Changes in ammonia oxidiser population during transition to low pH in a biofilm reactor starting with *Nitrosomonas europaea*

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Abstract Recent experiments in our laboratory using both biofilm and suspended biomass reactors have demonstrated high rate nitrification at low pH with known autotrophic nitrifying bacteria originating from wastewater treatment plants refuting previous assumptions that nitrification is significantly inhibited at low pH. Since much of the earlier microbiological work regarding ammonia oxidising bacteria (AOB) physiology was carried out using *Nitrosomonas europaea*, this model bacterium's capability for high rate nitrification at low pH in a continuous biofilm reactor was tested. A biofilm reactor filled with sintered glass particles was inoculated with a pure culture of *N. europaea*. The reactor was first operated to high nitrification rates under conditions favourable to *N. europaea* (pH $>$ 7; high ammonium concentrations). To eliminate inhibitory concentrations of nitrite at low pH, an enriched culture of *Nitrospira* (a nitrite oxidising bacterium) was then added. The transition from neutral to acidic conditions was attempted by sharply lowering the nitrification rate and by using a feeding solution containing insufficient buffer for complete nitrification. As opposed to other successful transitions, the pH in the *N. europaea/Nitrospira* reactor initially dropped only slightly and maintained pH $>$ 6 for over two weeks. The reactor reached pH 4.5 only after four weeks. FISH results showed that while the percent of AOB and *Nitrospira* to eubacteria remained relatively constant at 51.1 $\pm$ 8.2% and 40.8 $\pm$ 6.4%, respectively, the AOB community changed completely in 60 days from 100% *N. europaea* to 100% *Nitrosomonas oligotropha*. Even though *N. oligotropha* was not intentionally introduced into the reactor, it is apparently much better adapted to conditions of low pH.

Keywords Biofilm reactor; FISH; low pH; microbial populations; nitrification

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Introduction

Biological nitrification, the oxidation of ammonia to nitrite and nitrate, is a key microbial process in the global nitrogen cycle and is also a crucial stage in removing nitrogen compounds from wastewater to minimise eutrophication in receiving water bodies. Biological nitrification is known to be inhibited by low pH. Growth of pure cultures of autotrophic ammonia oxidising bacteria (AOB) in liquid culture is optimal within the pH range of 7.0–8.5 and typically does not occur below pH 6.5 (Burton and Prosser, 2001). The high sensitivity of nitrification to acidic environments has been attributed mainly to the exponential decrease in free ammonia (NH_3) with decreasing pH ($NH_3 + H^+ \leftrightarrow NH_4^+; pK_a = 9.25$) (De Boer and Kowalchuk, 2001). Free ammonia is considered to be the substrate for the primary enzyme, ammonia mono oxygenase (AMO) (Suzuki *et al.*, 1974) and the transport of free ammonia into the cells, unlike ammonium ions, is by passive diffusion.

In contrast, recent experiments in our laboratory using both attached (biofilm) and suspended biomass reactors have demonstrated high rate nitrification at low pH with autotrophic nitrifying bacteria originating from municipal wastewater treatment plants refuting previous assumptions that nitrification is significantly inhibited at low pH (Tarre and Green, 2004). In addition, we have shown under acidic conditions (pH $\approx$ 4) with microsensor measurements profiling pH and oxygen concentrations an active biofilm on

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both alkaline and inert carriers with a pH similar to that of the bulk pH and even lower (Tarre *et al.*, 2004; Gieseke *et al.*, 2006). These findings exclude the possibility of a favourable pH microenvironment increasing the availability of NH_3. Ammonia oxidising bacteria (AOB) identified using existing fluorescently labelled oligonucleotide probes were found to be from the monophyletic genus *Nitrosomonas* and *Nitrosospira* suggesting that autotrophic nitrification at low pH is more widespread than previously thought.

Since much of the earlier microbiological work regarding AOB physiology and pH inhibition was carried out using cultures of *Nitrosomonas europaea*, we wanted to test the capability of this model bacterium to do high rate nitrification at low pH in a continuous fluidised bed (FB) reactor.

