# High nitrification rate at low pH in a fluidized bed reactor with chalk as the biofilm carrier

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**Abstract** A typical steady state bulk pH of about 5 was established in a nitrifying fluidized bed with chalk as the only buffer agent. In spite of the low pH, high rate nitrification was observed with the nitrification kinetic parameters in the chalk reactor similar to those of biological reactors operating at pH>7. Various methods were used to determine the reasons for high rate nitrification at such low pH including (i) determination of bacterial species, (ii) microsensor measurements in the biofilm, and (iii) comparison of nitrification performance at low pH with a non-chalk fluidized bed reactor. Fluorescence in situ hybridization (FISH) using existing 16S rRNA-targeted oligonucleotide probes showed common nitrifying bacteria in the low pH chalk reactor. The prevalent nitrifying bacteria were identified in the *Nitrosomonas oligotropha, Nitrosomonas europeae/eutropha, Nitrosospira* and *Nitrospira* related groups, all well known nitrifiers. Microelectrode measurements showed that the pH in the biofilm was low and similar to that of the bulk pH. Finally, reactor performance using a non-chalk biofilm carrier (sintered glass) with the same bacterial inoculum also showed high rate nitrification below pH 5. The results suggest that inhibition of nitrification at low pH is highly overestimated.

**Keywords** Chalk dissolution; FISH; low pH; microsensors; nitrifying bacteria

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## Introduction

It is generally accepted that most strains of nitrifying bacteria are pH sensitive, with optimal growth in the narrow range of pH 7 to 8. Below values of pH 6.5, nitrification has been shown to be either severely inhibited or does not occur at all. Very low rate nitrification has been observed in acid soils and is usually attributed to the availability of other sources of non-ionic ammonia like urea or the proposed presence of alkaline micro-sites where favorable pH exists (Burton and Prosser, 2001; De Boer and Kowalchuk, 2001). In addition, nitrification activity at low pH was always seen in biofilms or aggregates (de Boer *et al*., 1991; Allison and Prosser, 1993).

During the last few years a nitrification fluidized bed reactor with chalk as the biofilm carrier and the only buffer agent was investigated at the Technion, Israel (Green *et al*., 2001; Green *et al*., 2002). The results showed high nitrification rate at a typical low bulk pH of 4 to 5. This paper investigates the possible reasons for the chalk reactor's characteristic high nitrification rate at low pH. Here, we present (i) results of fluorescence in situ hybridization (FISH) for identification of the major nitrifying groups, (ii) microelectrode measurements in the nitrifying biofilm for determination of a possible favorable microenvironment, and (iii) results of operation of a nitrifying biofilm reactor at low pH without chalk.

## Material and methods

### Experimental systems

*Chalk reactors*. The experimental systems consisted of a double column laboratory fluidized bed reactor. A one metre plexiglass column 90 mm in diameter with a total

volume of 6 litres containing 2,500 grams of screened chalk particles was used as the nitrifying column. A second column for aeration was made of a one metre plexiglass tube 50 mm in diameter and connected to the nitrifying column just below the overflow. Air or oxygen was bubbled at a constant flowrate through the second column and the oxygen saturated solution recirculated back to the first column. The influent flowrate was 36 ml·min$^{-1}$. The reactor operated at a high recirculation ratio that provided completely mixed conditions. The upflow velocity in the column was about 40 m·hr$^{-1}$ and the bed expansion was 60%. All the experiments were carried out at 25°C. Chalk was used in the low pH fluidized bed reactor for the dual purpose of biomass carrier and as the buffering agent. The reactor started from an inoculum originating from a similarly designed control nitrification reactor where chalk particles were used only as a biofilm carrier and sufficient alkalinity was added to maintain pH>7. An industrially produced chalk (United Lime Co., Haifa, Israel) was used as raw material for all the experiments and consisted of about 80% calcium carbonate, and a smaller fraction of about 20% silicon dioxide. The chalk was washed and screened (0.5 mm to 1 mm) to maintain a constant particle size and added daily to the reactor in quantities corresponding to the daily dissolution rate.

