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INVESTIGATION OF NITRIFYING BACTERIAL ACTIVITIES DURING WASTEWATER TREATMENT USING ACTIVATED SLUDGE PROCESS

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ABSTRACT : *The activity of nitrite oxidizing bacteria (NOB) during activated sludge wastewater treatment was investigated by monitoring nitrite oxidation, nitrate formation and carbon dioxide fixation. Nitrite oxidation, nitrate removal and carbon dioxide (CO_2) fixation assays in batch experiments were performed using mixed liquor samples obtained from a complete-mix, bench-scale, activated sludge reactor. Nitrite oxidation resulted in a 1:1 ratio (nitrite removal to nitrate formation) with equal rates in the batch samples. This ratio suggests conversion of all consumed nitrite to nitrate based on the stoichiometry of the reaction of nitrite oxidation in which 1 mol of nitrite-N is converted to 1 mol of nitrate-N. The mean assimilated inorganic carbon was 20.3 µg/L and the mean carbon dioxide fixation rate was calculated as 4.06 µg/L-hr. The ratio of carbon incorporated to nitrogen oxidized was 1.42 x 10^{-3}. Additionally, it was demonstrated that there was a connection between nitrite removal, nitrate formation and CO_2 fixation. Also, it was shown that uptake of inorganic carbon can be exploited as a method by which to demonstrate the in-situ activity of nitrifying bacteria.*

KEYWORDS : *Nitrite oxidation, nitrate formation, carbon dioxide fixation, activated sludge, wastewater treatment.*

AKTİF ÇAMUR PROSESİYLE ATIKSU ARITIMI SIRASINDA NİTRİFİKASYON BAKTERİLERİNİN AKTİVİTESİNİN ARAŞTIRILMASI

ÖZET *: Aktif çamur yöntemiyle atık su arıtımı sırasında, nitrit oksitleyen bakterinin aktivitesi, nitrit oxidasyonu, nitrat formasyonu ve CO_2 fiksasyonu gözlenerek araştırılmıştır. Nitrit oksidasyonu, nitrat giderimi ve karbon dioksit fiksasyonu kesikli reaktör sisteminde ölçülmüştür. Deneylerde kullanılan biyokütle örnekleri sürekli, tam karışımlı, laboratuar ölçekli aktif çamur reaktöründen alınmıştır. Nitrit oksidasyonunun nitrat formasyonuna eşit olduğu ve 1:1 oranında nitrojen dönüşümünün gerçekleştiği sonucu bulunmuştur. Sonuçlar ortalama inorganik karbon asimilasyonunun 20.3 µg/L ve ortalama CO_2 fiksasyonu oranının 4.06 µg/L-hr saat olduğunu göstermiştir. Asimile edilen karbonun oksitlenen nitrojene oranı 1.42 x 10^{-3} olarak bulunmuştur. Ayrıca, nitrit oksidasyonu, nitrat formasyonu ve CO_2 fiksasyonu arasında bir bağlantı olduğu da bu çalışmayla belirtilmiştir. Buna ek olarak, nitrit oksitleyen bakterinin aktivitesini ölçme metodu olarak inorganik karbon alımının kullanabileceği de gösterilmiştir.*

ANAHTAR KELİMELER : *Nitrit oksidasyonu, nitrat formasyonu, CO_2 fiksasyonu, aktif çamur, atık su arıtımı.*

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I. INTRODUCTION

The biological elimination of nitrogen in treatment of wastewater generally results from the combined processes of nitrification and denitrification. Reducing sewage nitrogen levels is necessary since discharges containing nitrogen can be toxic to aquatic life, cause oxygen depletion and eutrophication in receiving waters, and affect chlorine disinfection efficiency [1]. The key process in nitrogen removal during wastewater treatment is through the two-step oxidation of ammonia (NH_4^+) to nitrate (NO_3^-) via microbial mediated nitrification. Biological oxidation of ammonia to nitrate occurs primarily through the coordination of two distinct chemolitotrophic groups of bacteria: ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB). These microorganisms use ammonia and nitrite as an energy source and fix carbon dioxide as a source of carbon for cell material [2]. No known single autotrophic microorganism performs both ammonia oxidation and nitrite oxidation. The slow growth rate of these bacteria and their sensitivity to environmental factors including pH, temperature and oxygen concentration influence the minimum solid retention time (SRT) required to establish stable nitrification during wastewater treatment [3].

