INVESTIGATION OF NITRIFYING BACTERIAL ACTIVITIES BY MONITORING NITRITE OXIDATION, NITRATE FORMATION AND CARBON DIOXIDE FIXATION DURING ACTIVATED SLUDGE TREATMENT IN THE PRESENCE OF METABOLIC INHIBITORS ALLYLTHIOUREA AND AZIDE

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ABSTRACT

The effects of two metabolic inhibitors on nitrifying biomass during activated sludge wastewater treatment were investigated. The impact of allylthiourea [ATU ($C_4H_8N_2S$)] and azide (N_3) was measured by nitrite oxidation, nitrate removal and carbon dioxide fixation (CO_2) assays in batch experiments performed using mixed liquor obtained from a complete-mix, bench-scale, activated sludge system. ATU did not inhibit nitrite removal or nitrate formation rates, but did inhibit CO_2 fixation rates by approximately 50%. Azide did inhibit nitrite removal, nitrate formation and CO_2 fixation rates, as expected. Inhibition of CO_2 fixation by azide also indicated that the activity of nitrite-oxidizing bacteria (NOB) was inhibited since the bacteria were not able to fix inorganic carbon. Additionally, it was demonstrated that there was a connection between nitrite removal, nitrate formation and CO_2 fixation. Also, it was showed that it is possible to distinguish the activities of AOB and NOB in mixed cultures using these two inhibitors.

Keywords: Nitrite oxidation, nitrate formation, carbon dioxide fixation, activated sludge, wastewater treatment, allylthiourea (ATU), azide, ammonia-oxidizing bacteria (AOB), nitrite-oxidizing bacteria (NOB)

NİTRİFİKASYON BAKTERİLERİNİN AKTİVİTELERİNİN NİTRİT OKSİDASYONU, NİTRAT FORMASYONU VE KARBON DİOKSİT FİKSASYONU İZLENEREK AKTİF ÇAMUR YÖNTEMİ SIRASINDA ALLYLTHIOUREA AND AZİT METABOLİK İNHİBİTÖRLERİNİN VARLIĞINDA ARAŞTIRILMASI

ÖZET

İki metabolik inhibitörün nitrifikasyon bakterileri üzerindeki etkileri aktif çamur yöntemiyle atık su arıtımı sırasında araştırıldı. ATU ve azitin tesirleri kesikli reaktör sisteminde nitrit oksidasyonu, nitrat formasyonu ve karbon dioksit fiksasyonu deneyleriyle ölçüldü. Deneylerde kullanılan biyokütle örnekleri sürekli, tam karışımlı, laboratuar ölçekli aktif çamur reaktöründen alındı. ATU, nitrit oksidasyonu ve nitrat formasyonu hızlarını inhibe etmedi, fakat CO_2 fiksasyonu hızını %50 oranında inhibe etti. Azit, beklendiği üzere, nitrit oksidasyonu, nitrat formasyonu ve CO_2 fiksasyonu hızlarını inhibe etti. CO_2 fiksasyonunun azit tarafından inhibisyonu, nitrit oksitleyen bakterinin (NOB) aktivitesi de inhibe olduğunu gösterdi, çünkü bakteri enerji eksikliği nedeniyle inorganik karbonu bünyesine alma yeteneğini yitirdi. Ayrıca, nitrit oksidasyonu, nitrat formasyonu ve CO_2 fiksasyonu arasında bir bağlantı olduğu da bu çalışmayla gösterilmiş oldu. Karışık bakterilerin bulunduğu ortamlarda, AOB ve NOB aktivitelerinin ayırt edilebilmesinin mümkün olduğu da kanıtlamış oldu.

Anahtar kelimeler: Nitrit oksidasyonu, nitrat formasyonu, CO_2 fiksasyonu, aktif çamur, atık su arıtımı, allylthiourea (ATU), azit, amonyak oksitleyen bakteri (AOB), nitrit oksitleyen bakteri (NOB)

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INTRODUCTION

The biological elimination of nitrogen in wastewater treatment plants 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]. Nitrifying bacteria are characterized by low half-saturation constants that are typically approximately 1.0 mgN/L. Consequently low ammonia concentrations can be achieved in bioreactors whenever the SRT is long enough to ensure stable nitrifier growth. However, the ammonia concentration rises rapidly as the SRT is decreased to the point of washout [4], giving rise to the reputation of nitrification as an "all or nothing" phenomenon [2].

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 [5].

