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AMMONIA OXIDATION AT LOW pH BY ATTACHED POPULATIONS OF NITRIFYING BACTERIA

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Summary—Biofilm populations of *Nitrosomonas europaea* were formed in continuous flow sand columns supplied with defined inorganic medium containing 50 μg NH_4^+-N ml^{-1}. Steady-state nitrite concentrations in effluent from the columns decreased as the pH of the inflowing medium was reduced but ammonia oxidation occurred at a pH value of 6, even though the pH minimum for growth of the organism in liquid batch culture was 7. No evidence was obtained for NH_3 limitation at low pH values, resulting from ionization to NH_4^+. The pH minimum for ammonia-oxidizing activity was also reduced in continuous flow vermiculite columns supplied with medium of pH 5.4, with an effluent pH of 6.3, but detailed assessment of the effects of low pH on activity was prevented by the strong buffering capacity of vermiculite. A multispecies, nitrifying biofilm, formed as wall growth in an ammonia-limited chemostat, was capable of ammonia-oxidizing activity, but not growth, at pH 5 while growth was possible at pH 5.5. The data provide evidence of the potential for autotrophic nitrification in acid soils.

INTRODUCTION

The oxidation of ammonia and nitrite by autotrophic nitrifying bacteria growing in inorganic liquid media occurs optimally within the pH range 7.0–8.5. In natural environments, however, particularly the soil, significant nitrification rates can be measured at pH values as low as 3.7 (Boswell, 1955; Weber and Gainey, 1962; Olsen, 1929). Nitrification in acid soils may be explained in several ways. Heterotrophic nitrification is carried out by a wide range of bacteria and fungi (Focht and Verstraete, 1977), some of which are acid tolerant (Lang and Jagnow, 1986; Stroo *et al.*, 1986), although their specific nitrifying activity is low. The existence of urease activity in ammonia-oxidizing bacteria (de Boer *et al.*, 1989a; Allison and Prosser, 1991) provides for a mechanism linking ammonification and nitrification, in which urea hydrolysis leads to a localized increase in pH, favouring autotrophic nitrification (de Boer *et al.*, 1988). A third possibility is the existence of acidophilic strains of nitrifying bacteria, but, although an acidophilic strain of *Nitrobacter* has been characterized (Hankinson and Schmidt, 1988), acidophilic ammonia oxidizers have not yet been isolated in pure culture. Nevertheless, autotrophic ammonia oxidizers are present in significant numbers in acid soils (Hankinson and Schmidt, 1984; Walker and Wickramasinghe, 1979; de Boer *et al.*, 1989b; Allison and Prosser, 1991) and there is evidence that nitrification at low pH may be autotrophic (Killham, 1986).

Nitrifying bacteria in soil exist on the surface of particulate material and protection from low pH may be provided by surface attachment and biofilm formation. Growth of *Nitrobacter* on glass surfaces and on ion-exchange resins provides such protection, with a pH minimum for nitrite-oxidizing activity 1.5 units lower than that for freely-suspended cells (Keen and Prosser, 1987a). This may be due to production of extracellular polymeric material which completely covers attached nitrifying bacteria in glass bead columns.

We assessed the effect of pH on the activity of a laboratory strain of the autotrophic ammonia-oxidizer *Nitrosomonas europaea* colonizing sand and vermiculite in continuous-flow packed columns. In liquid batch culture, this strain has a pH minimum for growth of 7.0. In addition the pH response of a mixed species nitrification biofilm established on the glass walls of a chemostat vessel is described.

MATERIALS AND METHODS

A strain of *N. europaea* originally isolated from soil by R. M. Macdonald, Rothamsted Experimental Station, Harpenden, Herts was grown and maintained as described by Keen and Prosser (1987b) in modified Skinner and Walker (1961) medium (SW) containing 50 μg NH_4^+-N ml^{-1} as ammonium sulphate (Powell and Prosser, 1985).

Continuous flow columns

Continuous flow columns containing either sand or vermiculite were constructed. The former consisted of a glass cylinder, 1.5 cm i.d., length 40 cm, sealed at the base by a sintered glass plate and containing 40 g

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acid washed sand. A 1.5 cm dia glass microfibre filter (Whatman) was positioned on the upper surface of the sand to allow even distribution of liquid medium supplied from above. The column was closed at the top by a silicone rubber stopper through which were inserted Pasteur pipettes to allow entry of medium and filter sterilized air. The base of the column was tapered and connected to silicone rubber tubing for collection of effluent samples.