The major source of organic carbon nitrifying bacteria is carbon dioxide (CO_2) that is fixed to glucose via the Calvin cycle. All the reactions of CO_2 use ATP and reducing power from NADPH generated during oxidation of inorganic nitrogen compounds. In this cycle CO_2 is converted into a set of more complex sugars (like glucose), which are then assembled into the macromolecules comprising proteins, carbohydrates, lipids, nucleic acids, and other cell components [4].

In the first step of nitrification, AOB obtain energy by oxidizing NH_4^+ to NO_2^- according to the following reaction [5]:

$$NH_4^+ + O_2 + H^+ + 2e^- \text{ ----------> } NH_2OH + H_2O$$

$$NH_2OH + H_2O^- \text{ ----------> } NO_2^- + 5\,H^+ + 4e^-$$

The second step of the nitrification reaction is the oxidation of NO_2^- to NO_3^- by NOB using the nitrite oxidoreductase enzyme in the presence of oxygen. Nitrite oxidation results in equal molar accumulation of nitrate (i.e. nitrite oxidation equals nitrate formation). The nitrite oxidation reactions are shown in the following equations:

$$NO_2^- + H_2O \text{ ----------> } NO_3^- + 2H^+ + 2e^-$$

$$\underline{2H^+ + 2e^- + 0.5O_2 \text{ ----------> } H_2O}$$

$$NO_2^- + 0.5\ O_2 \text{ ----------> } NO_3^-$$

The stoichiometric relationship for the oxidation of nitrite to nitrate indicates that 1 mol of nitrite-N is converted to 1 mol of nitrate-N. Thus, if efficient nitrite oxidation occurs, equal molar removal of nitrite and formation of nitrate would hold.

Energy is derived from the oxidation of nitrite to nitrate providing NADPH and ATP for CO_2 fixation via the Calvin cycle. Figure 1 illustrates the relationship between nitrite oxidation and CO_2 fixation. The major source of organic carbon for NOB is carbon dioxide which is fixed via the Calvin cycle. It was expected that CO_2 fixation would be proportional to both nitrite removal and nitrate formation because NOB use energy gained from nitrite oxidation to fix CO_2. It was proposed that nitrification can be estimated indirectly by measuring CO_2 uptake by NOB [6, 7]; the underlying assumption being that there is a constant stoichiometric ratio between the rate of substrate oxidation and the rate of CO_2 uptake for a given species of nitrifier. It was found that there was a constant relationship between nitrite oxidation and chemoautotrophic CO_2 fixation for organic carbon production [6]. The relationship between substrate oxidation and CO_2 fixation was studied by Glover who found that the stoichiometric relationship between nitrification and carbon yield is dependent on the availability of the inorganic nitrogen substrate [8]. There is only a limited amount of information in the literature dealing with the stoichiometry of carbon uptake and nitrite oxidation. Additionally, no previous study showed the

relationship between nitrite removal, nitrate formation and CO_2 fixation in activated sludge reactors.

In contrast to textbook knowledge, *Nitrospira*-like bacteria, not *Nitrobacter* spp., are the dominant nitrite oxidizers both in most full-scale wastewater treatment plants and in laboratory scale reactors. Since many wastewater treatment plants suffer from repeated breakdowns of nitrification performance, more insight into the physiology of *Nitrospira*- like bacteria is required to find measures by which to stabilize this important step of nutrient removal in modern biological sewage treatment.

The primary objectives of this study are to investigate the stoichiometric relationship between nitrite oxidation and carbon yield in *Nitrospira* and to show that uptake of inorganic carbon can be used as a method to measure activity of nitrifying bacteria.

