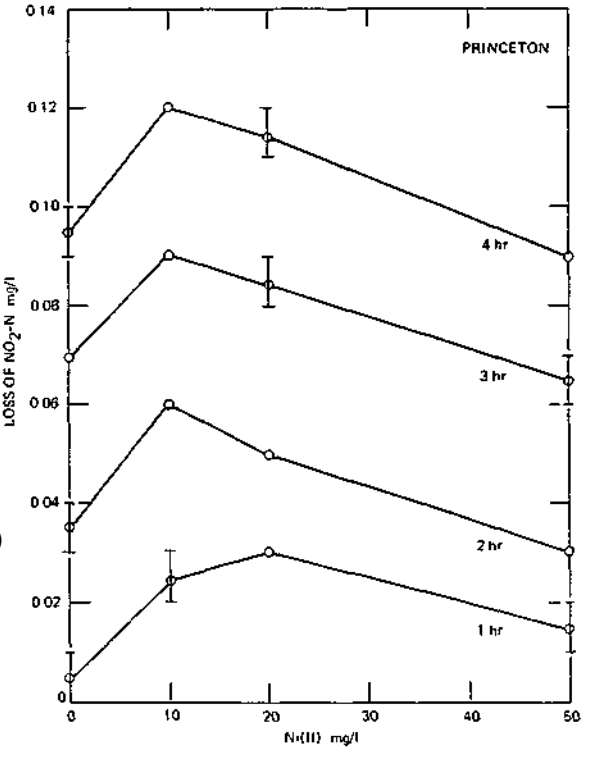


Figure 9. The effect of nickel on the Princeton sample (7/9/82)

Copper and zinc appear to be the least toxic of the metals tested. Figures 10 and 11 show that up to 50 mg/l the copper ion has no toxic effect on Nitrobacter. Instead, the stimulatory effect is readily apparent. All eight relationships in figures 10 and 11 fit into the pattern of general response (Bagby and Sherrard, 1981). Apparently, at 50 mg/l copper has not reached the cross-over point where a chemical shows neither stimulatory nor inhibitory effects.

The lead ion is moderately coxic, as shown in figure 12.

Organic Compounds Toxicity

The typical toxic effects of seven phenolic compounds on Nitrobacter activity are shown in figures 13 through 22. Phenol is slightly toxic to Nitrobacter. In fact, an attempt was made during the preliminary tests to suppress Nitrobacter with a large quantity of phenol, approximately 1-2 percent. The results show that phenol, even at this concentration, was not able to completely arrest nitrite utilization as formaldehyde did. In figures 13 and 14, it is clear that phenol did not have a strong toxic effect on Nitrobacter.

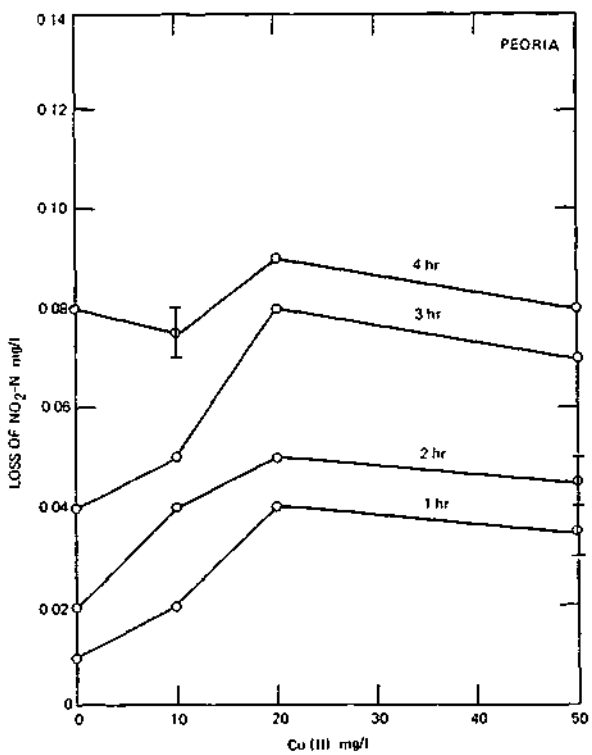


Figure 10. The effect of copper on the Peoria sample (7/9/82)

