NUCLEAR SCIENCE AND APPLICATIONS VOL. 1, NO. 2, JULY, 1989

Characterization and Categorization of *Azotobacter* and Related Microorganisms of Bangladesh Soils

S. I. KHAN, R. MANDAL, F. ALI AND A. H. M. AHMED

Department of Microbiology, University of Dhaka
Department of Soil Science, University of Dhaka
Dhaka, Bangladesh

Free-living nitrogen fixing bacterial isolates were purified from eleven soil samples representing eight typical zones of Bangladesh. The isolates were categorized on the basis of their colonial, morphological and physic-biochemical characteristics. Only one isolate could not be fitted into any recognized group. The rest were the members of *Azotobacter* and *Azomonas* genera.

Introduction

Nitrogenase, an inherent enzyme of a few free-living microorganisms, enables them to convert atmospheric nitrogen into utilizable organic form. This fact led many, notably, Kluyver and Becking[1] and Panosyan *et al*[2]. to explore the possibility of its agro-economic use. However, Szegi and Timar[3] unfolded the importance, activity and function of free-living nitrogen fixers. The genus *Azotobacter* is of wide occurrence in soil and water except in polar regions and are highly pleomorphic[4]. Several species of *Azotobacter* have so far been described[5-7]. The nitrogen fixing capacity of *Azotobacter* and related groups mainly depend on carbon and energy source[8]. Biological nitrogen fixation is a function of the type of the microorganisms, and the physical and chemical conditions of the soil.

Bangladesh being a tropical country, non-symbiotic nitrogen fixation and its impact on agriculture seems to be promising[9],[10]. However, till to-date, there is no published work on the characterization and categorization of the microorganisms of the genera *Azotobacter* and related groups in our soils. The present investigation is an attempt towards that end.

Experimental

Soil samples were collected (0-15 cm depth) from various agro-climatic zones of Bangladesh. The soil samples were air dried, ground and passed through 2mm seive. The soils were mostly calcarious and cultivated having pH range between 6.1 and 8.4. The soils were silty loam to silty clay loam in texture.

Ten gram soil with 90 ml sterile distilled water in a 500 ml Erlenmeyer flask was shaken for 10 minutes in a reciprocating shaker and ten fold dilutions were made up to 10^{-7}.

A modified Ashby's nitrogen free agar medium[11] in petridishes was inoculated with 0.2 ml of each dilution and incubated at 30°C for 7 days. Large (3-5 mm diameter) colonies were subcultured and the process continued until uniform and identical to the parent colonies were obtained. They were then transferred to slants using the same medium. The isolates from the slants were maintained as mother culture. Becking's medium[12] was used for the isolation of acid tolerant dinitrogen fixers. The plates were observed daily for growth. The isolates were designated A, B, C, D, E, F, G, H, I, J and K corresponding to soils of eleven different locations.

Characterizations were made by wet mounts for shape and size, hanging drop for motility, dilute iodine solution for starch granules, sudan black for lipid, 30% H_2O_2 for catalase. Gram reaction and sporulation were observed following standard methods[13].

Carbohydrate utilization and fermentation tests were performed using 1% each of glucose, sucrose, xylose, rhamnose, arabinose, sorbose, lactose, maltose, galactose, mannose, mannitol and starch. Citrate utilization and gelation hydrolysis tests were done using 0.2% and 0.5% respectively on the basal medium. Nitrate reduction and indole production were observed following methods as detailed by Harrigan and McCance[13]. The tolerance of hydrogen ion concentrations at pH 5.5, 6.5, 7.5 and 9.0 was studied by assessing growth.

Results and Discussion

Colonial, morphological and various physio-biochemical characteristics of eight isolates of the *Azotobacterin* group were examined (Table I and II).