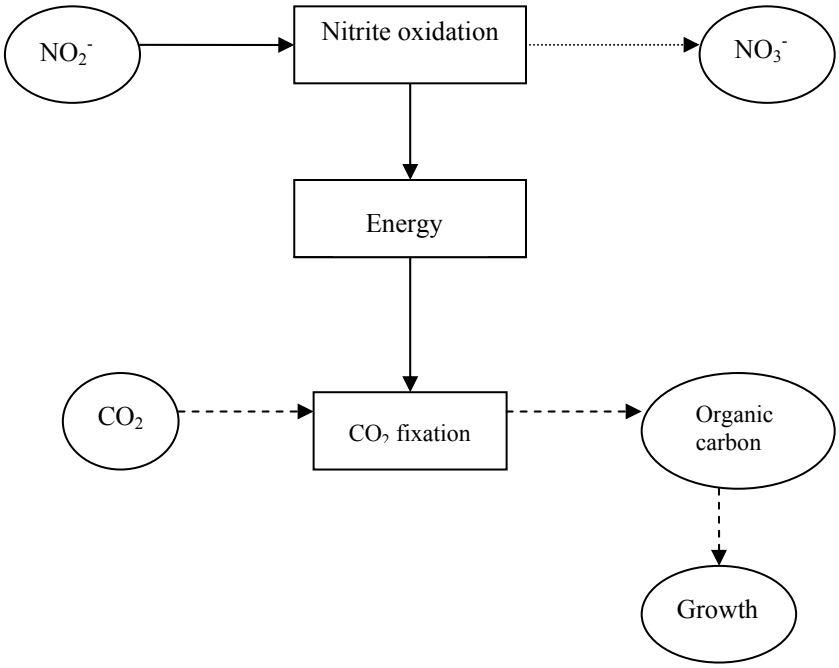

Figure 1. Relationship between nitrification and CO_2 fixation.

II. MATERIALS AND METHODS

II.1. Experimental Approach

Nitrite removal during nitrification and the NOB activity of activated sludge were studied in laboratory batch experiments. A continuous-stirred tank reactor (CSTR) was selected to provide activated sludge samples. Nitrite and nitrate were measured to demonstrate nitrite removal and nitrate formation. Activity of NOB was investigated based on the measurements of CO_2 fixation.

II.2. Bench-scale continuous-stirred tank reactor (CSTR)

The bench-scale treatment system consisted of a complete mix reactor at an SRT of 10 days and an external secondary clarifier with biomass recycle operated in a constant temperature room at 20^{o}C. Influent was collected from a large municipal treatment plant (primary clarifier effluent) and fed to the reactor at a rate of 19mL/min, which provided a hydraulic retention time (HRT) of 8.8 hr. The average ammonia concentration in the influent feed was 17 ± 1.5 mg N/L, while the nitrite and nitrate levels were consistently below the detection limit of 1.0 mg N/L. The dissolved oxygen concentration was maintained at 3.0 mg/L using a control system. Solids retention time was maintained via direct wastage from the aerated reactor. Mixing was provided with a magnetic stirrer. Filtered laboratory air was supplied to ensure adequate aeration. Reactor performance was monitored via influent and effluent NH_4^+-N, NO_2^--N and NO_3^--N concentrations. Both ammonia oxidation and nitrite oxidation were 100% in the reactor.

II.3. Batch Experiments

Nitrite removal, nitrate formation and CO_2 fixation assays were carried out in triplicate in 40-ml serum vials containing 5 ml of mixed liquor samples. 25 mg-N/L of nitrite as sodium nitrite ($NaNO_2$) (final concentration) was used as the primary electron donor for the NOB. Radiolabelled 2.5 μL (1,375,000 dpm or 0.625 μCi per vial) $NaH^{14}CO_3$, specific activity 6.3 mCi/mmol (Sigma, St. Louis, Missouri), was also added to vials used in the CO_2 fixation experiments at a concentration of 20 μM (final concentration). Initial samples were taken for nitrite and nitrate measurements from the liquid phase using a 0.45 μM Gelman glass fiber syringe filter and dispensed into auto-sample vials. Two mL subsamples acidified with 2N H_2SO_4 were initially collected from the vials

containing $H^{14}CO_3$ and were transferred to 20 mL scintillation vials and flushed with nitrogen gas for 30 min to remove inorganic ^{14}C as $^{14}CO_2$. After initial samples were taken, all remaining vials were sealed with a teflon/silica septum and cap and incubated at 20°C on a shaker (approx. 200 rpm) for 5h. Nitrite, nitrate and CO_2 samples were taken every 1 to 2 hours and at the end of the experiments using the same procedures described above. All assays were terminated after 5 h of incubation. The nitrite removal and nitrate formation rates were calculated from the concentrations of nitrite and nitrate in samples taken initially and every 1 to 2 hours in batch experiments. The CO_2 fixation rates were determined from the amounts of radiolabel incorporated into bacterial biomass during the 5-hr incubation period in batch experiments. The rates of nitrite removal, nitrate formation and CO_2 fixation were determined based on least squares regression analysis (linear fit) using the Sigma Plot computer program (SPSS, Inc., Chicago, IL). Measured concentrations were plotted over time and the slope was equal to the rate. These experiments were repeated several times on different days to determine the reproducibility of the results and then the data was averaged.