*Non-chalk reactor*. A similar double column fluidized bed reactor system was used for experiments using a non-chalk carrier. Sintered glass (Schott, Germany) was chosen as the inert biofilm carrier and had a particle size of 1–2 mm, with a pore diameter of 60 to 300 microns. Reactor start-up was at pH >7 and bacterial inoculum from the control chalk reactor (also operated at pH>7) was used.

*Reactor feeding solution*. Tap water enriched with ammonium and phosphate was used in all experiments to make up the feeding solution for the reactors: $NH_4^+$-N 100 to 250 mg·l$^{-1}$; 0.5 to 2 mg·l$^{-1}$ $H_2PO_4^{-1}$-P; pH of 7.5 to 7.6; $Ca^{+2}$ – 110 to 150 mg·l$^{-1}$ as $CaCO_3$; alkalinity 100–130 mg·l$^{-1}$ as $CaCO_3$. No additional alkalinity was added to the feeding solution of the chalk reactor operating at low pH. Alkalinity in excess of the stoichiometric requirement for full nitrification was added to the control chalk reactor feed water and during start-up of the sintered glass reactor to maintain reactor pH>7. When the sintered glass reactor operated at low pH, alkalinity was added to the influent at 10% less than the stoichiometric requirement.

*Chemical analysis*. Nitrate, nitrite, and phosphate concentrations were determined using a Metrohm 761 ion chromatograph equipped with a Metrosep Dual 1 anion separating column and suppressor using a carbonate/bicarbonate eluent. Ammonium and calcium concentrations were determined using a second Metrohm 761 ion chromatograph equipped with a Metrosep C2 cation separating column using a dipicolonic acid eluent. pH was measured using a daily calibrated portable Eutech pH meter equipped with a standard combined electrode and temperature probe. Alkalinity was measured using the Gran titration procedure (Gran, 1952).

*FISH analysis*. Sample suspensions were prepared by removing chalk particles from the low pH chalk reactor and control chalk reactor (pH 7) and gently shaking them in reactor water to detach nitrifying biofilm. The suspension was then fixed in fresh 4% paraformaldehyde, washed twice with PBS and immobilized on gelatin covered slides. Hybridization was performed according to Manz *et al*. (1992), with a hierarchical set of fluorescently labeled oligonucleotide probes targeting 16S rRNA (Gieseke *et al*., 2001). Samples were analyzed by standard epifluorescence microscopy on a Zeiss Axioplan II microscope and by confocal scanning electron microscopy on a Zeiss LSM 510 microscope.

*Microsensor measurements*. Microsensor measurements were performed on virgin and biofilm covered chalk particles freshly removed from the nitrifying reactor at low pH. The particles were transferred to a 100 × 50 × 50 mm flow cell coupled to the recirculation of the reactor. The flow was about 1 cm $s^{-1}$ and laminarized by a sieve insert at the inlet of the flow cell (Gieseke *et al*., 2003). Concentration microprofiles in the biofilm were measured for oxygen with Clark-type microsensors (Revsbech, 1989) with tip diameter of 10 microns. For pH concentration microprofiles, potentiometric glass microelectrodes with a tip diameter of 10 microns and the pH sensitive glass at the tip not longer than 50 microns were used (Revsbech *et al*., 1983). Multiple microprofiles were taken at different positions on each particle. Individual profiles were taken at 25 micron increments.



## Results and discussion

### Chalk reactor performance

The consumption of alkalinity during nitrification together with the shortage in alkalinity in the influent resulted in a drop in the bulk pH of the chalk reactor. The low pH allowed for chalk dissolution which otherwise would not have occurred. The influent water alkalinity (~100 mg/l) was sufficient only to nitrify about 14 mg·$l^{-1}$ $NH_4$-N while nitrification in the chalk reactor was between 150–250 mg·$l^{-1}$ N. The additional alkalinity required was supplied by chalk dissolution. Chalk dissolution in the reactor always followed the stoichiometric ratio of one mole of $CaCO_3$ dissolved for each mole of $NH_4^+$ oxidized and the nitrite concentration was always close to zero. A comparison between reactor performance using different chalk types (similar particle surface area but different dissolution rates) showed that the typical steady state bulk pH was around 5 with fluctuations between 4.4 to 6.6, depending on the type of chalk used and on the frequency of chalk addition (Green *et al*., 2001). Nitrification rate was found to be limited by $CO_2$ concentration with much higher volumetric rates observed in reactors with lower $CO_2$ degassing (Green *et al*., 2002).