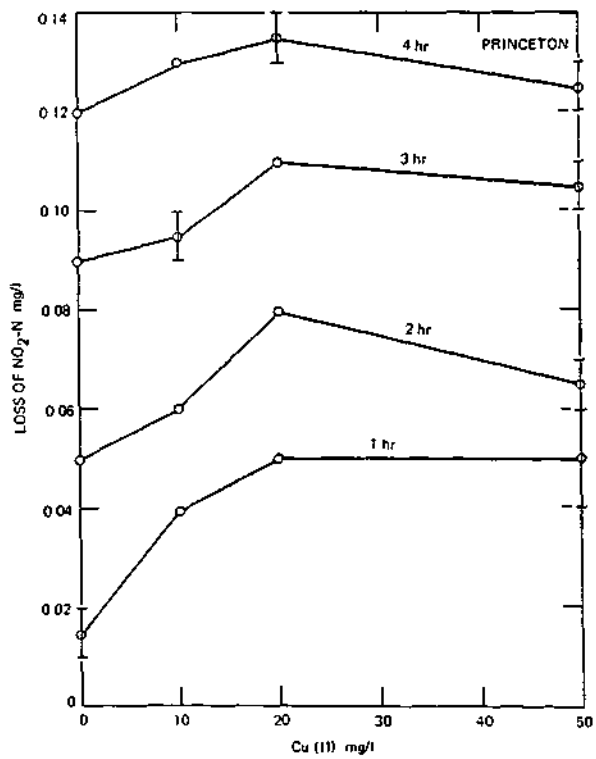


Figure 11. The effect of copper on the Princeton sample (7/8/82)

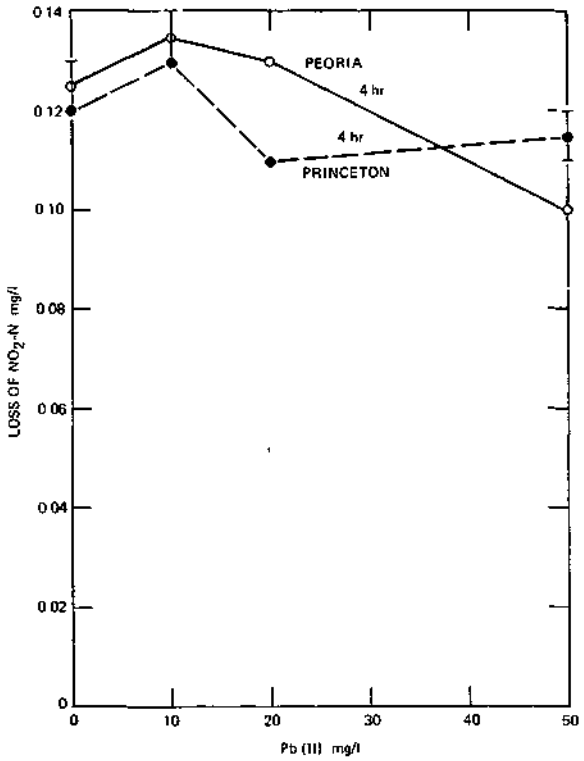


Figure 12. The effect of lead on the Peoria and Princeton samples (7/15/82)

Three derivatives of chlorophenol up to 50 mg/l were tested against Nitrobacter. Chlorophenol is less soluble in water than phenol, and consequently a small amount- (less than 2 percent) of acetone was added to make it into solution. This small quantity of carrier solvent is unlikely to influence the toxicity effect (Barera and Adams, 1981). Of the three derivatives, o-chlorophenol appears to be most toxic (figure 15). The Peoria sample did not show a toxic effect from either m-chlorophenol or p-chlorophenol, while the Princeton sample showed a slight toxic effect from these two derivatives, particularly at higher concentrations (figures 16 and 17).

The upper concentration limit for 2,3- and 2,4-dichlorophenol was set at 30 mg/l. This is because these compounds are even less soluble than phenol, and also more toxic. A comparison of figures 18 and 19 makes it obvious that 2,3-dichlorophenol is more toxic than 2,4-dichlorophenol. For example, after exposure to 30 mg/l toxicant for 4 hours, the microbial activity decreases were 96 percent (0.25 to 0.01 mg/l) and 21 percent (0.095 to 0.075 mg/l) for 2,3- and 2,4-dichlorophenol, respectively. In the lower concentration range, 10 mg/l or less, the less toxic compound 2,4-dichlorophenol showed a biostimulatory effect. The effect is particularly obvious at the shorter exposure time of 1 to 2 hours (figures 19 and 20).