Characteritics of the isolates

Colony : The colony size of the isolates varied from 1.5-4.0 mm in diameter (Table I). The elevations of the colonies were umbonate for isolate A, pulvinate for isolates B and K, convex for isolates C, D and H, and flat for isolates E and G. The consistency and optical character of the colonies varied widely (Table I). The colony of A was somewhat different from others ; its consistency was lentyrous with dull white appearance. Colonies of B and K were respectively viscous-transparent and highly viscous-translucent. The isolate K particularly produced tenacious gum-like substance and when pulled by a needle, the growth followed like a thread. The colonies of isolate C was less spreading and dull white with soft consistency. The isolates D and H were near opaque with copious slime while E and G were opaque with moderate slime.

Pigment : Variations in pigmentation of the colonies reflected the possible variability in the isolates[7] (Table I). The colour intensity reached maximum at around 20-day incubation with no appreciable change thereafter. The colonies of B and K were conspicuously nonpigment formers. The pigments ranged from light brown to dark brown except for C which, however, imparted light brown to pale pink shades that diffused into agar slants.

Morphology : The cells of the isolates varied widely in shape and size where B was a perfect rod, A and C were coccoid to oval while the rest were coccoid (Table I). However, all of them were non-spore former and noncapsulated except for B and C. The isolates showed variability in motality while the isolate K was highly motile.

Physio-biochemical reactions : The utilization of carbohydrates by the isolates varied to a degree that manifested distinguishable metabolic character intrinsic to a particular isolate. Xylose and mannose were found to be poor carbon sources for all the isolates (Table II). The isolates C, E and G on rhamnose and B, C, D and H on starch failed to grow while rest of the isolates utilized most of the carbohydrates. All of them were catalase positive and Gram negative, reduced nitrate to nitrite (but not to elemental N), produced no indole, utilized citrate, hydrolyzed starch (isolates A, C, E, G) and gelatin (Table II) and except for B and K, they were devoid of lipid granules (Table II).

The isolates were found to be sensitive to low pH and seemed to be more susceptible to acidity than to alkalinity (Table II). Most of them could not survive at pH 5.5 grow well at pH 7.5. Only two isolates (H and K) survived at pH 9.0. It is suggested that the isolates could form diverse species of the genus *Azotobacter* or may represent separate genera.

It could be reckond from the colonial morphology and growth characteristics of the isolates that much heterogeneity was manifested amongst the isolates. Based on colonial, morphological and biochemical evidences, the isolates were classified into five different groups.

Group I and II : The isolates A, D, E, G and H synthesized water insoluble brown pigment and may possibly be placed under the genus *Azotobacter*. Among them, isolates A, E and G utilized starch, formed cyst, failed to grow on rhamnose which could be considered as a distinguishing feature of *Azotobacter chroococcum*[7]. However, the isolate A could be a variant of *Azotobacter chroococcum* as manifested from its slightly different colony character and poor utilization of rhamnose.

Table I. Colonial and morphological characteristics of the isolates.

Isolates	A	B	C	D	E	G	H	K
				Colony				
Count ($x10^3$/g soil)	2.0	1.0	6.6	10.0	12.0	6.0	5.0	6.0
Diameter (mm)	3.0-4.0	1.5-2.5	2.0-3.0	3.0-3.5	3.0-3-5	3.0-3.5	1.5-2.0	1.5-2.5
Form	irregular	circular	circular	circular	circular	circular	circular	circular
Elevation	umbonate	pulvinate	convex	convex	flat	flat	convex	pulvinate
Margin	undulate	entire	entire	entire	entire	entire	entire	entire
Surface	contoured	smooth	smooth	smooth	smooth	smooth	smooth	smooth
Optical character	dull white	transparent	dull white to opaque	nearly opaque	opaque	opaque	opaque	translucent
Consistency	butyrous	viscous	soft	copious slime	thick slime	thick slime	copious slime	highly viscous
Pigment	moderate	colourless	light brown to pale pink	light brown	dark brown	dark brown	grey brown	colourless
				Morphology				
Shape	coccoid to oval	rod	coccoid to oval	coccoid to oval	coccoid	coccoid to oval	coccoid to oval	coccoid
Size (μm)	1.70-2.00	6-7x1.45	1.50-2.00	1.75-2.00	1.50-1.85	1.45-2.00	1.80-2.00	1.45-1.75
Motility	moderate	nonmotile	poor	poor	moderate	moderate	vigorous	vigorous
Sporulation	–	–	–	–	–	–	–	–
Capsulation	–	+	+	–	–	–	–	–
Growth on nutrient agar	good	poor	poor	moderate	moderate	moderate	moderate	moderate