In spite of the low pH, kinetic parameters for nitrification in the chalk reactor were similar to typical values in biological reactors operating at pH>7 (Table 1). An average cell yield of 0.1 ± 0.01 gram cells per gram N removed was measured in the low pH chalk reactor. A similar yield coefficient was measured during the start-up period, when bicarbonate buffer was added and the pH in the reactor was above 7.

In the chalk reactor, much lower yield coefficients were expected due to the negligible concentration of free ammonia (the energy source for ammonia oxidizers) at the typical low pH values observed. Oxidation of ammonia under these conditions probably depends on energy consuming mechanisms, like active transport of ammonium. This should result in much less energy available for cell anabolism, and usually almost no growth is found below pH 7 (Allison and Prosser, 1993). The results from the chalk reactor indicated that the energy fraction allocated by the cells for growth purposes did not decrease at low bulk pH values.

### Nitrification at low pH

The major reasons for inhibition of nitrification at low pH have been suggested to be: 1) changes in the spatial structure of proteins and other cell macromolecules inactivating main

**Table 1** Chalk reactor operation and kinetic parameters

| Aeration | D.O. conc. mg·$l^{-1}$ | $CO_2$ conc. mmol·$l^{-1}$ | pH | Vol. oxid. rate g N·$l^{-1}$·$d^{-1}$ | Specific oxid. rate g N·g prot$^{-1}$·$d^{-1}$ | Yield coeff. g cell·g N oxid.$^{-1}$ |
|---|---|---|---|---|---|---|
| Air | >2 | 0.019 ± 0.008 | 4.7–6.6 | 1–1.4 | 1.9–3.5 | 0.1 |
| Pure oxygen | >5 | 0.26 ± 0.05 | 4.4–5.6 | 1.3–2.7 | 1.25–2.6 | 0.1 |

metabolic pathways, and 2) low $NH_3$ concentrations (the substrate for the primary enzyme ammonia mono-oxygenase) under acidic conditions. Other possible reasons like high concentrations of $HNO_2$, $CO_2$ limitations and toxicity of heavy metals were eliminated in this research project.

The two most likely explanations for the high nitrification rate in the low pH chalk reactor are:

1. Bacterial species different from the more common nitrifiers (acid-tolerant bacteria).
2. Favorable pH microconditions surrounding the nitrifying biomass attached to the chalk due to chalk dissolution.

To test the possible explanations for the characteristic high nitrification rate in the chalk reactor at low pH, the following subjects were investigated: identification of the major bacterial groups, determination of a possible favorable microenvironment using microelectrodes, and the performance at low pH of a non-chalk biofilm reactor.

*Identification of the major bacterial groups*. Using existing fluorescent 16s rRNA targeted probes, identification of the dominant nitrifying bacteria was carried out for the chalk reactor (pH~5) and the results were compared to those of a control chalk reactor operating at pH 7. The two reactors operated under these pH conditions for more than two years and were both originally inoculated from the same nitrifying chemostat operating at pH>7. In both neutral and low pH reactors, the prevalent ammonia oxidizing bacteria were found to be affiliated with the monophyletic groups *Nitrosomonas oligotropha*, *Nitrosomonas europeae/eutropha,* and *Nitrosospira*. The nitrite oxidizing bacteria were affiliated to the *Nitrospira* group. These well known groups of nitrifiers are not considered acid-tolerant bacteria, although *Nitrosospira* has been isolated from acid soils and *Nitrosomonas oligotropha* does show very low $K_s$ values of 1.9 to 4.2 μM $NH_3$ (Koops and Pommerening-Röser, 2001). *In situ* activity of the individual groups is not possible to measure, but the presence of a heterogeneous population gives an indication that the ability to nitrify at low pH is not restricted to one monophyletic group.