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

$NH_4^+ + O_2 + H^+ + 2e^-$ ----------> $NH_2OH + H_2O$
$NH_2OH + H_2O^-$ ----------> $NO_2^- + 5 H^+ + 4e^-$ $\Delta G^{0'}$ = -10.82 Kcal / e^- eq
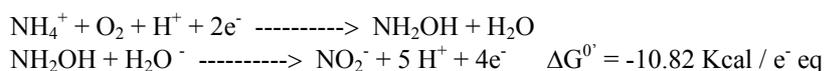
$\Delta G^{0'}$ is the standard free energy to transform each electron equivalent for the reaction when the pH is set to 7.0. The reaction, as written, contains 6 electron equivalents. Besides consuming oxygen, the oxidation of ammonium produces five equivalents of hydrogen per mole of ammonium [6].

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:

$NO_2^- + H_2O$ ----------> $NO_3^- + 2H^+ + 2e^-$
$2H^+ + 2e^- + 0.5O_2$ ----------> H_2O $\Delta G^{0'}$ = -9.25 Kcal / e^- eq
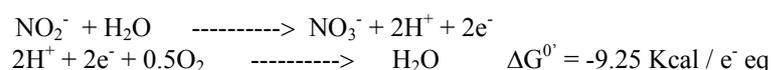
Energy is derived from the oxidation of nitrite to nitrate providing NADPH and ATP for CO_2 fixation via the Calvin cycle. Figure 1 illustrates the relationship between nitrification and CO_2 fixation.

Traditionally, nitrification has been mathematically treated as one composite biochemical process, with the assumption that NH_4^+ to NO_2^- oxidation limits the overall transformation of NH_4^+ to NO_3^- [2]. However, if NH_4^+ and NO_2^- oxidation both limit overall nitrification (at different stages of the process), the single-step representation is inadequate. Under such circumstances, individual characterization of both NH_4^+ and NO_2^- is necessary. An accurate knowledge of the kinetics of each step is required to formulate appropriate design and control strategies for achieving desired NH_4^+ and NO_2^- removal. Thus, for mixed cultures, such as activated sludge, selective inhibitors that allow separation of the different activities are needed. Ideally, inhibition should be instantaneous and complete for the targeted population and should not affect other populations. Three compounds (allylthiourea, chlorate and azide) have been extensively used to study nitrification inhibition.
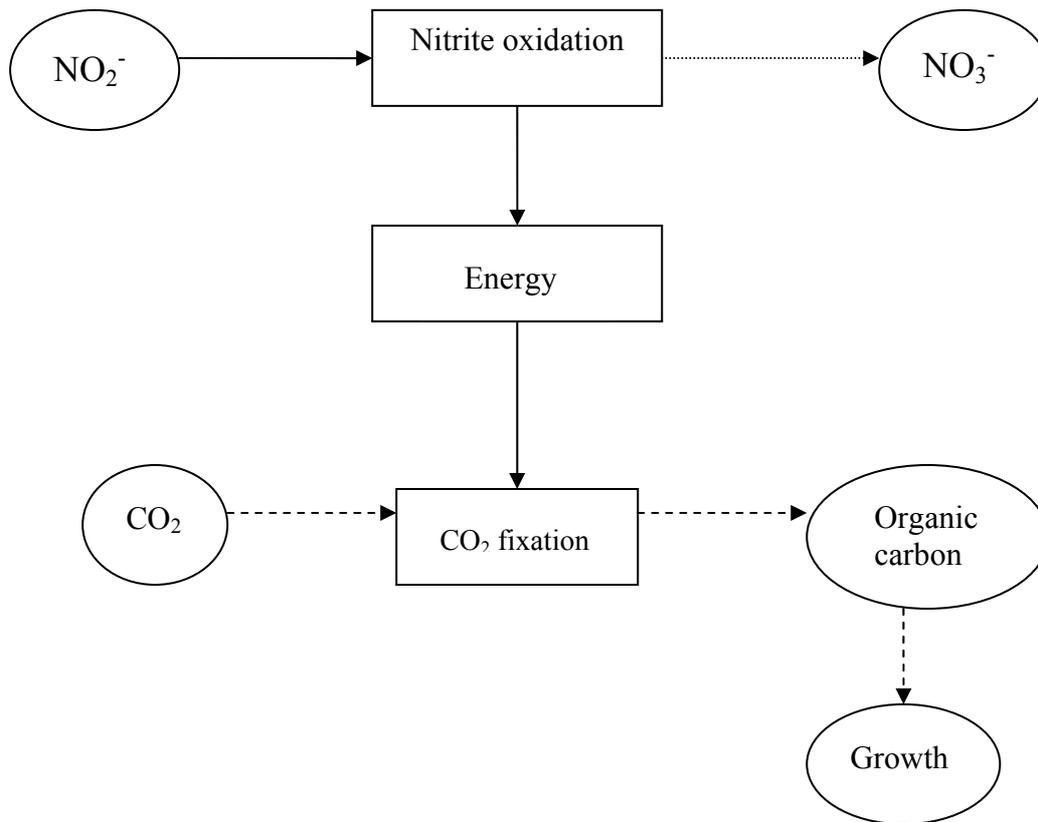
Figure 1. Relationship between nitrification and CO_2 fixation.