– = negative ; + = positive

Table II. Physio-biochemical characteristics of the isolates

Isolates	A	B	C	D	E	G	H	K
				Carbohydrate utilization				
Glucose	A ++	A ++	A ++	A ++	A ++	A ++	A ++	A ++
Sucrose	A ++	Al ++	Al ++	N ++	Al ++	A ++	Al ++	N ++
Arabinose	A ++	A ++	A ++	A +	A ++	A ++	A ++	A ++
Rhamnose	A +	A ++	A−	A ++	A —	A —	N ++	A +++
Maltose	A ++	A ++	A ++	A +	A ++	N +++	Al +++	N +++
Lactose	A +++	A ++	A ++	A ++	A ++	A ++	N +++	N +++
Mannose	A +	A +	A +	A +	A +	A +	Al +	A +
Starch	Al +++	Al −	Al −	N ++	Al ++	N ++	Al −	Al+++
Mannitol	A +++	A ++	Al +++	A ++	A +++	A +++	A +++	Al+++
Xylose	A +	A +	A +	A +	A +	A +	A +	A +
Galactose	A ++	A +	A ++	A ++	A ++	A ++	A ++	A ++
				Biochemical reactions				
Gram reaction	—	—	—	—	—	—	—	—
Lipid	—	+	—	—	—	—	—	+
Catalase	+	+	+	+	+	+	+	+
Nitrate reduction	+	+	+	+	+	+	+	+
Indole	—	—	—	—	—	—	—	—
Citrate	+	+	+	+	+	+	+	+
Gelatin hydrolysis	—	—	—	—	—	—	—	—
Starch hydrolysis	+	—	+	—	+	+	—	—
				pH tolerance				
5.5	—	—	—	—	—	—	—	—
6.5	+	+	+	+	+	+	+	++
7.5	+++	+++	+++	+++	+++	+++	+++	++
9 0	—	—	—	—	—	—	+	+

A = acidity ; Al = alkalinity ; N = neutral ;
— = negative ; + = positive

The isolates D and H could not utilize starch, but typically grew on rhamnose suggesting their similarity to *Azotobacter vinelandii*, the only species that is capable of utilizing rhamnose[7]. Light brown to grey brown diffusible pigment formation by them was also a characteristic feature of *Azotobacter vinelandii*[7].

Group III : The isolate B was a non-motile, contained granules, produced profuse slime than those of A, E and G, but its nonpigmented dome-shaped colonies and pH growth in acidic pH indicated that it might probably be a member of the species *Azotobacter beijerinckii*[5].

Group IV: Formation of light brown to pale pink water soluble pigment was an interesting feature of the capsulated isolate C. It appears from its failure to utilize starch and rhamnose, that this isolate might represent the genus *Azomonus* and the species *A, macrocytegenes*[5,7].

Group V : The isolate K produced certain unique properties different from those described earlier. The isolate had rounded ends, contained intracellular granules of PHB and was vigorously motile. The colonies were transparent and raised, produced copious tenacious and elastic slime. The elastic and tough colonies were difficult to remove by pulling with a needle. Unlike *Azotobacter* and *Azomonas*, the isolate K did not grow on plain peptone agar. From the characteristics manifested in this investigation, it is suggested that the isolate K resembles the characteristics of the genus *Beijerinckia*[7]. Pending more data and further developments, the genus *Beijerinckia* is at the moment listed under the noncommital category "Other genera"[7]. Further studies on genetics and molecular biology of the isolates are warranted before confirming genus/species status to the isolates.

