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IMPACT OF ARSENICALS ON NITRIFICATION IN AQUEOUS SYSTEMS

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ABSTRACT

The impact of both arsenate and cacodylic acid (at arsenic concentrations of 0, 0.1, 1, 10, 100, and 1000 mg/liter) on mixed populations of nitrifiers in model aqueous systems containing ammonia was determined by measuring levels of ammonia and nitrite over a 24-day incubation period. Arsenate decreased the rate of oxidation of ammonia by *Nitrosomonas* only at high concentrations (100 and 1000 mg/liter) of arsenic; low levels of arsenate (0.1, 1, and 10 mg As/liter) had no effect on the oxidation rate, in comparison with arsenic-free controls. The oxidation of nitrite to nitrate by *Nitrobacter* was affected by all concentrations of arsenic added as arsenate; low concentrations (0.1, 1, and 10 mg/liter) delayed the oxidation of nitrite and high concentrations (100 and 1000 mg/liter) inhibited the process. The only impact of cacodylic acid on nitrification occurred at 1000 mg As/liter. The oxidation of ammonia by *Nitrosomonas* was delayed by the arsenical, but the *Nitrobacter* population was not affected. Although cacodylic acid is not toxic to the nitrification process, its degradation product, arsenate, can inhibit nitrification if it is in an available form. This inhibition of the *Nitrobacter* population may promote the accumulation of nitrite in the environment.

An understanding of the impact of pollutants on nutrient cycling in aquatic ecosystems is important to decision makers in industry and to surveillance and enforcement personnel in local, state, and federal government. These individuals need to know (1) how a pollutant affects an environment, (2) what the consequence of an altered environment might be, and (3) how the environment influences the fate of a pollutant.

Economic factors prevent the use of individual field studies to answer these questions for every pollutant. Instead, smaller laboratory-associated studies must be completed and the data ultimately extrapolated to a field situation. To meet this end, we have focused much of our laboratory's research on the fate and impact of pollutants in different environmental systems. Our environmental system studies largely involve mixed microbial populations that are important in the nutrient cycling process.

For this study, we investigated the impact of two arsenicals, cacodylic acid (hydroxydimethylarsine oxide) and sodium arsenate, on the nitrification process in aqueous systems. Cacodylic acid, which is representative of the organic arsenicals, is used in the cotton-growing states as a defoliant (Versar, Inc., 1976). Sodium arsenate was chosen because it is a common metabolic product of the organic arsenicals (Woolson and Kearney, 1973).

Oxidation of nitrogen from a reduced state, such as ammonia, to a more oxidized state, such as nitrite and nitrate, is called nitrification (Hardy and Holsten, 1972). These nitrogen transformations are usually mediated by two types of chemosynthetic, autotrophic bacteria. Ammonia oxidizers, typified by *Nitrosomonas*, get energy for growth by oxidizing ammonia to nitrite; nitrite oxidizers of the *Nitrobacter* type complete the process by oxidizing nitrite to nitrate.

The objectives of this research were to develop simple systems to study the impact of pollutants on nitrification and to determine whether arsenicals, represented by sodium arsenate and cacodylic acid, significantly alter nitrification rates. These objectives were met by the following steps: (1) Mixed populations of nitrifiers were cultured in an aqueous medium; (2) the reproducibility and reliability of the system was determined; and (3) the impact of arsenicals on nitrification was determined by measuring the oxidation of ammonia and nitrite.

MATERIALS AND METHODS

Growth Medium

Nitrification rates were determined in a chemically defined medium containing micronutrients and macronutrients. In a final volume of 1000 ml, the filter-sterilized micronutrient stock contained EDTA—Na_2, 500 mg; $FeSO_4 \cdot 7H_2O$, 200 mg; $ZnSO_4 \cdot 7H_2O$ 10 mg; $MnCl_4 \cdot 4H_2O$, 3 mg; H_3BO_3, 30 mg; $CoCl_2 \cdot 6H_2O$, 20 mg $CuCl_2 \cdot 2H_2O$, 1 mg; $NiCl_2 \cdot 6H_2O$, 2 mg; and $Na_2MoO_4 \cdot 2H_2O$, 3 mg. Each liter of the chemically defined medium contained NH_4Cl,

38.2 mg; K_2HPO_4, 1.1 mg; $MgCl_2$, 5.7 mg; $MgSO_4 \cdot 7H_2O$, 1.9 mg; $CaCl_2 \cdot 2H_2O$, 4.4 mg; $NaHCO_3$, 150 mg; and micronutrient stock, 10 ml. The medium was sterilized by filtration, and 100-ml aliquots were added to sterilized 250-ml Erlenmeyer flasks.