The vermiculite column was constructed from autoclavable plastic, 4 cm i.d., length 20 cm, closed at both ends by silicone rubber stoppers enabling entry of medium and filter sterilized air, and exit and collection of effluent. The column contained 12.5 g medium grade horticultural vermiculite (Silva perl), with high cation-exchange capacity, packed between two 4 cm dia glass microfibre filters (Whatman) to allow even distribution of inflowing medium and to prevent washout of particulate material.

Sand and vermiculite columns were sterilized, along with connecting tubing, by autoclaving for 30 min at 121°C. After cooling, SW medium, autoclaved in the same manner and adjusted to pH 8, was supplied to the column at a rate of 5 ml h^{-1} for 24 h prior to inoculation with 30 ml of a mid-exponential phase culture of *N. europaea*. The inoculum was allowed to drain to the level of the column packing material at which point the effluent tubing was clamped and the column was kept at 30°C in the dark for 5 d. After batch incubation, SW medium was supplied continuously at a constant flow rate using a LKB 2132 Microperpex peristaltic pump and effluent from the base of the column was collected in a LKB 2070 Ultrorac II fraction collector. Activity in the samples was prevented by addition of the nitrification inhibitor potassium ethyl xanthate to a final concentration of 20 μg ml^{-1}. Samples were analysed for ammonia and nitrite using a Technicon Autoanalyser II System.

The pH of SW medium was adjusted to the required value by aseptic addition, after sterilization, of 5% (w/v) Na_2CO_3. Stepwise changes in pH were achieved by switching supply ports at the top of the column between reservoirs of media at appropriate pH values. SW medium supplied to vermiculite columns contained 50 μg NH_4^+-N ml^{-1} as $(NH_4)_2SO_4$, while sand columns were supplied with 5, 50 or 500 μg NH_4^+-N ml^{-1}.

Chemostat culture

Chemostat studies were carried out in a modified LH 500 Series Modular Fermenter System (LH Engineering) with automated pH control and 1 litre glass vessel with an operating volume of 700 ml. Silicone rubber tubing was used throughout and the vessel was covered with aluminium foil to prevent photoinhibition. The culture vessel was autoclaved at 121°C for 20 min containing 700 ml distilled water which was replaced, after cooling, with SW medium at pH 8. Agitation was achieved using a magnetic impeller (500 rev min^{-1}) and the temperature was maintained at 30°C. Filter sterilized, humidified air was delivered by HyFlo Model C Air pump (Medcalf Bros. Ltd) at a rate of 500 ml min^{-1} and SW medium containing 50 μg NH_4^+-N ml^{-1} was supplied using a LKB 2132 Microperpex peristaltic pump.

The medium was inoculated with 0.5 g of a Ranker soil (Hatton–Tomintoul–Kessock association, pH 4.1) and the culture was incubated in batch until formation of nitrite. SW medium was then supplied to give a dilution rate of 0.014 h^{-1} and effluent samples were analysed for ammonia, nitrite and nitrate by autoanalysis. Significant wall growth became visible after several days and the dilution rate and stirrer speed were reduced to 0.007 h^{-1} and 250 rev min^{-1}, respectively. Step changes in pH were imposed by automatic addition of Na_2CO_3 at concentrations of 5% (w/v) above pH 6 and 0.5% (w/v) at lower pH values. In one experiment, the pH of the medium was adjusted using 0.05% (w/v) NaOH.

For both chemostat and continuous-flow column studies, the establishment of steady states in substrate or product concentrations was determined as described by Keen and Prosser (1987b). Statistical comparisons between steady-state values were carried out using the Student's *t*-test. However, steady states in different columns, and in the same column but separated by long time intervals and differing conditions, may not be directly comparable. This is due to long-term development of biofilm populations and variations in their distribution within the column. The majority of comparisons are therefore made between values for adjacent steady states, where population changes will have been minimal.