References

1. A. V. Kluyver and J. H. Becking, Derx. Soumalais. Tiedeakat. Toimituks, Ser. A. **11 (60)**, 367 (1955).
2. A.K. Panosyan. R.Sh. Arutyunyan, N.A. Avetisyan and S.V. Zakharyan, Izv. Akad. Nauk. Arm. SSR, Biol. Nauki. **15(2)**, 13 (1961).
3. J. Szegi and E. Timar, Acta. Agron. Acad. Sci. Hungariease. **13** (3-4), 337 (965).
4. W.D.P. Stewart, *Nitrogen Fixation in Plants*. The Althlene Press, Univ. London, p 67, 1966.
5. J. R. Norris and H. M. Chapman, *In Identification Methods* for Microbiologists. Part B. (Ed.) Gibbs, B.M. and Shapton, D.A. Academic Press. London, p 19, 1968.
6. E.N. Mishustin and V.K. Shilnikova, Soil Biol. Review Res., UNESCO, p 37, 1969.
7. N.R. Krieg (ed.), Bergy's Manual of Systematic Bacteriology, Vol. 1. Williams and Wilkins, Baltimore, 1984.
8. N.B. Krylova, Dokl. TSHA. **84**, 293 (1963).
9. A.H.M. Ahmed, S.I. Khan, R. Mandal and F. Ali, Int. J. Trop. Agric. IV (3 & 4), 110 (1987).
10. S. I. Khan, R Mandal, F. Ali and A.H.M. Ahmed, Dhaka University Studies, **Part E, 3 (2)**, 109 (1988).
11. N. A. Hegazi and S.I. Niemela, J. Appl. Bacteriol. **41**, 311 (1976).
12. J. H. Becking, Plant and Soil, **14**, 49 (1961).
13. W.F. Harrigan and M.E. McCance, Laboratory Methods in Microbiology, Academic Press, London, 1966,

Pak. j. sci. ind. res. vol. 32, no. 9, September 1989

EFFECT OF CHLORIDES OF COBALT, NICKEL AND COPPER ON NITRIFICATION IN PEAT

R. Mandal* and J. W. Parsons

Department of Soil Science, University of Aberdeen, AB9 2UE, Scotland, U.K.

(Received April 3, 1989; revised September 27, 1989)

Mineralization of N in the presence of 0 to 2000 mg kg^{-1} each of Co, Ni and Cu as chlorides in peat was studied. These salts of metals did not produce any significant change in the process of nitrification and ammonification up to 40 and 50 days respectively. However, thereafter the general trend was for NH_4-N to decrease up to 110 days as (NO_2+NO_3)-N accumulated. Nitrification was increasingly suppressed as the Co, Ni and Cu were increased, especially with the highest level of Cu where NH_4-N remained high and (NO_2+NO_3)-N remained at about the initial level suggesting that nitrification was virtually prevented. Between 110 and 130 days of incubation ammonification increased slightly in all treatments but nitrification showed an unexplained flush at the highest level of applied Cu.

Key words: Peat, Nitrification, Heavy metals.

Introduction

It is well established that heavy metals are highly toxic and can cause serious hazards in the soil-plant-animal system. Heavy metals may originate from deliberate application to correct deficiencies, as pesticides or from industrial waste and sewage [1]. As no information is available it may be interesting to see what could be the course of their reactions on nitrification in soil.

However, studies on the role metal ions in soils are difficult to plan and put into practice in selecting a correct soil environment conditions. In soils these elements are strongly chelald by organic matter and their availability is chiefly regulated by pH. At normal pH values in mineral soils they become insoluble while at low pH the solubility is at maximal. An acid peat was chosen as the suitable medium because of its low metal content to study the effect of chlorides of Co, Ni and Cu on nitrification.

Materials and Methods

Commercially available Fisons peat (II) originally derived from Somerset was air-dried and passed through 2 mm sieve. The physical and chemical characteristics of the peat are presented in Table 1.