Appropriate amounts of sodium arsenate or cacodylic acid were added to the medium before filter sterilization. Reagent grade chemicals were used to prepare the growth medium. Mixed nitrifying populations (containing both *Nitrosomonas* and *Nitrobacter* types) from local garden soil were used as inocula for the systems. Although the functional populations were not identified, oxidation of ammonia to nitrate indicated that both *Nitrosomonas* and *Nitrobacter* were present.

Ammonia and nitrite concentrations in each flask were measured at selected intervals during the incubation period using a Technicon Autoanalyzer unit (AAII). Ammonia was measured using the automated colorimetric phenate method (Technicon Corp., 1971). Nitrite concentrations were measured using the automated cadmium reduction method (Environmental Protection Agency, 1974).

Experimental Design

Enrichment of nitrifying populations was completed by adding 1 g of garden soil to 250-ml Erlenmeyer flasks containing 100 ml of the growth medium. The flasks were incubated in the dark at 25°C at a shaking rate of 125 rpm. At 2- to 3-day intervals, samples were taken, diluted, and analyzed for ammonia and nitrite. After populations had oxidized all the ammonia to nitrate, 1-ml inocula were transferred into fresh media, and the sampling cycle was repeated. Inocula from the second and third transfers were used for the arsenic studies.

The reproducibility of the nitrification system was measured in seven replicate flasks, each receiving a 5-ml inoculum from a culture transferred twice previously. Biological activity was determined within each flask by measuring the oxidation of ammonia to nitrate over a 20-day incubation period.

The impact of both sodium arsenate and cacodylic acid (at arsenic concentrations of 0, 0.1, 1, 10, 100, and 1000 mg/liter) was measured on mixed cultures of nitrifiers in aqueous systems containing 10 mg/liter nitrogen as ammonia. Duplicate flasks were set up for each treatment, and nitrification rates were determined in each flask by measuring levels of ammonia and nitrite over a 24-day period.

The hours required for the oxidation of ammonia by *Nitrosomonas* and nitrite by *Nitrobacter* to a nitrogen concentration of 1

mg/liter were the quantities chosen for statistical analysis. Data were analyzed using analysis of variance and Tukey's ω procedure (Steel and Torrie, 1960).

RESULTS

Nitrifying populations were easily cultured from the garden-soil inoculum. For the initial enrichment, ammonia was oxidized to nitrite by *Nitrosomonas* organisms within 2 weeks, and the nitrite formed in the first step was oxidized to nitrate by *Nitrobacter* within 3 weeks. In successive transfers of 1-ml inocula, however, the rate of oxidation of ammonia to nitrate was significantly increased. By the second transfer, all the ammonia was oxidized to nitrite within 1 week. The entire nitrification process, involving the oxidation of 10 mg/liter ammonia nitrogen to 10 mg/liter nitrate nitrogen, was shortened from 24 days to 10 days by the third transfer.

No counts of nitrifiers were made as the ammonia was being oxidized to nitrate. *Nitrosomonas* probably outnumbered *Nitrobacter* in each flask, however, because the oxidation of ammonia to nitrite provides more energy for growth of cells than does the oxidation of nitrite to nitrate (Tuffey, 1973; Curtis, Durrant, and Harmon, 1975).

These nitrification systems provide reproducible results. Data in Table 1 were collected from seven replicate flasks, each receiving a 5-ml inoculum from a culture transferred twice previously. These results demonstrate that aqueous systems in flasks can be used to study the impact of soluble pollutants on nitrification. An estimate of homogeneity of data, the coefficient of variation (CV), which is 100(standard deviation/mean), calculated from the data in Table 1 shows that these systems have CV values of 20% or less when the ammonia or nitrite nitrogen concentration is above 1 mg/liter.