*Favorable pH micro-conditions surrounding the nitrifying biomass attached to the chalk due to chalk dissolution*. The most natural explanation for the higher performance of the chalk reactor is the presence of favorable pH micro-conditions for bacterial growth on the dissolving chalk particles. Indeed, Kowalski and Lewandowski (1984) speculated that such conditions exist when reporting on a nitrifying fixed bed reactor operating at low pH using marble chips. Theories of pH-neutral micro-sites have also been advanced to explain autotrophic nitrification in acid soils (De Boer and Kowalchuk, 2001). Diffusion controlled dissolution of $CaCO_3$ does offer an increasing pH gradient from the bulk solution to the dissolving surface that may provide some protection for the bacteria. The effect was demonstrated in this study on virgin chalk particles dissolving in bulk reactor water at low pH using microelectrode measurements (Figure 1). However, since the biofilm is the source of protons for $CaCO_3$ dissolution in the nitrifying reactor, the pH in (at least part of) the biofilm must be lower than that of the surrounding bulk solution.

Modeling a nitrifying biofilm on a $CaCO_3$ carrier using literature diffusion constants gave only a slightly increasing pH gradient approaching the carrier surface (results not shown). Typical microsensor pH profiles on 100 micron thick biofilm covered chalk particles showed a more typical decline in pH (Figure 1) for nitrifying biofilms or, in the case of microprofiles on thinner biofilm, a pH close to bulk was found. In spite of the low pH, the biofilm was surprisingly active as shown by the sharp decline in oxygen concentration in the microsensor profile (Figure 2).

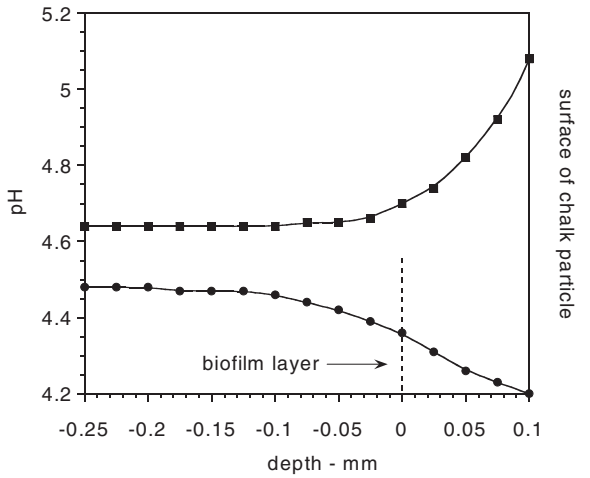


**Figure 1** Microsensor pH profiles of virgin chalk (■) and biofilm covered chalk particles (●)

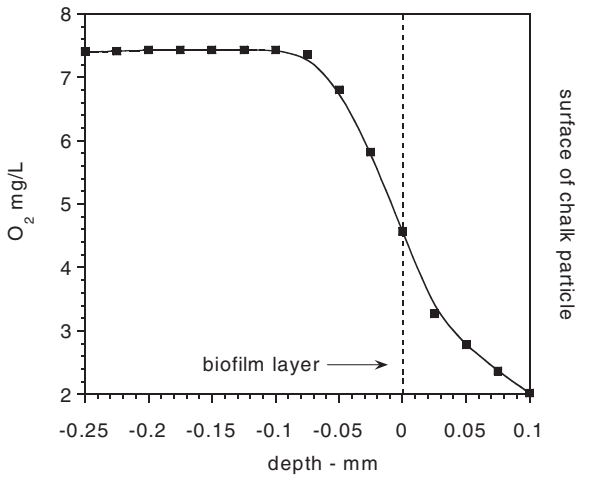


**Figure 2** Microsensor oxygen profile of biofilm covered chalk particle

*Performance of a non-chalk biofilm reactor at low pH*. Establishing that the biofilm in the chalk reactor contains common nitrifying bacteria and is highly active at low pH, the performance of a non-chalk, sintered glass biofilm reactor was tested at similar low pH for comparison purposes. Here, $NaHCO_3$ buffer in the influent rather than $CaCO_3$ dissolution in the low pH chalk reactor would supply alkalinity. During the start-up period (pH>7; Figure 3), the reactor received excess alkalinity and reached a relatively high nitrification rate similar to that of the chalk reactor operating at low pH (2.3 g N/litre reactor/day; Figure 4). Subsequently, the feeding solution was changed to ensure that the bulk pH of the reactor would decrease. The ammonium load of the reactor was arbitrarily reduced to 20% and instead of the excess alkalinity concentrations prevailing during start-up, an insufficient quantity of alkalinity (90–95% of the alkalinity requirement) was added to neutralize the protons released by complete nitrification of the ammonium concentration in the influent. The lack of alkalinity in the influent solution together with the lower ammonium load produced the desired effect of a significant decrease in reactor pH. As shown in Figure 3, after the first day of operation the pH dropped to pH 5, but the bacteria remained active albeit at a much lower rate (Figure 4). The next day the pH further declined signaling a slight increase in nitrification activity and warranting an increase in the ammonium load. In order to deter-