For this experiment a different commercial sample of peat was used with an initial pH of 4.4 compared to that of 3.4 for the sample used in the previous two papers. So comparisons are not feasible. Its organic matter content of 72% (C x 1.72) is similar and its C/N ratio is 30 compared to 36 for the Red Moss peat but its (NO_2+NO_3)-N is considerably higher 90 compared to 7. Its available P is also considerably lower 0.28 compared to 1.58.

Three concentrations of each of Co, Ni and Cu as chloride in addition to one control treatment, in duplicate, were used. Aqueous solution of each salt of metal was applied separately at the rate of 500, 1000 and 2000 mg kg^{-1} peat. Samples treated with salts received lime as estimated from pH lime curve to maintain the initial pH of 4.40. The experiment was arranged according to a randomized block design.

Portions of air-dry peat (50 g) were weighed into a series of clean-dry 500 ml conical flasks and incubated at 50% WHC at 25° with "clingfilm" covering. A constant moisture content was maintained gravimetrically removing the "clingfilm" cover for 5 minc each day in order to make up the loss of water and provide aeration.

Peat samples (5 g) were collected, in duplicate, every 10 days up to 70 followed by 20 days intervals up to 130 days. These samples were extracted with 1M KCl and the extracts analyzed for NH_4-N and (NO_2+NO_3)-N using a Technicon Auto-Analyzer.

Table 1. General Characteristics of the Peat Examined.

Sample	pH	WHC	Org.C	Total N	C/N ratio	CEC meq kg^{-1} peat
			Percent			
Fisons Peat (II)	4.40	287	41.9	1.39	30	928.5

Available			KCl exch. cations		
NH_4-N	NO_2+NO_3)-N	P	Co	Ni	Cu
mg kg^{-1} peat					
18.9	90.0	0.28	bdl	bdl	2.66

bdl = below detection limit.

* C/o Dr. A.H. Moinuddin Ahmed, 28/E Issa Khan Road, Dhaka University Staff Quarters, Ramna, Dhaka-1000, Bangladesh.

pH was measured with a combined glass/calomel electrode using a pH meter from a saturation past in the beginning and at the end. Organic carbon was determined by wet oxidation method [2], total N by Kjeldahl procedure, CEC by 1M NH_4OAc (pH 7.0) and 1M KCl exchangeable Co, Ni and Cu by atomic absorption spectrophotometer (Shandon Southern Model A 3400). 0.5 M acetic acid [3] extractable P was estimated colorimetrically using a Cecil Spectrophotometer (Model E 272).

Results

Figure 1A (Co), 2A (Ni) and 3A (Cu) show that release of NH_4-N increased rapidly up to 20 days of incubation, declined slightly up to 30 days in most of the treatments and then decreased gradually up to 90 days until it became nil for each salt of metals in two treatments (0 and 500 mg kg^{-1}). The decrease in NH_4-N continued up to 110 days in samples treated with 1000 mg salt of metal kg^{-1}. Addition of 2000 mg salt of metal kg^{-1} showed almost a steady state in NH_4-N between 50 and 110 days. After 110 days a significant flush in NH_4-N in all the treatments was observed. None of the salts of metals showed any significant effects up to 50 days.

Figure 1B (Co), 2B (Ni) and 3B (Cu) show that the trend of accumulation of (NO_2+NO_3)-N up to 40 days was similar to