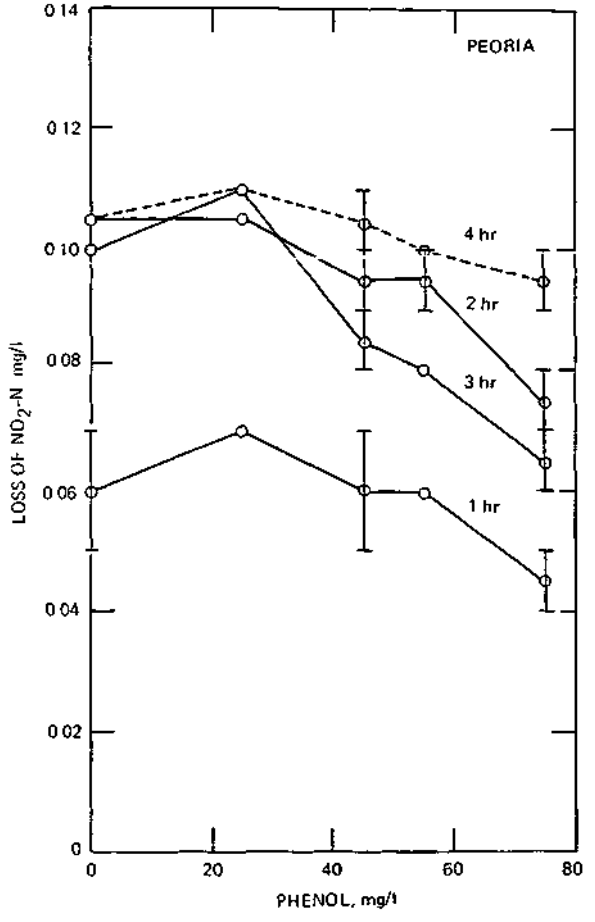


Figure 13. The effect of phenol on the Peoria sample (7/30/82)

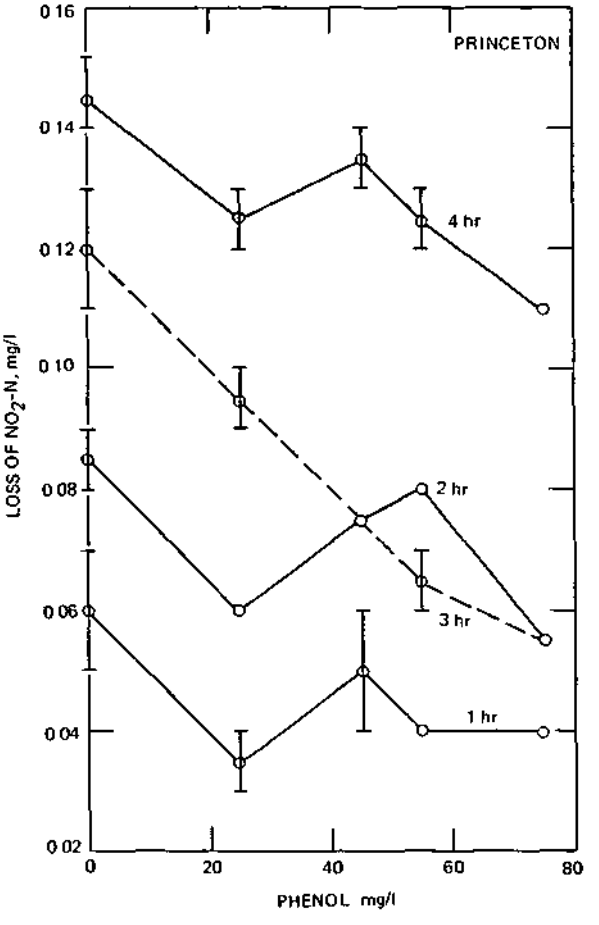


Figure 14. The effect of phenol on the Princeton sample (7/30/82)

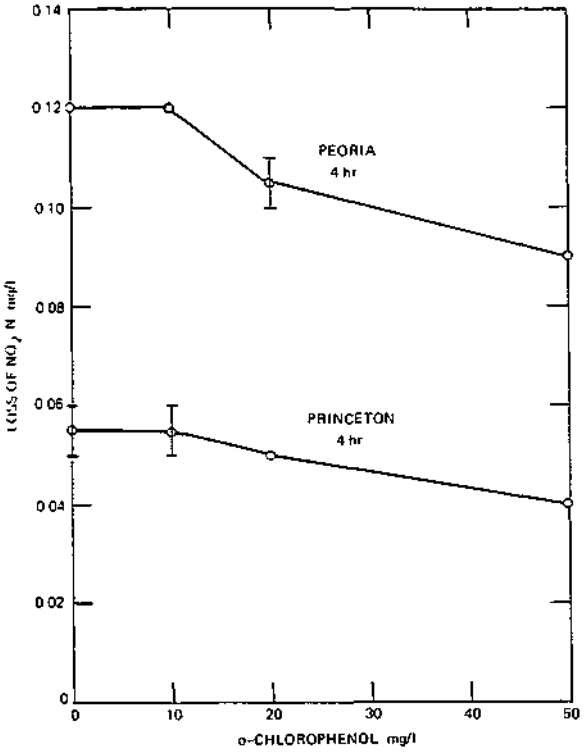


Figure 15. The effect of o-chlorovhenol on vhe Peoria and Princeton samples (6/11/82)