Using these test systems, we observed that *Nitrosomonas* organisms are not sensitive to arsenicals. At low levels of arsenate (10 mg As/liter), the rate of ammonia oxidation was identical to that of the arsenic-free controls (Fig. 1). The rate of ammonia oxidation was significantly different from the control only at 1000 mg As/liter added as arsenate, determined with Tukey's ω procedure ($\alpha = 0.05$). These results show that arsenate should not have an inhibiting action on *Nitrosomonas* in the natural environment because of the high concentrations necessary before any effects are seen.

Likewise, cacodylic acid should not have a significant direct effect on *Nitrosomonas*. *Nitrosomonas* oxidized ammonia at normal rates in media containing as much as 100 mg As/liter as cacodylic

TABLE 1

CONCENTRATIONS OF NITROGEN IN AQUEOUS NITRIFICATION SYSTEMS*

Incubation, days	NH_4^+—N, mg/liter	NO_2^-—N, mg/liter
0		
1	9.35† (S = 0.38)	
3	9.23 (S = 0.38)	0.04† (S = 0.01)
6	8.42 (S = 0.36)	0.71 (S = 0.13)
8	5.14 (S = 0.97)	4.35 (S = 0.78)
10	0.12 (S = 0.22)	8.93 (S = 0.51)
13		7.81 (S = 0.46)
15		6.06 (S = 0.82)
17		4.79 (S = 0.99)
20		0.43 (S = 0.49)

*The growth medium containing 10 mg/liter NH_4^+—N was inoculated with 5 ml of a mixed nitrifier population. Abbreviation S is standard deviation of seven flasks.

†Mean of seven observations.

acid. There was a small decrease in the oxidation rate at concentrations of 1000 mg As/liter (Fig. 2).

Nitrobacter-type organisms used in this study are sensitive to arsenate (Fig. 3). Apparently there is a progressively longer lag phase in the arsenic concentration range from 0.1 to 10 mg/liter, but the only treatment statistically different from the control (based on analysis of variance, $\alpha = 0.05$) is 10 mg/liter. Because only one point from each oxidation curve was used for the statistical analysis and only two replicate curves were obtained for each treatment, the analyses for differences are probably conservative. At very high levels of arsenate (100 and 1000 mg As/liter), *Nitrobacter* was inhibited, and nitrite accumulated.

Cacodylic acid does not affect *Nitrobacter*. Arsenic concentrations as high as 1000 mg/liter as cacodylic acid did not alter the oxidation rate of nitrite (Fig. 4).

DISCUSSION

The technique we adapted for this study is useful for studying the impact of pollutants on specific functional groups of bacteria. In this study we enriched the medium for chemosynthetic autotrophs important in the nitrification process. There are several advantages to