Allylthiourea [ATU ($C_4H_8N_2S$)] selectively inhibits ammonia oxidation at concentrations between 8 and 80 µM [7-9]. The mechanism for inhibition appears to be chelation of copper from the active site of the ammonia monooxygenase [10]. Ginestet et al. [11] performed a comprehensive investigation of ammonia oxidation using a mixed nitrifier culture from an activated sludge and found that instantaneous selective inhibition was achieved at an allylthiourea concentration of 86 µM.

Chlorate (ClO_3) has been used to inhibit nitrite oxidation in soils, sediments, and activated sludge systems [12-14]. However, doubts concerning the slow and nonspecific action of chlorate limit its usefulness in discriminatory assays with mixed cultures [11-13].

Azide (N_3) is a selective bacteriostatic agent active in gram-negative bacteria and has been shown to inhibit nitrite oxidation in activated sludge. Azide is believed to complex with the molybdenum atoms of the nitrite oxidoreductase [11]. Chandran and Smets [15] found that an optimum azide concentration of 24 µM enabled selective inhibition of nitrite oxidation without affecting ammonia oxidation. This optimum concentration was also consistent with a previous report by Ginestet et al. [11]. The inhibition was independent of the nitrite concentration and was reversible after azide was removed by biomass washing.

Based on literature results, ATU and azide were chosen to study ammonia and nitrite oxidation inhibition in this research. The primary objectives of this study are to distinguish biological activities performed by ammonia oxidizers and nitrite oxidizers and to determine how nitrite removal, nitrate formation and CO_2 fixation rates were affected during nitrite oxidation in the presence of ATU and azide.

1. MATERIALS AND METHODS

1.1 Experimental Approach

The effects of ATU and azide on 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 during ATU and azide exposure to examine effects on nitrite removal and nitrate formation. Activity of NOB was investigated based on the measurements of CO_2 fixation.

1.2 Bench-scale continuous-stirred tank reactor (CSTR)

The bench-scale treatment system consisted of a complete mix reactor and an external secondary clarifier with biomass recycle operated at an SRT of 10 days. Influent was collected from a large municipal treatment (primary clarifier effluent) plant and fed to the reactor at a rate of 19mL/min. 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. Solids retention time was maintained via direct wastage from the aerated reactor.

1.3 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 and either ATU or azide or both. Control samples, in the absence of the inhibitors, were also analyzed. 25 mg-N/L of nitrite as sodium nitrite ($NaNO_2$) (final concentration) was used as the primary electron donor for the NOB. 2.5 µL (1,375,000 dpm or 0.625 µCi per vial) $NaH^{14}CO_3$, specific activity 6.3 mCi/mmol (Sigma) was added to each sample vial (final concentration 20 µM $NaH^{14}CO_3$) for CO_2 fixation measurements. All assays were terminated after 5 h of incubation at $20^{o}C$ on a shaker (200 rpm). 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. These experiments were repeated six times on different days to determine the reproducibility of the results and then the data was averaged. Total nitrogen mass after 5 hrs was summed and the rates of nitrite removal, nitrate formation and CO_2 fixation were determined via least squares regression analysis (linear fit).

1.4 Analytical techniques

Ammonia concentration in the influent and effluent was measured using Standard Method 4500 D, Ammonium Selective Electrode Method [16]. Nitrite and nitrate concentrations were measured according to Standard Method 4110 B, Ion Chromatography with Chemical Suppression of Eluent Conductivity [16] using a Dionex DX 500 Ion Chromatograph (IC) outfitted with an Ionpac® AS4A 4mm anion exchange column. 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.