RESULTS

Ammonia oxidation in continuous flow sand and vermiculite columns

A sand column was enriched with *N. europaea*, by incubation in batch culture for 5 d, and was then supplied with medium of pH 8.0 containing 50 μg NH_4^+-N ml^{-1} at a rate of 1 ml h^{-1}. A steady state in effluent nitrite concentration was established 400 h after monitoring began (Fig. 1), following a period of 160 h during which flow rate varied due to pump failure. This was rectified at 300 h and the steady-state nitrite concentration at 400 h was 17.3 μg NO_2^--N ml^{-1} (Table 1). A reduction in the pH of the inflowing medium to 7.0 resulted in a decrease in effluent NO_2^- concentration within 20 h, slightly longer than 13 h, the residence time for liquid passing through the column. A new steady state of 6.8 μg NO_2^--N ml^{-1} was established and further reductions in pH to 6.5 (720 h) and 6.0 (1060 h) reduced steady-state NO_2^- concentrations to 3.35 and 1.66 μg NO_2^--N ml^{-1}, respectively (Table 1). All reductions in NO_2^- concentration occurred within 30 h of the change in pH, except for the reduction to pH 6.0, where establishment of the new steady state occurred after several hundred hours (Fig. 1). A subsequent reduction in

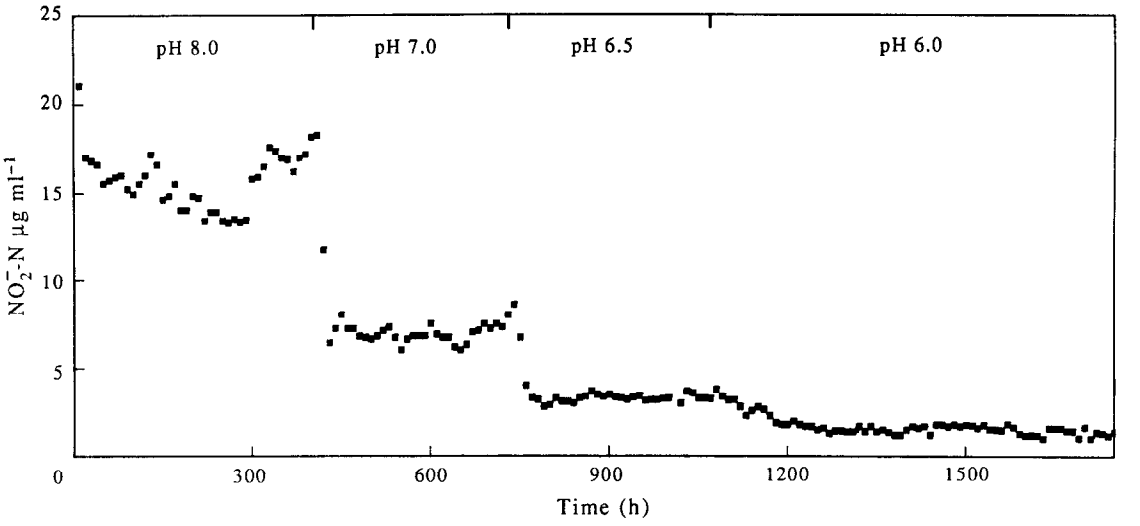

Fig. 1. Effect of pH on nitrite concentration in the effluent from a continuous flow sand column inoculated with *N. europaea*. The pH of the inflowing medium is indicated along the upper axis.

pH to 5.5 resulted in a sharp decrease in NO_2^- concentration within 20 h, followed by a gradual decrease to negligible amounts within 350 h.

Similar experiments using a column containing vermiculite inoculated with *N. europaea* were complicated by the stronger ion-exchange properties of the clay mineral. Thus, while the pH of the inflowing medium was reduced in several steps from 8.0 to 5.0, vermiculite provided significant buffering and the pH of the effluent did not fall below 6.3 (Table 2). Despite this, steady states in NO_2^- concentration were established at each inflowing pH value (Fig. 2a, b) although at the lower pH values fluctuations were greater than in the sand column. Medium was initially supplied at a flow rate of 4 ml h^{-1} and reductions in pH from 8.0 to 7.0 (362 h) and subsequently to pH 6.0 (695 h) reduced steady-state NO_2^- concentration to 13.19 and 3.01 μg NO_2^--N ml^{-1} respectively. At 850 h the flow rate was reduced to 2 ml h^{-1} to increase effluent NO_2^- concentrations. Subsequent reductions in pH to 5.6, 5.4, 5.2 and 5.0 reduced activity but did not prevent ammonia oxidation because of the existence of regions of higher pH within the column. Changes in NO_2^- concentration occurred within 20 and 30 h for flow rates of 4 and 2 ml h^{-1} respectively, after periods 2–3 times greater than the residence times.