Materials and methods

Biofilm reactor

The biofilm reactor consisted of a double column fluidised bed made of transparent 40 mm diameter PVC tubes. The main nitrifying column was 110 cm long (1 L) and was filled with 350 g (700 mL) of 1–2 mm porous sintered glass particles used as the biomass carrier (Schott AG, Germany). The main column was interconnected to a 50 cm long aeration column. Pure oxygen was bubbled at a constant flow rate through the aeration column and the oxygen saturated solution recirculated via pump back to the main column. The reactor operated at a high recirculation ratio to provide completely mixed conditions. The upflow velocity in the column was between 50 and 60 m hr^{-1} and the bed expansion was 30%. All the experiments were carried out at 30°C. The reactor was operated with continuous feeding made from tap water spiked only with varying concentrations of ammonium chloride (200–1,500 mg L^{-1}), potassium phosphate mono basic (2–5 mg L^{-1}) and sodium bicarbonate buffer (600–3,000 mg L^{-1}).

FB reactor operation at low pH contained two parts:

- *Start-up period*. The inoculum of the continuous reactor was a pure culture of *Nitrosomonas europaea* (ATCC 19178 kindly supplied by Prof. Lisa Stein, University of California, Riverside) grown batch-wise in a 2 L flask (ATCC medium 221). The reactor was operated at conditions known to be favourable to *N. europaea*, i.e. pH > 7 and with bulk ammonium concentrations close to 250 mg $N{\cdot}L^{-1}$.

- *FB reactor transition to low pH*. To eliminate inhibitory concentrations of nitrite at low pH, a large amount of an enriched culture of *Nitrospira*, a nitrite oxidising bacterium (grown in a similar separate reactor on sintered glass particles using only nitrite as substrate at a low pH of 4.5) was first added to the reactor. The combined *N. europaea/Nitrospira* reactor was then put into transition to low pH conditions by lowering the nitrogen load and the alkalinity relative to the ammonia concentration.

Chemical analysis

Daily samples from the reactors were analysed for ammonium, nitrate, nitrite and phosphate concentrations. Nitrate, nitrite and phosphate were determined using a Metrohm 761 ion chromatograph (IC) equipped with a Metrosep Dual 1 anion separating column and suppressor using a CO_3^{2-}/HCO_3^- eluent. Ammonium was determined using a second IC equipped with a Metrosep C2 cation separating column using a dipicolonic acid eluent. pH measurements were taken 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. Biomass content was measured weekly using the volatile suspended solids method.

Analysis of nitrifying population in the biofilm and suspended reactors

Biofilm samples from the reactor throughout the experimental period were analysed for AOB and nitrite oxidising bacteria (NOB) populations using fluorescent *in situ* hybridisation (FISH). The samples were tested using Cy3 and Cy5 fluorescently labelled oligonucleotide probes targeting 16S rRNA for eubacteria (EUB338), betaproteobacteria (Bet42a), AOB from the *N. europaea/eutropha* (Nse1472), *Nitrosococcus mobilis* (NmV), *N. oligotropha* (Nmo218), *Nitrosospira* (Nsv443) groups and the nitrite oxidiser, *Nitrospira* (Ntspa662) (Loy *et al.*, 2003). Sintered glass particles were first placed in a vortex for 2 minutes. The biofilm that sloughed off from the carrier was separated and fixed in 4% paraformaldehyde for 2 hours at 4°C. The sample was subsequently washed three times in PBS and a thin layer was immobilised and dehydrated on a gelatin-coated slide. Hybridisation was performed as described previously (Manz *et al.*, 1992). To determine the percentage of AOB and NOB groups relative to eubacteria or betaproteobacteria, the relative areas occupied by each group in a micrograph were derived using the image analysis software, ImageJ (Briones *et al.*, 2002). The red (Cy3) and blue (Cy5) images were first changed to grey scale and the highest background value of fluorescence intensity in each image was determined manually. This was set as the minimum threshold value to eliminate blank spaces and background fluorescence. The grey scale image was then converted to black and white pixels to determine the area of each target group. Ten different areas on each slide were analysed and the cumulative area was converted into relative percentages of each population.