II.4. Analytical Techniques

Ammonia concentration in the influent and effluent was measured using Standard Method 4500 D, Ammonium Selective Electrode Method (APHA, 1998). Nitrite and nitrate concentrations were measured according to Standard Method 4110 B, Ion Chromatography with Chemical Suppression of Eluent Conductivity (APHA, 1998) using a Dionex DX 500 Ion Chromatograph (IC) outfitted with an Ionpac® AS4A 4mm anion exchange column (Dionex, Sunnyvale, California). The radioactive bicarbonate in the biomass was quantified by a Packard liquid scintillation counter Model 2900 TR (Packard Instrument Company, IL) using 10 ml of Ultima Gold XR (Packard Instrument Company, IL) as the scintillation cocktail. General linear model (GLM) univariate analyses were performed on the data generated in this study, using the SPSS 12.0 (SPSS, Inc., Chicago, IL) statistical analysis software package, to determine statistical differences. The probability was maintained at 5% (α = 0.05).

III. RESULTS AND DISCUSSION

III.1. Demonstration of nitrite oxidation in batch samples from the CSTR

In the batch experiments, samples were analyzed to demonstrate nitrite oxidation. Two separate calculations were performed. First, the total mass of nitrite removed and nitrate formed were calculated by subtracting the final concentrations of nitrite and nitrate from the initial concentrations. Second, nitrite oxidation and nitrate formation rates were determined based on the slope of a linear regression analysis of the time dependent data.

Nitrite oxidation in batch samples was consistent throughout the course of this study. An initial nitrite concentration of 22.4 ± 1.68 mg-N/L was lowered to 8.23 ± 3.15 mg-N/L after 5 hours of incubation in 13 separate control experiments. As a result, 14.2 ± 3.18 mg-N/L of nitrite was removed, on average, representing a 63% decrease in concentration. The rate of nitrite removal was calculated as 2.85 mg-N/L-hr (Figure 2).

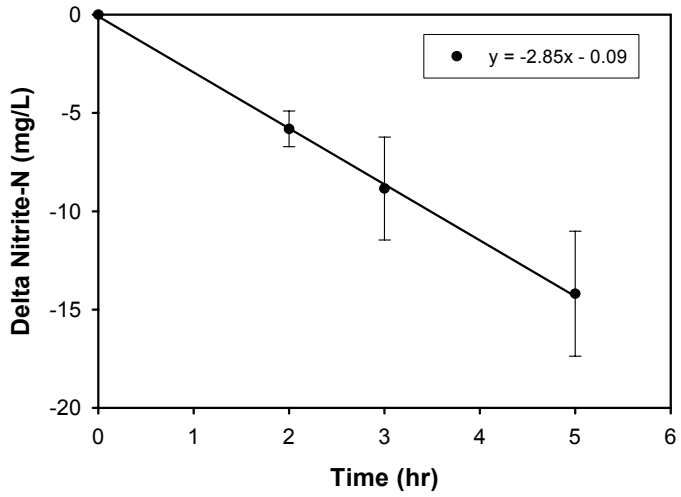

Figure 2. Average nitrite removal in control samples over time in batch samples (n = 13). The slope represents the average nitrite removal rate.

Alternatively, initial nitrate levels varied throughout each experiment because concentrations were dependent upon ammonia levels entering the CSTR and nitrification efficiency of the 10-day SRT reactor. The mean initial nitrate

concentration was 8.98 ± 3.53 mg-N/L and the mean amount of nitrate accumulated after 5 hours was 22.8 ± 4.26 mg-N/L. As a result 13.82 ± 3.8 mg-N/L of nitrate was formed, on average, during the control experiments. The average nitrate formation rate was 2.79 mg-N/L-hr (Figure 3).