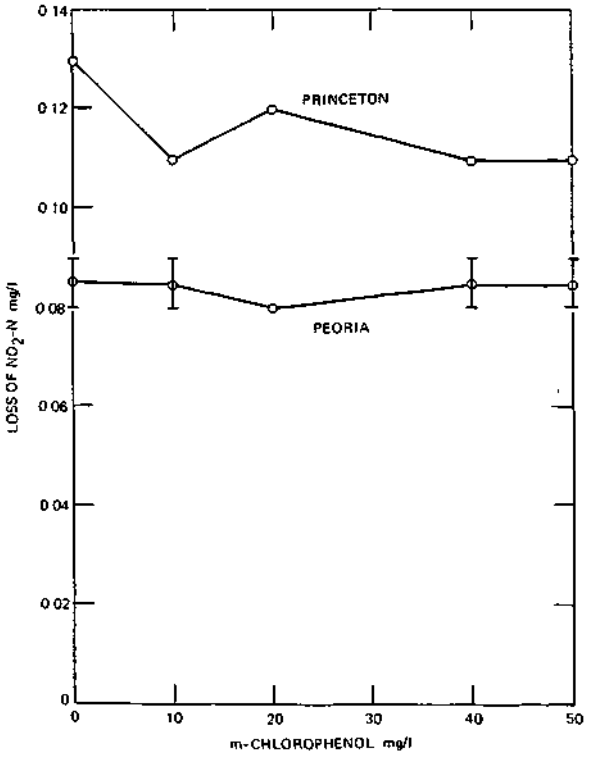


Figure 16. The effect of m-chlorophenol on the Peoria and Princeton samples (7/28/82)

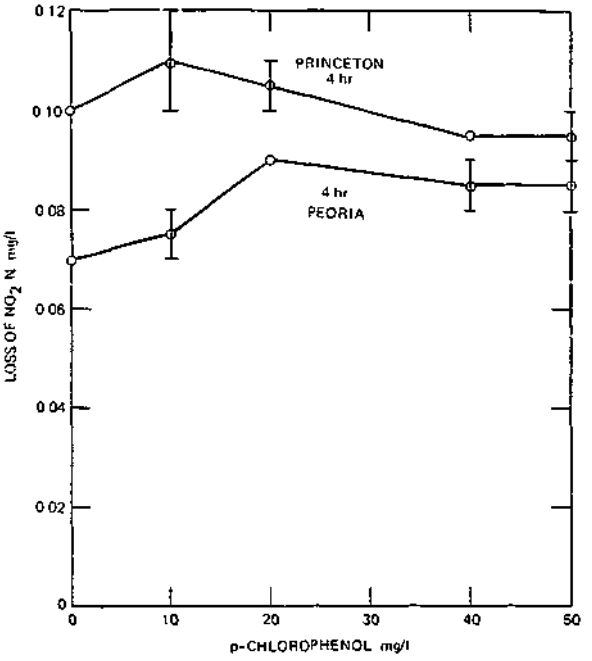


Figure 17. The effect of p-chlorophenol on the Peoria and Princeton samples (7/26/82)

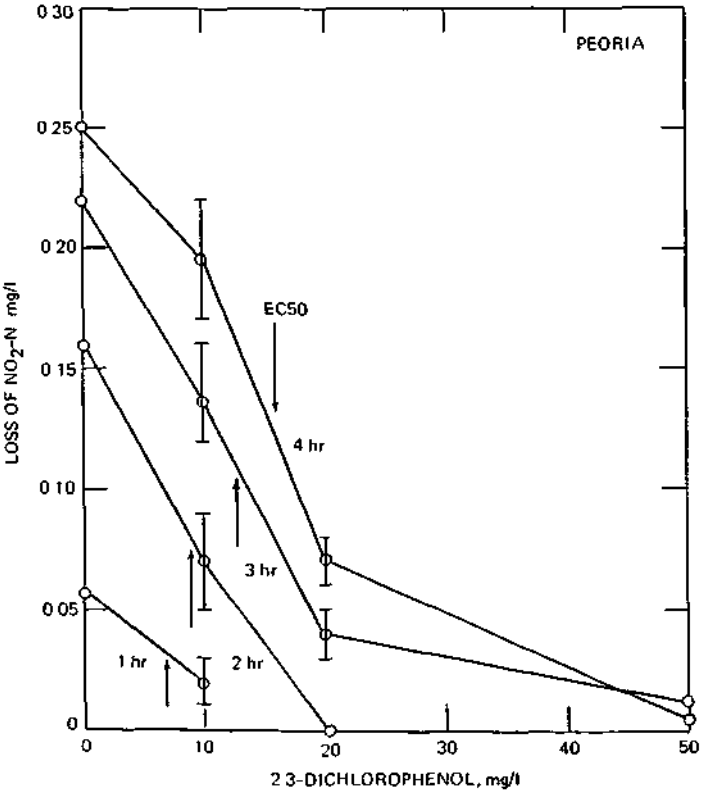


Figure 18. The effect of 2,3-dichlorophenol on the Peoria sample (6/17/82)