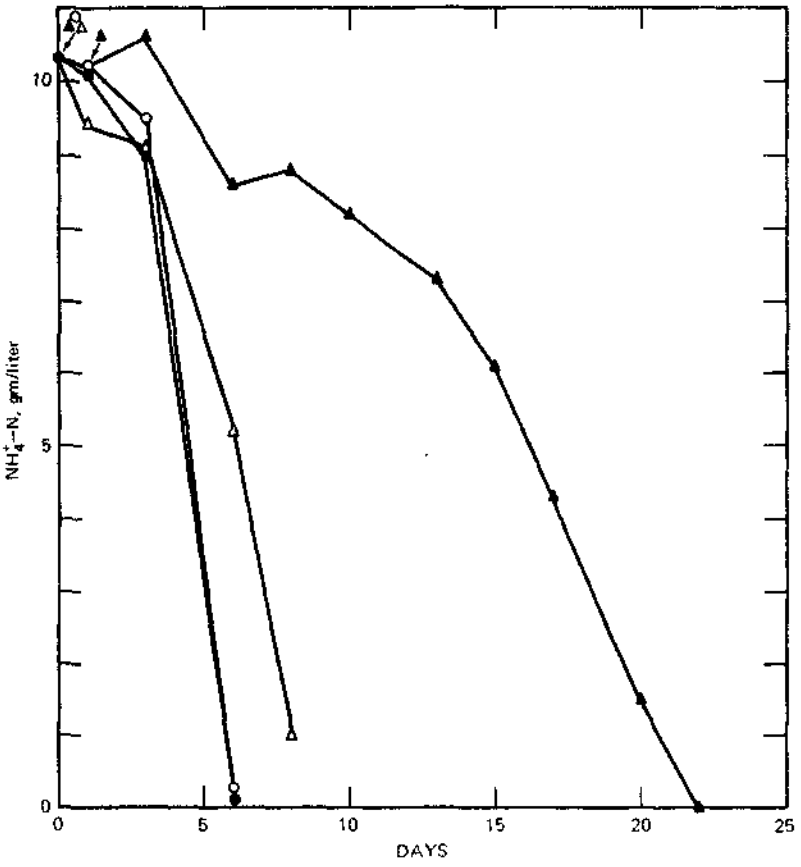

Fig. 1 Impact of arsenate on *Nitrosomonas*. ●, no arsenic; ○, 10 mg As/liter; △, 100 mg As/liter; and ▲, 1000 mg As/liter.

this approach. First, mixed cultures growing in a selective medium provide a larger genetic pool than do pure cultures. This large genetic pool may allow the system to adapt to the toxicant much as populations do in nature. Second, enrichment of the mixed population assures the researcher uniform cultures to be used for a given set of toxicity tests (Table 1). This assurance usually is not present in studies of functional groups using natural samples or colonized slides. Third, monitoring ecosystem function is usually faster and easier than measuring specific bacterial populations. For example, the study by Fliermans, Bohlool, and Schmidt (1974) using fluorescent antibodies for counting nitrifiers, is very time and labor consuming.

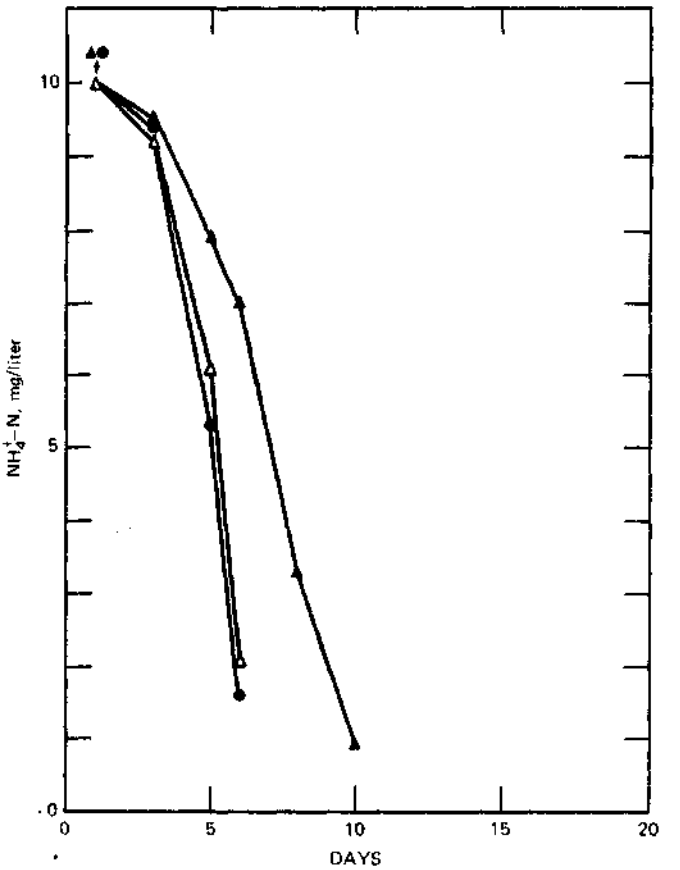

Fig. 2 Impact of the organic arsenical, cacodylic acid, on *Nitrosomonas*. ●, no arsenic; △, 100 mg As/liter; and ▲, 1000 mg As/liter.