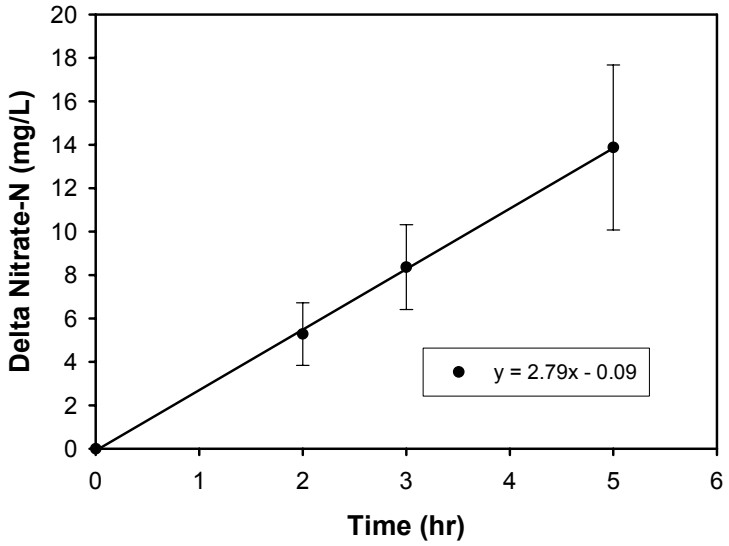

Figure 3. Nitrate formation in control samples over time in batch experiments (n = 13). The slope represents the average nitrate formation rate.

Although there was slight variability in the starting concentration of nitrite, the mean nitrite decrease was 14.2 ± 3.18 mg-N/L while the mean nitrate increase was 13.82 ± 3.8 mg-N/L suggesting the 1:1 ratio expected when nitrite is oxidized to nitrate by NOB. Additionally, the nitrite removal and nitrate formation rates were similar in the control experiments (2.85 mg-N/L-hr and 2.79 mg-N/L-hr, respectively) (Figures 2 and 3).

In summary, nitrite oxidation resulted in a 1:1 ratio (nitrite removal to nitrate formation) with equal rates in the control batch samples. This ratio suggests conversion of all consumed nitrite to nitrate based on the stoichiometry of the reaction of nitrite oxidation in which 1 mol of nitrite-N is converted to 1 mol of nitrate-N.

III.2. Demonstration of CO_2 fixation in batch samples from the CSTR

All the data indicated that the amount of inorganic carbon assimilated into the biomass was consistent in 9 separate experiments showing good reproducibility in the samples. The mean assimilated inorganic carbon was 20.3 µg/L after 5 hours of incubation and the mean carbon dioxide fixation rate was calculated as 4.06 µg/L-hr (Figure 4). The ratio of carbon incorporated to nitrogen oxidized was 1.42×10^{-3} in the experiments. There is only a limited amount of information in the literature dealing with the stoichiometry of carbon uptake and substrate oxidation. Additionally, no previous study showed the relationship between nitrite removal, nitrate formation and CO_2 fixation in activated sludge reactors. The ratio of CO_2 fixed to nitrate produced was shown in pure *Nitrobacter* sp. and *Nitrococcus mobilis* cultures. Belser found a ratio of 1 to 0.02 with *Nitrobacter* sp [9]. In a study with *Nitrococcus mobilis*, the ratio was between 0.014-0.031 [8]. The ratio found in this study was much lower than those, but note that it was expected that lesser amounts of carbon may be fixed because these experiments were performed using a mixed culture. Heterotrophic bacteria generate CO_2 through respiration which would provide a source of unlabeled CO_2 for NOB.

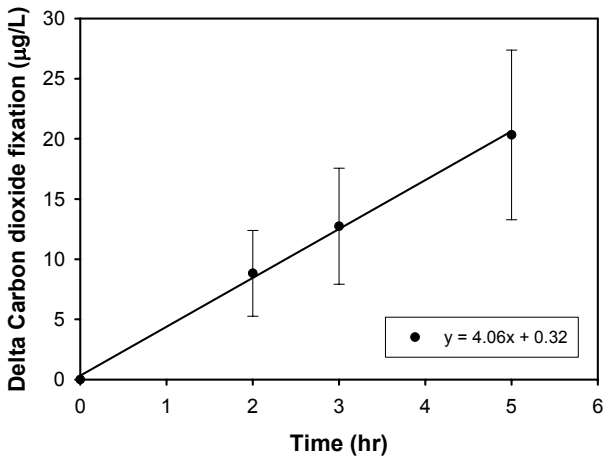

Figure 4. Carbon dioxide fixation in control experiments over time in batch experiments (n = 9). The slope represents the average carbon dioxide fixation rate.

IV. CONCLUSION

Nitrite removal rates were equal to nitrate formation rates in the experiments which indicated that all of the nitrite removed was converted to nitrate. It was demonstrated that there was a connection between nitrite removal, nitrate formation and CO_2 fixation. Furthermore, uptake of inorganic carbon can be exploited as a method by which to demonstrate the in situ activity of nitrifying bacteria.

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