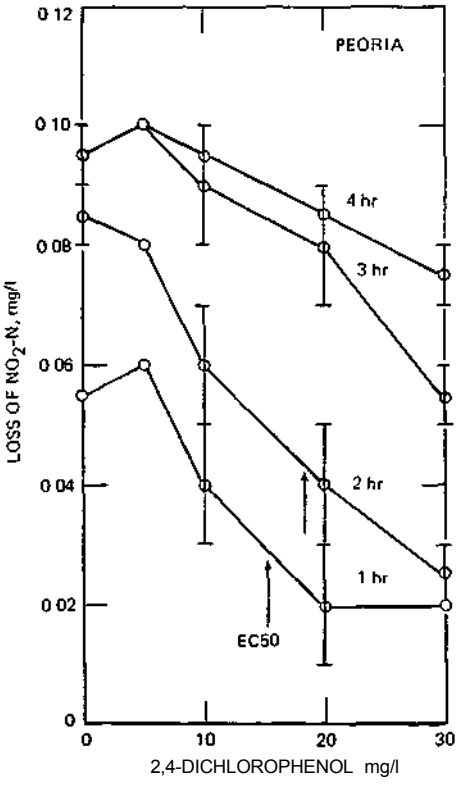


Figure 19. The effect of 2,4-dichlorophenol on the Peoria sample (7/22/82)

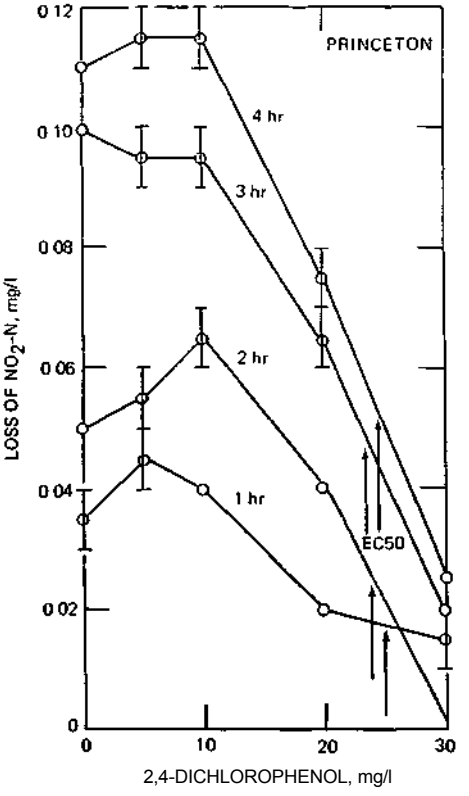


Figure 20. The effect of 2,4-dichlorophenol on the Princeton sample (7/22/82)

The compound 2,4,6-trichlorophenol, the most toxxc among the chemicals tested, was tested repeatedly as a measure for determining reproducibility. The Peoria and Princeton samples were both tested seven times with 4-hour incubation times, and the median effective concentrations (in mg/l) were as follows:

	Date tested (all in 1982)								
	5/27	6/1	6/3	6/4	6/7	6/9	6/14	Ave.	SD
Peoria sample	3.0	3.8	4.3	6.5	5.0	4.2	6.2	4.7	1.3
Princeton sample	3.4	3.3	1.3	3.7	3.7	2.4	0.7	2.6	1.2

For the Peoria sample, the average is 4.7 mg/l and the standard deviation 1.3 mg/l; for Princeton the average is 2.6 and the standard deviation is 1.2 mg/l. The coefficients of variation are 28 and 46 percent for the Peoria and Princeton sources, respectively. The lower median effective concentration for the Princeton sample indicates that this sample is more susceptible to 2,4,6-trichlorophenol toxicity than the Peoria sample. Typical responses of Nitrobacter to this toxicant are shown in figures 21 and 22.

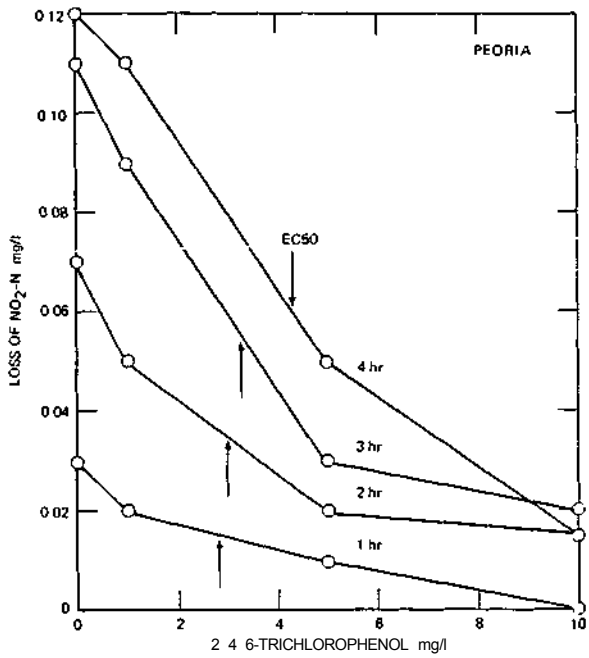


Figure 21. The effect of 2,4,6-trichlorophenol on the Peoria sample (6/3/82)