The relative proportions of NH_4^+ and NH_3, the substrate for ammonia oxidation, depend on pH, ionization increasing at lower pH values. Reduced activity at low pH may therefore result indirectly from ammonia limitation. This was tested using a sand column, inoculated as described above, and supplied initially with medium containing 50 μg NH_4^+-N ml^{-1} at pH 6.5. A steady state was established after 300 h with a NO_2^- concentration of 4.46 μg NO_2^--N ml^{-1} (Table 3). Effluent NO_2^- concentration was not significantly affected by supply, at 550 h, of 500 μg NH_4^+-N ml^{-1}, nor by a return to supply of 50 μg NH_4^+-N ml^{-1} at 1000 h (Table 3). A reduction in inflowing ammonium concentration to 5 μg NH_4^+-N ml^{-1} led to a 23% reduction in activity and a steady-state NO_2^- concentration of 3.42 μg NO_2^--N ml^{-1}.

Table 1. Steady-state nitrite concentrations in effluent from a continuous-flow sand column inoculated with *N. europaea* provided with inorganic medium containing 50 μg NH_4^+-N ml^{-1} at a range of pH values

pH of inflowing medium	Steady-state nitrite concn (μg NO_2^--N ml^{-1})	SE
8.0	17.30	0.19
7.0	6.80	0.11
6.5	3.35	0.04
6.0	1.66	0.04

Nitrification by a mixed culture biofilm

An ammonia-limited chemostat, inoculated with soil, was used to enrich for nitrifying bacteria as a preliminary to isolation of pure cultures of ammonia oxidizers. The chemostat was operated at pH 8 and a dilution rate of 0.014 h^{-1} and a multispecies nitrifying biofilm developed as wall growth on the chemostat vessel. Dilution rate was then reduced to 0.007 h^{-1} and changes in ammonia, nitrite and nitrate concentrations following several step changes in pH

Table 2. Steady-state nitrite concentrations in effluent from a continuous-flow vermiculite column inoculated with *N. europaea* provided with inorganic medium containing 50 μg NH_4^+-N ml^{-1} at a range of pH values

pH of inflowing medium	pH of effluent	Steady-state nitrite concn (μg NO_2^--N ml^{-1})	SE
8.0	7.2	29.15	0.08
7.0	6.4	13.11	0.08
6.0	6.3	3.01	0.05
5.6	6.3	3.23	0.08
5.4	6.5	2.51	0.04
5.2	6.7	2.35	0.09
5.0	7.0	1.31	0.03