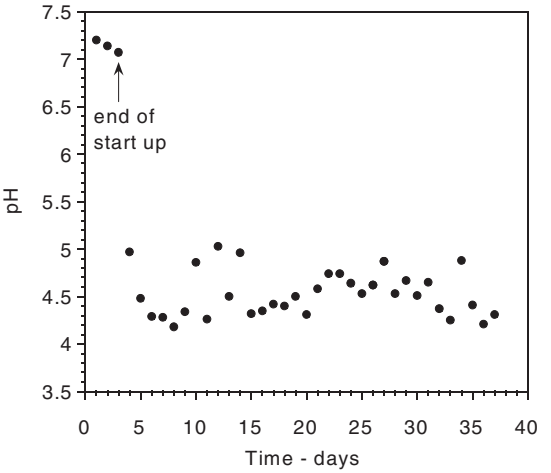


**Figure 3** Sintered glass reactor pH

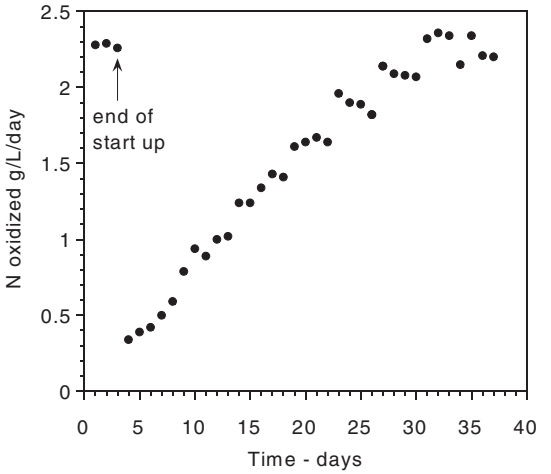


**Figure 4** Sintered glass reactor nitrification performance at low pH

mine how much the reactor could recover nitrification capacity at low pH, the ammonium and corresponding insufficient alkalinity load was steadily increased by 10 to 20% per day as long as the pH in the reactor remained below pH 5 (Figure 3). Maintaining a low pH of 4.5, the full former pH 7 nitrification capacity was attained after 3 weeks (Figure 4). This demonstrates that in spite of the extremely low bulk $NH_3$ concentrations (about 0.01 μM) and low pH conditions, the nitrifying bacteria acclimatized relatively quickly and operated at high volumetric rates providing that near stoichiometric amounts of alkalinity were added to the influent solution.

## Conclusions

A typical steady state bulk pH of about 5 was established in a nitrifying fluidized bed with chalk as the only buffer agent. In spite of the low pH, high rate nitrification was observed with the nitrification kinetic parameters in the chalk reactor similar to those of biological reactors operating at pH>7. The prevalent nitrifying bacteria were identified as *Nitrosomonas oligotropha*, *Nitrosomonas europeae/eutropha, Nitrosospira,* and *Nitrospira* related bacteria, all well known nitrifiers. Results from microsensor measurements demonstrated an active biofilm with very low pH close to bulk values. In addition, it

was shown that in a non-chalk carrier reactor nitrification reached the same high performance at low pH as the chalk based carrier reactor providing that alkalinity was supplied in the influent. These results indicate that the major mechanism responsible for high performance in the chalk reactor at low pH was due to the large neutralizing capacity of the chalk carrier. The chalk dissolves on a stoichiometric basis controlled by the nitrification rate but does not provide favorable microconditions. The results presented in the paper suggest that inhibition of nitrification at low pH is highly overestimated.



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