Our approach also has some disadvantages. First, selection of new variants or species may occur, but this is not monitored. In fact, the selection process may have occurred during the enrichment phase of the study since the nitrification rate increased on successive transfer. This phenomenon may also have occurred when populations of *Nitrobacter* were exposed to arsenicals (Fig. 3). Second, interactions of populations from different segments of the nutrient cycle are not possible with enrichment techniques. This contrasts with work by Laveglia and Dahm (1974), who used soil systems to monitor the activity of several functional groups of bacteria after the test systems were dosed with pollutants.

Inhibition of *Nitrosomonas* and, consequently, inhibition of the total nitrification process, can be economically and ecologically

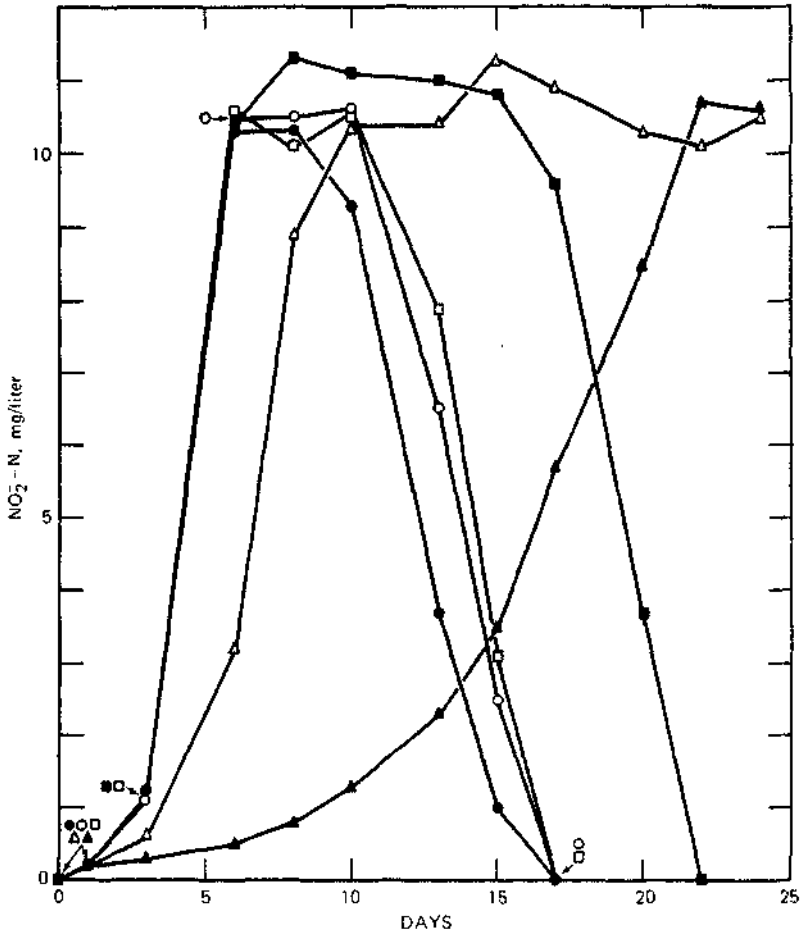

Fig. 3 Impact of arsenate on *Nitrobacter*. ●, no arsenic; ○, 0.1 mg As/liter; □, 1 mg As/liter; ■, 10 mg As/liter; △, 100 mg As/liter; and ▲, 1000 mg As/liter.

desirable. The agricultural industry prolongs the availability of nitrogen fertilizer by applying N-SERVE [2-chloro, 6-(trichloromethyl) pyridine], an inhibitor specific for *Nitrosomonas* (Campbell and Aleem, 1965). When the oxidation of ammonia to nitrite is inhibited, the nitrogen remains in a relatively immobile form, ammonia, but it is still available for plants. Pesticides such as lindane, Malathion, and Baygon also inhibit nitrification at this first stage (Garretson and San Clemente, 1968). It may also be desirable to control nitrification in streams and lakes because it contributes a significant oxygen demand in some waters. Theoretically, 4.57 mg of