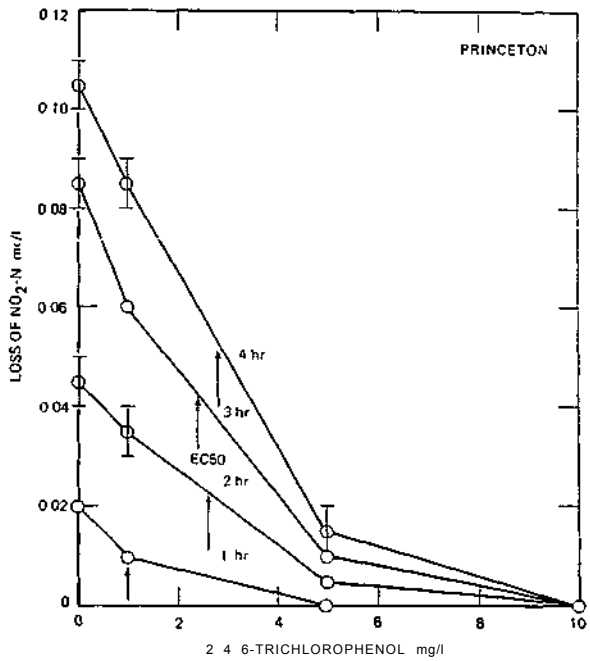


Figure 22. The effect of 2,4,6-trichlorophenol on the Princeton sample (6/3/82)

Time Effect

Two compounds, 2,3,-dichlorophenol and 2,4,6-trichlorophenol, are so toxxc that median effective concentrations can be derived. A casual observation shows that the median effective concentration is not consistent from hour to hour but rather increases with time. The results as plotted in figures 23 through 25 show a linear relation.

-DISCUSSION

The Nitrobacter bioassay for evaluating aquatic toxicity is a relatively new approach. The results produced here suggest that the bacteria are less sensitive than the fish used in standard fish bioassays. For example, for channel catfish the median lethal concentration of the copper ion for 14 days of exposure is 1.2 mg/l (Richey and Roseboom, 1978). For duckweed, the median effective concentration of the copper ion for 4 days of exposure is 1.2 mg/l (Wang, to be published). In contrast, the response of Nitrobacter to copper ions shows negligible toxicity (figures 10 and 11). Also, in tests with duckweed the median effective concentration of the cadmium ion is 0.2 mg/l (Wang, to be published), while in the Nitrobacter tests it is 40-50 mg/l (figures 6 and 7).

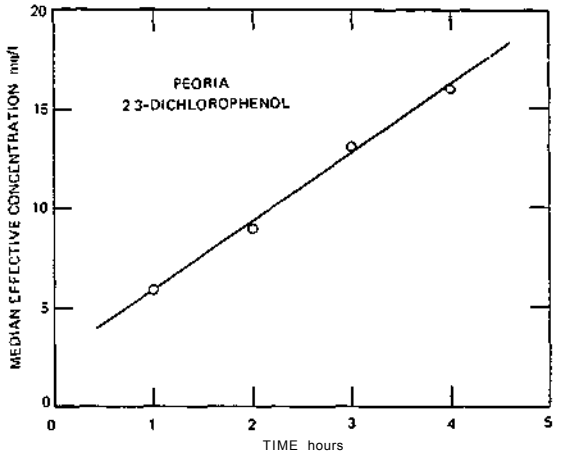


Figure 23. The time effect of 2,3-dichlorophenol on the median effective concentration (Peoria, 6/17/82)

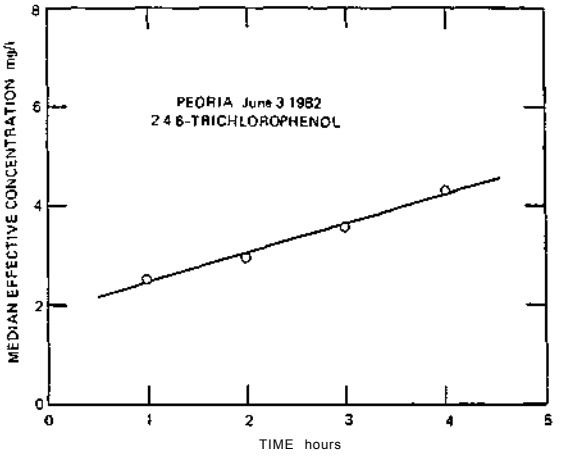


Figure 24. The time effect of 2,4,6-trichlorophenol on the median effective concentration (Peoria, 6/3/82)