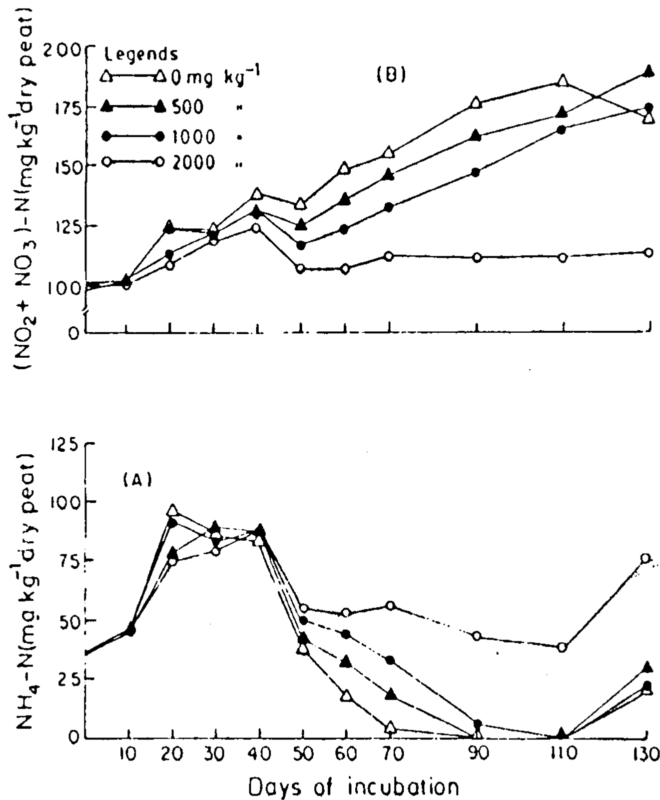

Fig. 1. Changes in NH_4+N (A) and (NO_2+NO_3)-N (B) as influenced by $CoCl_2$ during aerobic incubation of Fisons peat (II).

1M KCl was used because no signifi between 1M and 2M KCl results was observed.

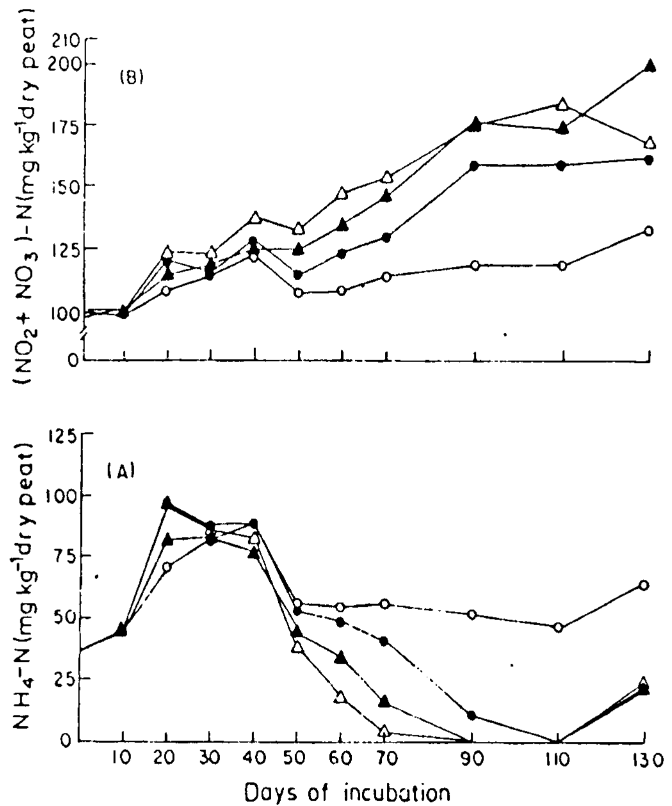

Fig. 2. Changes in NH_4-N (A) and (NO_2+NO_3)-N (B) as influenced by $NiCl_2$ during aerobic incubation of Fisons peat (II). Legends : see Fig. 1.