Results and discussion

At the end of the startup period, a high nitrification rate of over 5 g N oxidized·L reactor^{-1}·d^{-1} (Figure 1) at pH 7 (Figure 2) was achieved. The transition attempt from neutral to acidic conditions was carried out by sharply reducing the ammonium load to the reactor, lowering the nitrification rate to approximately 0.9 g N oxidized·L reactor^{-1}·d^{-1} and by changing the feeding solution to contain insufficient buffer for complete nitrification of the influent ammonium concentration. The ammonium concentration in the effluent was between 10 and 15 mg·L^{-1} N, in contrast to the much higher ammonium concentration used in reactor startup. Also, unlike the startup phase where ammonium was converted only to nitrite, nitrite concentrations were negligible due to the

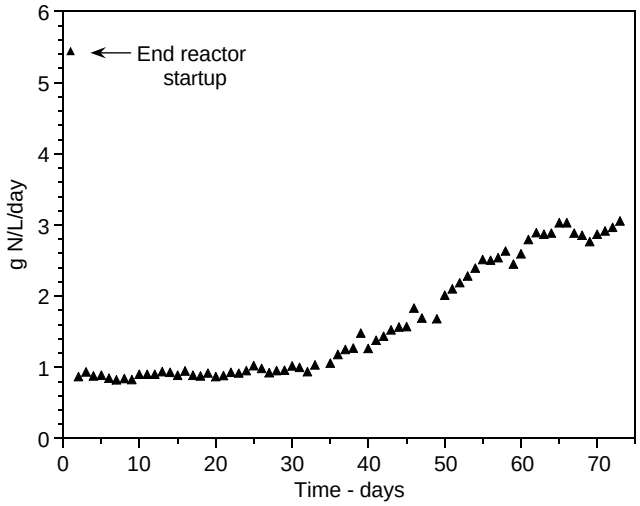

Figure 1 Nitrification rate in biofilm reactor during transition and operation at low pH

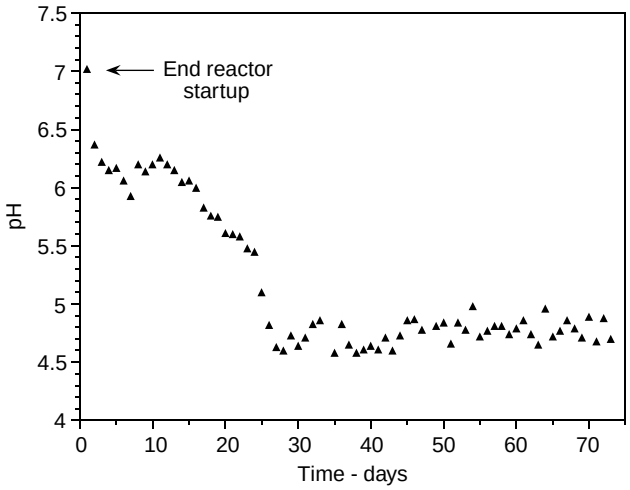

Figure 2 Bulk pH in biofilm reactor during transition and operation at low pH

addition of NOB (*Nitrospira*). As opposed to other successful transitions carried out in our laboratory (Tarre and Green, 2004), the pH in the *N. europaea/Nitrospira* reactor dropped only slightly from 7.0 to 6.4 (Figure 2) on the first day and maintained pH $>$ 6 for over two weeks. The reactor ammonium load was maintained at approximately 0.9 g N oxidised·L reactor^{-1}·d^{-1} for 4 weeks until all of the influent alkalinity was finally destroyed (Figure 3) and the pH reached 4.5. The ammonium loading rate of the reactor was then steadily increased over a period of 5 weeks to over 3 g N oxidised·L reactor^{-1}·d^{-1} (Figure 1) while maintaining low pH and close to zero alkalinity (Figure 3) indicating that high rate nitrification at low pH was achieved.

As expected, FISH results at the end of the startup period showed highly enriched cultures of *N. europaea* and *Nitrospira* (greater than 95% of eubacteria). In addition, batch tests on the enriched cultures showed no production of nitrate for particles covered with *N. europaea*, while *Nitrospira*-covered particles were unable to oxidise ammonium. After introducing *Nitrospira* into the reactor containing *N. europaea*, the initial ratio was equal (each about 47% of eubacteria). FISH results showed that during reactor transition to low pH, the ratio of AOB and NOB to eubacteria remained relatively unchanged at