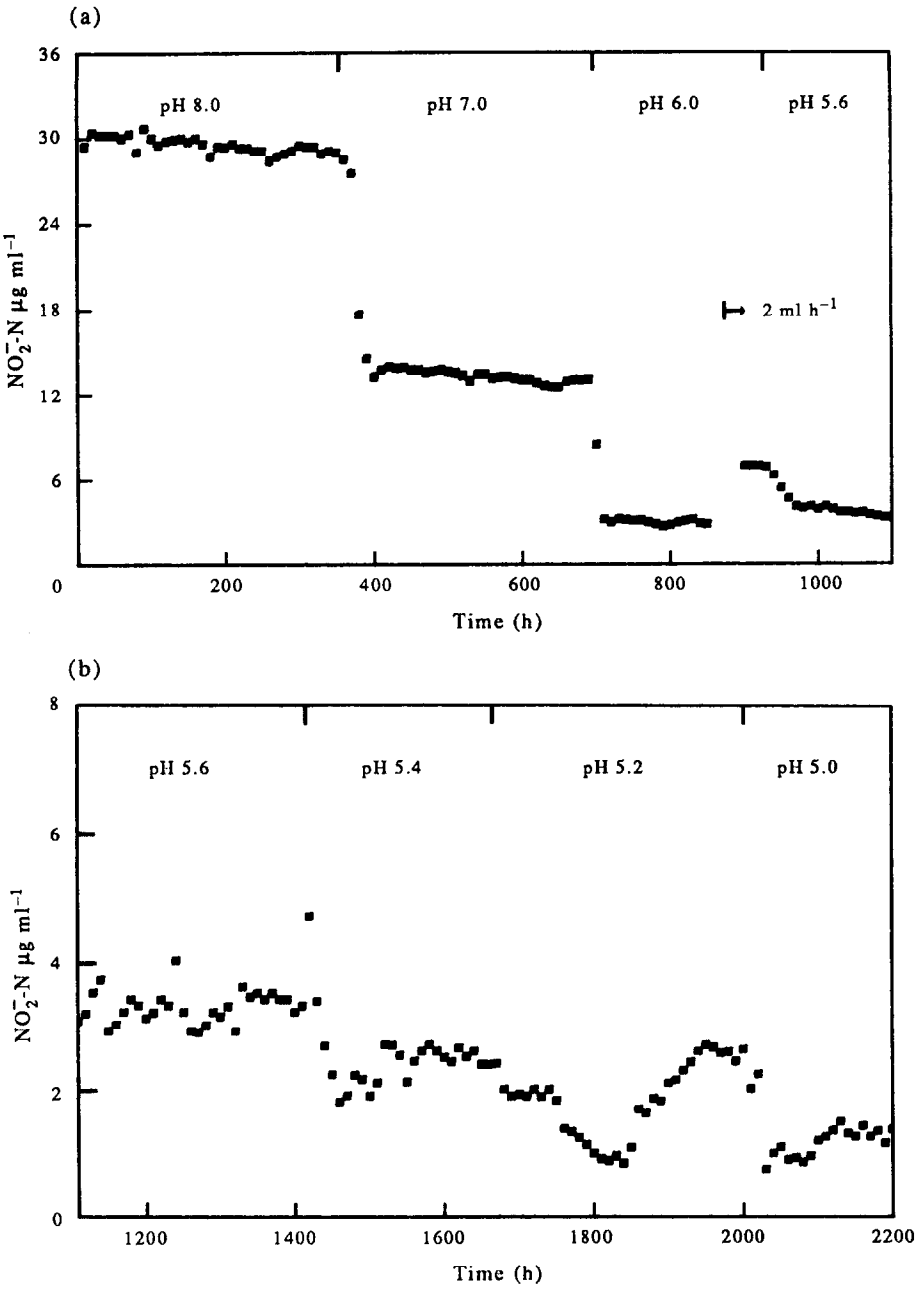

Fig. 2. Effect of pH on nitrite concentration in the effluent from a continuous flow vermiculite sand column inoculated with *N. europaea*. The pH of the inflowing medium is indicated along the upper axis.

values were measured. Initially ammonia oxidation dominated with negligible concentrations of nitrate. True steady states were not established during this experiment as the biofilm, and in particular the nitrifier population, increased continuously in size and species composition changed.

Table 3. Steady-state nitrite concentrations in effluent from a continuous-flow sand column inoculated with *N. europaea* provided with inorganic medium containing 500, 50 or 5 μg NH_4^+-N ml^{-1} at pH 6.5

Ammonia concn in inflowing medium (μg NH_4^+-N ml^{-1})	Steady state nitrite concn (μg NO_2^--N ml^{-1})	SE
50	4.46	0.07
500	4.50	0.03
50	4.18	0.05
5	3.42	0.03

At 600 h, development of a nitrite-oxidizing population was indicated by the appearance of NO_3^- and a decrease in NO_2^- concentration. By 1000 h (Fig. 3) NO_2^- concentrations were negligible and NO_2^- was not detected during the remainder of the experiment. Following a reduction in pH to 5.5 (1220 h), NO_3^- concentration decreased for 50 h but then increased to an apparent steady-state value higher than at pH 6. At 1495 h pH was reduced to 5.0, resulting in a 50%