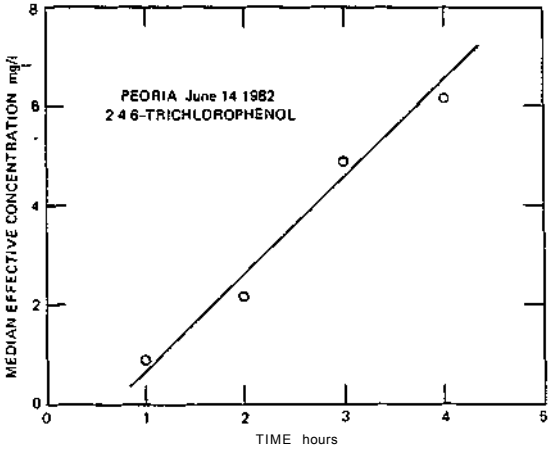


Figure 25. The time effect of 2,4,6-trichlorophenol on the median effective concentration (Peoria, 6/14/82)

Presumably, the apparent insensitivity of Nitrobacter to various chemical compounds is due to both the inherent nature of the slow growth of this microorganism and the short exposure time used in this study. These two factors work together so that any toxic effect on enzyme synthesis and/or cell reproduction is not apparent (Hockenbury and Grady, 1977). Consequently, because of their apparent low sensitivity, Nitrobacter are not a good candidate among nitrifying bacteria for the monitoring of aquatic toxicity.

One babic question that arises when a relatively new bioassay technique is employed is the reproducibility of the bioassay method. The reproducibility of the Nitrobacter bioassay can be seen from the results of the repeated tests of 2,4,6-trichlorophenol, listed previously. Repeated experiments show that the coefficients of variation are 28 and 46 percent for Peoria and Princeton samples, respectively. Considering that the experiments were conducted with uncontrolled natural bacterial populations, the variation is not excessive.

Williamson and Johnson (1981) described the Nitrobacter bioassay as simple, sensitive, rapid, and inexpensive. One model toxicant they tested is 2,4,6-trichlorophenol. They reported that the median effective concentration of this compound in tests with pure bacterial cultures was 3.5 mg/l. In comparison, as shown previously, the average median effective concentrations for the Peoria and Princeton sources with the same compound were 4.7 and 2.6 mg/l, respectively. The results obtained from pure culture and mixed culture methods are surprisingly close. This study tested many more model chemical compounds with many more repetitions than Williamson and Johnson reported. The conclusion based on this study is that the Nitrobacter bioassay is relatively insensitive to toxicity in contrast to what they described.

During an earlier study of fractionating sediment oxygen demand, it was found that 1-2 percent phenol stopped microbial respiration in sediment, thus permitting the separation of biological from chemical oxygen demand (Wang, 1980). The same amount of phenol, however, had little effect on Nitrobacter as shown in this study. The inevitable conclusion is that microbial respiration in sediment is due primarily to heterotrophic bacteria.

A summary of the toxicity of metals and organic compounds to Nitrobacter is given in table 1. For metal ions the order of toxicity is Cd(II)>Ni(II), PB(II)>Cu(II). Sposito (1981) summarized the general toxicity order of metal ions as: Hg(II)>Cu(II)>Pb(II)>Cd(II)>Cr(III)>Zn(II)>Ni(II)>Al(III). This order was based on ionic properties such as size, electronegativity, polarizability, etc. The general order determined on the basis of duckweed bioassays is somewhat different: Cd(II)>Ni(II)>Cu(II)>Se(IV)>Fe(III)>Pb(II)>Zn(II)> Ba(II)>Mn(II) (Wang, to be published).

For phenolic compounds, the order of toxicity is trichlorophenol>dichlorophenol>monochlorophenol>phenol, a result consistent with the literature (Liu, 1981).

Table 1. Nitrite-Nitrogen Losses for Various Metal Ions and Phenols

(All losses in milligrams per liter after 4 hours exposure)

	Concentration	*Peoria sample*			*Princeton sample*		
	(mg/l)	*Control*	*Treated*	*% decrease*	*Control*	*Treated*	*% decrease*
Cd	50	0.125	0.06	52	0.13	0.08	38
Ni	50	0 125	0.10	20	0.095	0 09	5
Cu	50	0.08	0.08	0	0.12	0.125	0
Pb	50	0.125	0.10	20	0.12	0.115	4
phenol	75	0.115	0.095	17	0.145	0.11	24
o-Cl phenol	50	0.12	0.09	25	0.055	0.04	27
m-Cl phenol	50	0.085	0.085	-	0.13	0.11	15
p-Cl phenol	50	0.070	0.085	-	0.10	0.095	5
2,3, -Cl_2 phenol	30	0.25	0.01	96	0.11	0.03	73
2,4,-Cl_2 phenol	30	0.095	0.075	21	0.11	0.025	77
2,4,6-Cl_3 phenol	10	0.12	0.015	88	0.105	0	100