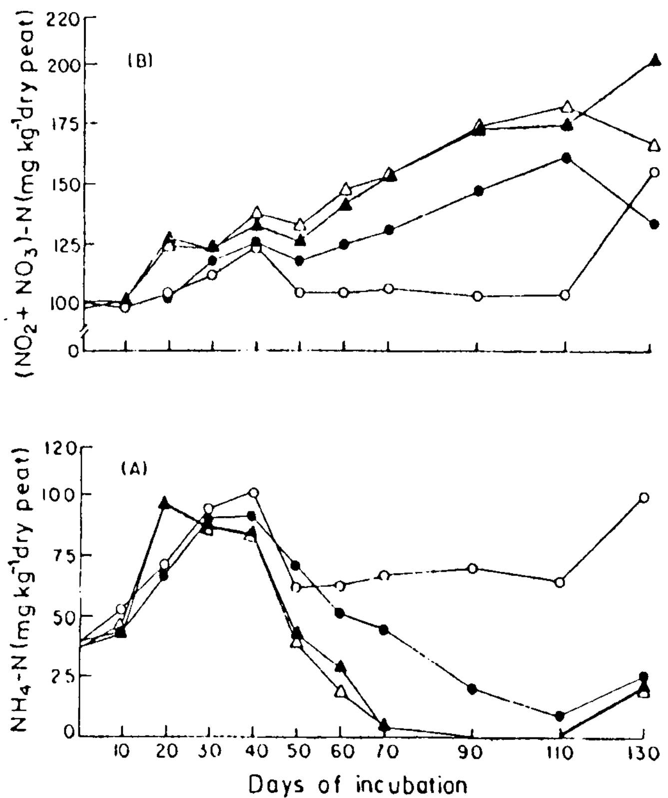

Fig. 3. Changes in NH_4-N (A) and NO_2+NO_3-N (B) as influenced by $CuCl_2$ during aerobic incubation of Fisons peat (II). Legends : see Fig. 1.

that of NH_4-N (Figs. 1A-3A), there being no significant suppression of nitrification (Table 2). Between 50 and 110 days nitrification increased steadly in the 0 and 500 mg salt of metal treatments but it was significantly and increasingly inhibited at the higher levels (Table 2). None of the salts of metals at the concentration 2000 mg kg^{-1} completely curtailed nitrification which remained nearly constant from 50 to 110 days but Ni showed a slight increase and Cu a greater increase in nitrification at 130 days. By the end of the experiment in the control and 1000 mg kg^{-1} of Cu the amount of (NO_2+NO_3)-N declined after 110 days.

TABLE 2. STATISTICAL TREATMENT OF THE DATA (LSD) AT 5%.

Treat-ment	N	Days of incubation 50	60	70	90	110	130
Co	NH_4-N	ns	2.50	2.00	1.81	**	1.95
	(NO_2+NO_3)-N	2.41	4.92	3.2	1.11	3.41	4.25
Ni	NH_4-N	ns	3.78	3.01	4.00	**	2.44
	(NO_2+NO_3)-N	2.55	2.88	5.26	1.81	6.55	7.12
Cu	NH_4-N	ns	4.05	5.62	3.78	**	3.71
	(NO_2+NO_3)-N	4.61	5.55	6.66	7.51	7.22	5.55

0 to 40 days not significant, ns = not significant, ** = not done.

Discussion

As regards Cu toxicity, Peremi and Cornfield [4] reported that excess of Cu (10000 mg kg^{-1}) caused significant reduction in nitrification. Similar views were also expressed by Tyler [5]. Lipman and Bericks [6] showed that a tolerable range of Cu was 100 mg kg^{-1} soil and above that was detrimental for nitrification. It has been noted that mineral-N showed an unusual flush at 130 days in salt treated samples except one treatment where 1000 mg kg^{-1} Cu was added.

Acknowldegement. R. Mandal was supportd by a scholarship from the Association of Commonwealth University.

References

1. F.A.M. De Haan and G.H. Bolt, *Pollution in the Encyclopedia of Soil Science*, ed. R.W. Fairbridge and C.W. Finkl. Jr. (Dwoden, Hutchinsons and Ross, Inc., 1979), Part 1., pp. 386-390.
2. J. Tinsley, *A Manual of Experiments* (Department of Soil Science, University of Aberdeen, Scotland, U.K., 1970).
3. E. G. Williams and A.B. Stewart, J. Soc. Chem. Indust., 60, 291 (1941).
4. P. R. Premi and A. H. Cornfield, Geoderma., 3, 233 (1969-70).
5. G. Tyler, Nature., **255**, 701 (1975).
6. C.B. Lipman and W.F. Gericke, Univ. Calf. Publ. Agric. Sci., **1**, 495 (1917).