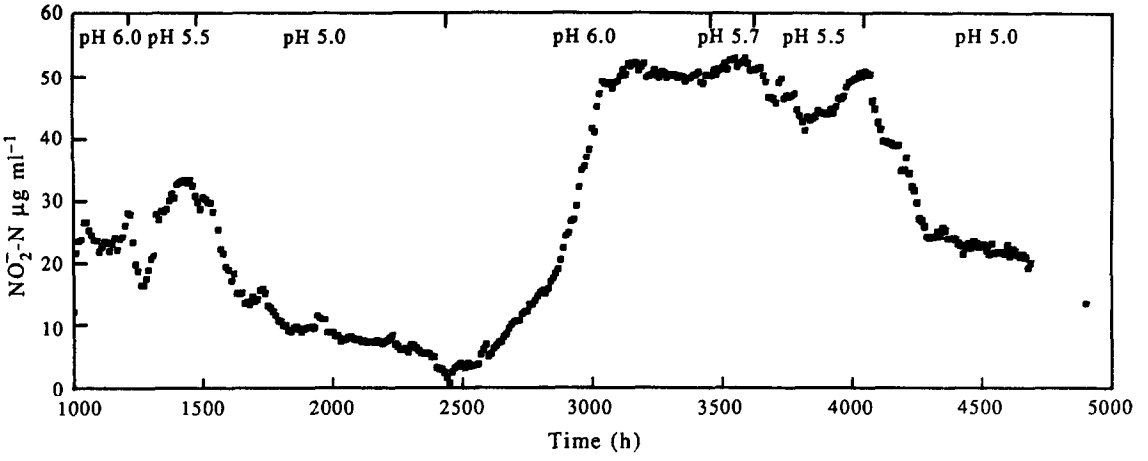

Fig. 3. Changes in nitrate concentration in effluent from a chemostat colonized by a nitrifying biofilm previously operated for 1000 h. The pH of the inflowing medium is indicated along the upper axis.

decrease in NO_3^- concentration during the following 200 h and a subsequent slower decline to negligible concentrations.

When the pH was returned to 6.0 conversion of ammonia was complete, indicating growth of the ammonia-oxidizer biofilm population after 1200 h and at pH values less than 6.0. Ammonia did not appear when pH was reduced to 5.7 (3470 h) and a slight increase at pH 5.5 (3600 h) was followed again by complete conversion. A return to a pH of 5.0 at 4050 h produced a 50% reduction in activity over 200 h followed by a more gradual reduction at a rate which suggested eventual washout of NO_3^- at this dilution rate. Nevertheless, nitrification occurred at a significant rate at this pH for 40 h, producing 0.14 μg NO_3^- h^{-1}.

The requirement for CO_2 was tested by adjusting the pH of the medium using 0.05% (w/v) NaOH rather than Na_2CO_3. A pH of 6 was maintained until total ammonia conversion occurred, when pH was reduced to 5.5. Nitrate concentration then decreased reaching a minimum concentration of 3.5 μg NO_3^- ml^{-1} after 500 h (Fig. 4). During the following 450 h NO_3^- concentration increased to 20 μg NO_3^--N ml^{-1}, demonstrating the potential for growth of nitrifiers at low pH in a mixed community containing heterotrophic bacteria in the absence of high concentrations of supplied CO_2.

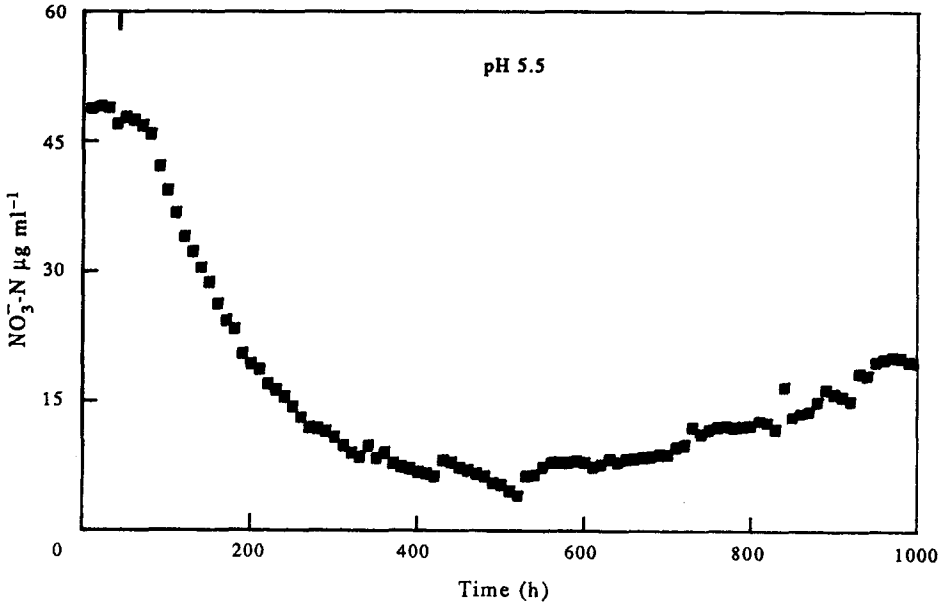

Fig. 4. Changes in nitrate concentration in effluent from a chemostat colonized by a nitrifying biofilm previously operated for 5000 h. The pH of the medium was adjusted using NaOH and was reduced from 6.0 to 5.5 at time = 0.