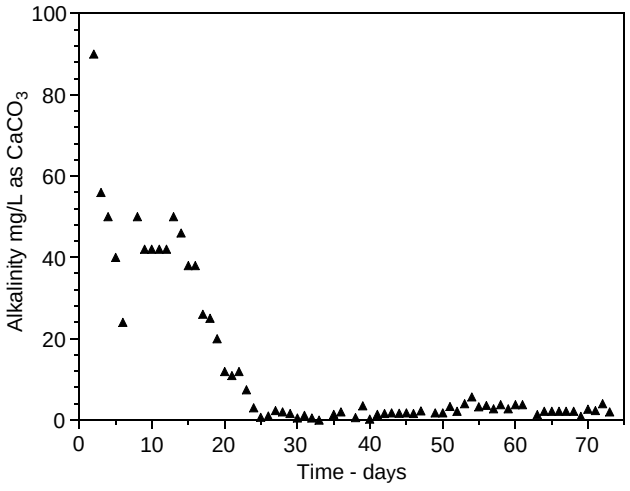

Figure 3 Alkalinity in biofilm reactor during transition and operation at low pH

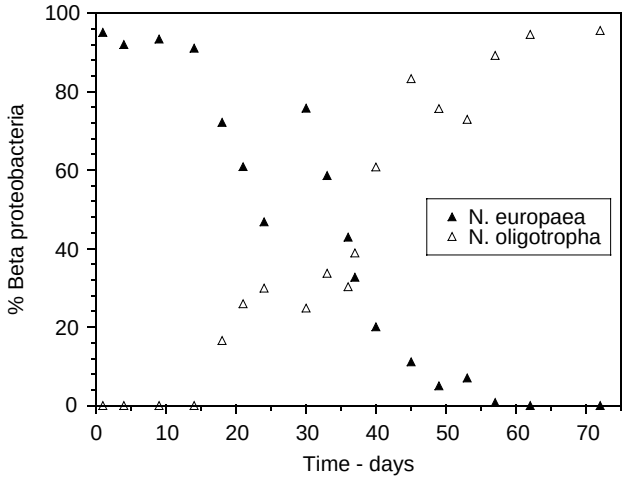

Figure 4 Change in ammonia oxidising bacteria population from *N. europaea* to *N. oligotropha* during transition and operation at low pH in biofilm reactor

51.1 ± 8.2% and 40.8 ± 6.4%, respectively. While the species of NOB population did not change (*Nitrospira*), the microbial community of AOB changed significantly throughout the transition period to low pH from 100% *N. europaea* at the beginning to 100% *N. oligotropha* at the end (Figure 4). The changes in AOB were observed to begin after 15 days when the pH started to decrease below pH 6 and increased dramatically when the reactor operated below pH 5 (day 25).

The complete and total change in the reactor AOB population demonstrates that while *N. europaea* can be successfully established at high bulk ammonium concentrations at neutral pH, it does not compete well under conditions of low pH and low ammonium concentrations. *N. oligotropha* is apparently better able to adapt to low pH and low concentrations of ammonium than *N. europaea* and other species like *Nitrosospira* that were observed before at low pH (Tarre *et al.*, 2004; Gieseke *et al.*, 2006). Although the biofilm reactor was not run under strictly sterile conditions, this point is especially true considering that *N. oligotropha* was not knowingly introduced into the reactor and was not found positive by FISH in either the *N. europaea* or *Nitrospira* reactor before the transition to low pH commenced. The results suggest that the much stronger NH_3 substrate affinity (K_s) reported for *N. oligotropha* (2–4 μM as opposed to 30–60 μM for *N. europaea*) may be a significant characteristic in determining the nitrifying strains capable of operating at low pH (Koops and Pommerening-Roser, 2001).

Conclusions

In this paper the ability of an enriched culture of *N. europaea* to perform high rate nitrification at low pH in a biofilm reactor was tested. As opposed to other successful transitions to low pH carried out in our laboratory, *N. europaea* did not adapt well to conditions of decreasing alkalinity and could not force the bulk pH of the reactor to drop below pH 6. Only when the reactor became significantly contaminated with *N. oligotropha* did the reactor successfully achieve low pH with good nitrification rates. These results support earlier contentions that ecophysiological differences exist between the various AOB species allowing one species to out-compete another under different environmental conditions, even though the basic metabolism for ammonia oxidation is the same.

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