As"'a general rule, the toxic effect becomes more pronounced as the exposure time increases. Fish bioassays show that the lethal concentration decreases as exposure time increases, particularly if toxicant concentration is high. Obviously the bioaccumulation of a toxicant by fish during prolonged exposure makes the fish more sensitive to the toxicant. The case of Nitrobacter is more complex. The time factor of metal toxicity to Nitrobacter is not conclusive due to low metal toxicity. Nitrobacter, however, appeared to develop resistance to 2,3-dichlorophenol and 2,4,6-trichlorophenol as shown in figures 23-25. It took an increasingly larger dose of the chemicals to inhibit Nitrobacter activity as time progressed. It can be postulated that during exposure Nitrobacter and/or other microorganisms are detoxifying this compound in addition to developing a resistance. This is strictly speculation and deserves a follow-up by microbiologists.

Another interesting result is that when Nitrobacter were treated with metal ions at low concentrations and with short exposure times, the nitrite consumption increased noticeably in many cases (figures 6-12) . With higher metal concentrations, the toxicity overcame the biostimulation effect, resulting in a decrease iff nitrite consumption. This fits the general pattern of toxic response shown in figure 3 (Bagby and Sherrard, 1981).

Two hypotheses can be advanced to explain this result. First, during exposure to toxins, microorganisms under stress are undergoing 'vigorous activity to cope with this metal toxicity. This results in a greater consumption of nitrite by Nitrobacter , as nitrite is their sole energy source. Second, the metal treatment resulted in a greater permeability of bacterial cell membranes so that nutrient uptake increased (P. Seyfried, University of Toronto, personal communication, 1983).

The results in figures 6-12 can be illustrated in a conceptual model that goes one step further than the model in figure 3. There are two important parameters influencing toxic effect: toxic concentration and exposure time. In figure 3, the model is restricted to toxic concentration. If the exposure time is also considered, the model can be transformed into figure 26, where exposure time $t_1<t_2<t_3$.

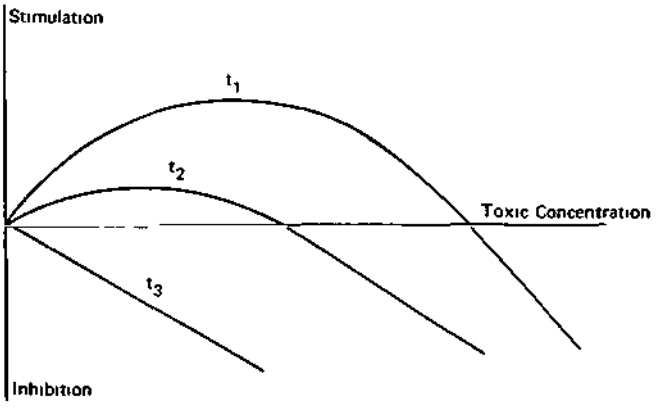


Figure 26. Conceptual model of toxic response (I)

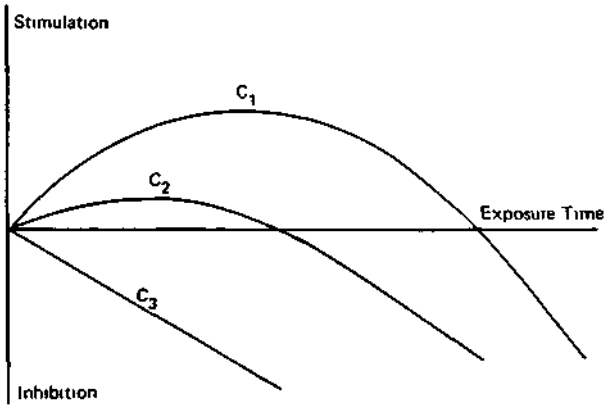


Figure 27. Conceptual model of toxic response (II)

A literal translation of the results is that the longer the exposure time is, the greater is the toxic effect. This statement seems contradictory to the results of the time effect for 2,3-dichlorophenol and 2,4,6-trichlorophenol (figures 23-25). It should be mentioned that metal ions are refractory, so they cannot be destroyed. On the other hand, organic compounds are more likely to be susceptible to microbial decomposition and detoxification.

Figure 26 is based on the use of toxic concentration as an independent variable against microbial activity as a dependent variable, with exposure time as a third factor. This figure can also be transformed, with toxic concentration and exposure time exchanging positions, as in figure 27. In this model, toxic concentration $c_1<c_2<c_3$.

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