DISCUSSION

Our studies provide further evidence for the activity of autotrophic nitrifying bacteria in acid soils. The laboratory strain of *N. europaea* has a pH minimum for growth in liquid culture of 7 but was active in both sand and vermiculite continuous flow columns at pH values significantly lower than this. Significant activity was seen in the sand column at pH 6. Activity decreased at pH 5 but even this decrease occurred gradually, suggesting either a gradual decline in cellular ammonia-oxidizing activity or a decrease in the number of microenvironments of higher pH. These differences may merely highlight the distinction between growth and activity of ammonia-oxidizing bacteria. In liquid-batch culture, energy from ammonia oxidation will result in measurable growth. At low pH values, energy obtained may be sufficient for maintenance of cells only, and growth may not be possible.

The existence of stable steady states in NO_2^- concentration in continuous flow columns indicates either establishment of maximum 'saturating' populations, possibly limited by available space for attachment, or a balance between growth and washout of cells. Keen and Prosser (1987a) found similar steady states in an air-lift column fermenter in which *Nitrobacter* cells grow on ion exchange resin beads. Prolonged operation of the fermenter, however, indicated these to be 'quasi-' or short-term steady states, similar to those found in the chemostat culture, and nitrate productivity increased with time due to slow, long-term colonization. This process may also have occurred in the continuous-flow columns, and steady states may have been short term. Nevertheless, the variation in NO_2^- productivity of the sand column with pH indicates a shifting of the pH profile by 1.3 units.

Armstrong and Prosser (1988) provide evidence for a mechanism for ammonia oxidation by cells attached to clay particles at low pH involving exchange of NH_4^+ and H^+ and consequent local buffering at the clay surface. Activity will therefore not be directly related to the pH of the medium, as for suspended cells. One effect of pH on suspended cells is ammonia limitation due to increased ionization of NH_3, the substrate for ammonia monooxygenase (Suzuki *et al.*, 1974), leading effectively to ammonia limitation. This was tested in the continuous flow sand column at pH 6.5 by increasing and decreasing the supplied concentration of NH_4^+ by a factor of 10. The increase did not affect steady NO_2^- concentration indicating that, at this pH, activity was not limited by ammonia concentration. This may imply that the cells do not rely on passive diffusion of NH_3 for substrate uptake and that active transport of NH_4^+ is involved, although further experimentation would be required for confirmation. The 23% reduction in steady-state NO_2^- concentration following the reduction in supply concentration may be explained on the basis of the K_s value for ammonia. This was determined by Keen and Prosser (1987b) to be 3.65 μg NH_4^+-N ml^{-1} at pH 8. Precise calculation of the effluent NO_2^- concentration expected from columns supplied with low ammonia concentration is not possible as ammonia concentration will vary throughout the column and the K_s value will presumably be different at pH 6.5.

Although ammonia oxidation occurred at low pH in vermiculite columns, analysis of data is complicated by the ability of the mineral to buffer the growth medium and consequent establishment of gradients in pH throughout the column. Thus, activity when supplied with medium of pH 5 is likely to result from areas of neutral pH near the base of the column. The greater variation in NO_2^- concentrations, and difficulties in establishing steady states may have resulted from movement of populations within the column to more favourable regions.

The biofilm formed on the glass walls of the chemostat vessel provided significant ammonia-oxidizing activity at a pH of 5.5. This further reduction in pH minimum may be due to the presence of different strains of ammonia oxidizers, although no pure cultures were obtained from this soil and enrichment was possible at pH 8 but not pH 6. More likely, the presence of other organisms in the biofilm may have provided protection. In particular the presence of NO_2^- oxidizers will have reduced NO_2^- toxicity and heterotrophs may have reduced CO_2 limitation.

In conclusion, our results demonstrate the ability of surface-attached ammonia oxidizers to maintain activity at pH values significantly lower than that possible by suspended cells and provide further evidence for the potential for autotrophic ammonia oxidation in acid soils.

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