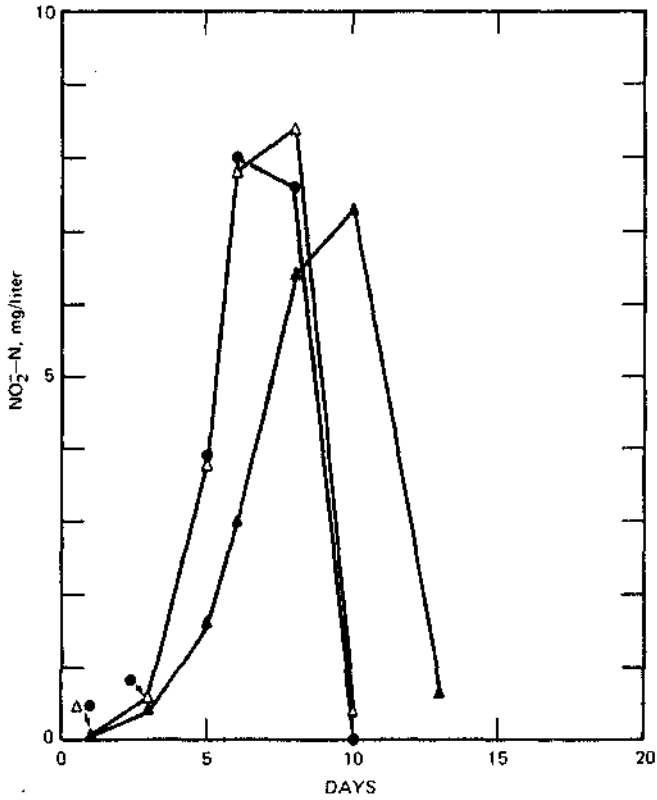

Fig. 4 · Impact of the organic arsenical, cacodylic acid, on *Nitrobacter*. ●, no arsenic; △, 100 mg As/liter; and ▲, 1000 mg As/liter.

molecular oxygen are required to oxidize 1 mg of ammonia nitrogen to nitrate nitrogen (Young, 1969).

Our results show that arsenate inhibits the second step of nitrification to a greater degree than it does the first stage. This was reflected in a delay in the oxidation of nitrite at low concentrations of arsenate; at high levels no nitrite was oxidized within 25 days. The mechanism of this inhibition was not studied. Button and Dunker (1971), however, showed that arsenate interrupts phosphate metabolism in some microorganisms. Torstensson (1974) reported that MCPA, 2,4,5-T, and Linuron inhibit *Nitrobacter* and cause an accumulation of nitrite.

Nitrite in the environment is undesirable because of its toxicity to biota (National Academy of Sciences, 1973) and its possible implication in the formation of carcinogenic nitrosamines (Lijinsky

and Epstein, 1970; Elespuru and Lijinsky, 1973; Eisenbrand, Ungerer, and Preussmann, 1975).

Generally organic arsenicals are reported to be between 10 and 100 times less toxic to biota than inorganic arsenicals (Versar, Inc., 1976). Our results showed this to be the case. Although arsenic in the form of arsenate increased the lag phase of *Nitrobacter* at concentrations as low as 0.1 and 1 mg/liter, cacodylic acid affected the nitrifiers only at arsenic concentrations of 100 mg/liter and above. Bollen, Norris, and Stowers (1977), studying forest floors and forest soils treated with either cacodylic acid or MSMA, concluded that these organic arsenicals should not have a significant adverse effect on nitrogen metabolism of forest-floor and soil microorganisms.

In aerobic systems, cacodylic acid is readily degraded to arsenate (Woolson and Kearney, 1973). This fact, coupled with our observation of arsenate toxicity towards nitrifiers, points toward a potential temporary buildup of nitrite in environments receiving high levels of the nontoxic arsenical.

In summary, our study showed (1) that these nitrification systems provide reproducible results, (2) that *Nitrobacter*-type organisms used in these systems are sensitive to arsenate, and (3) that cacodylic acid is not directly toxic to nitrifiers. Field investigations should be completed to test whether arsenicals inhibit *Nitrobacter* in field situations.

DISCLAIMER

Mention of tradenames of commercial products does not constitute endorsement or recommendation for use.

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