2. RESULTS AND DISCUSSION

Inhibitor concentrations utilized in this study (ATU = 86 µM; azide = 24 µM) were similar to those used in experiments performed by Ginestet et al. [11] and Chandran and Smets [15]. However, initial experimental results indicated that azide did not completely inhibit nitrite oxidation at a concentration of 24 µM; therefore, a level of 50 µM was utilized in all subsequent experiments. ATU was not expected to inhibit nitrite removal and nitrate formation rates because ammonia oxidizers do not play a direct role in nitrite oxidation. However, it was anticipated that CO_2 fixation rates would be reduced since ammonia oxidizers also fix CO_2. Azide was predicted to inhibit nitrite removal, nitrate formation and CO_2 fixation rates since this compound is a specific inhibitor for NOB.

2.1 Effect of Allylthiourea and Azide on Nitrite Removal and Nitrate Formation

a. Allylthiourea

The mean changes in nitrite concentrations over time in the 2 separate control and ATU experiments were plotted to determine the impact of ATU on nitrite removal (Figure 2). After 5 hr of incubation, the mean nitrite decrease was 11.71 ± 1.25 mg-N/L in the control and 11.50 ± 0.86 mg-N/L in the ATU experiments. Approximately 50% of the initial nitrite added was removed in both experiments indicating that the inhibitor had no effect on nitrite oxidation. Additionally, the average rates of nitrite removal were 2.35 mg-N/L-hr and 2.33 mg-N/L-hr in the controls and in the samples with added ATU, respectively. Again, these results implied that the presence of ATU had no negative impact on nitrite removal. Statistical analysis of the data also indicated no significant difference in nitrite removal rates between the control and ATU tests after five hours of incubation ($\alpha = 0.05$).

The impact of ATU on nitrate formation was also examined. After 5 hours of incubation, 11.23 ± 1.33 mg-N/L nitrate accumulated in the controls and 11.88 ± 0.52 mg-N/L nitrate was formed in the experimental tests (Figure 3). The mean nitrate formation rates were calculated as 2.24 mg-N/L-hr and 2.12 mg-N/L-hr in the control and ATU experimental samples, respectively. The similar rates and extent of the reactions suggested that ATU had little effect on nitrate formation, as well. Statistical analysis of the data also showed that ATU did not significantly impact nitrate formation ($\alpha = 0.05$).

In control experiments, the mean nitrite decrease and the mean nitrate increase were comparable (11.71 ± 1.25 mg-N/L and 11.23 ± 1.33 mg-N/L, respectively) confirming the stoichiometric 1:1 nitrite to nitrate ratio. Similar to the controls, the mass of nitrite removed (11.50 ± 0.86 mg-N/L) equaled the mass of nitrate formed in ATU experimental samples (11.88 ± 0.52 mg-N/L) also yielding a 1:1 ratio. Additionally, the rates of nitrite removal and nitrate formation were similar in both control and experimental samples. It can be concluded that nitrite was oxidized to nitrate in both control and ATU samples (Figures 2 and 3).

These results indicated that NOB in the ATU experimental samples behaved similarly to NOB in the controls, as anticipated, since ATU does not affect nitrite oxidation (i.e. nitrite removal = nitrate formation). These findings are in agreement with Ginestet et al. [11] and Chandran and Smets [15] who illustrated that ATU selectively inhibited ammonia oxidizers without affecting nitrite oxidizers.
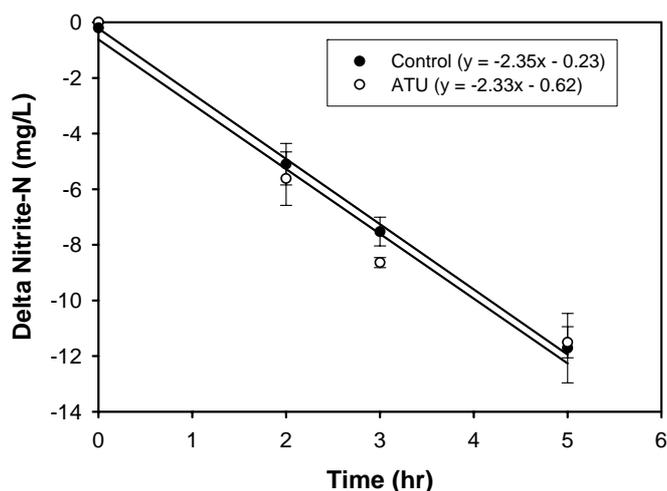
Figure 2. Nitrite removal in the control and ATU experimental samples over time in batch experiments (n = 2). The slopes represent the average nitrite removal rates.
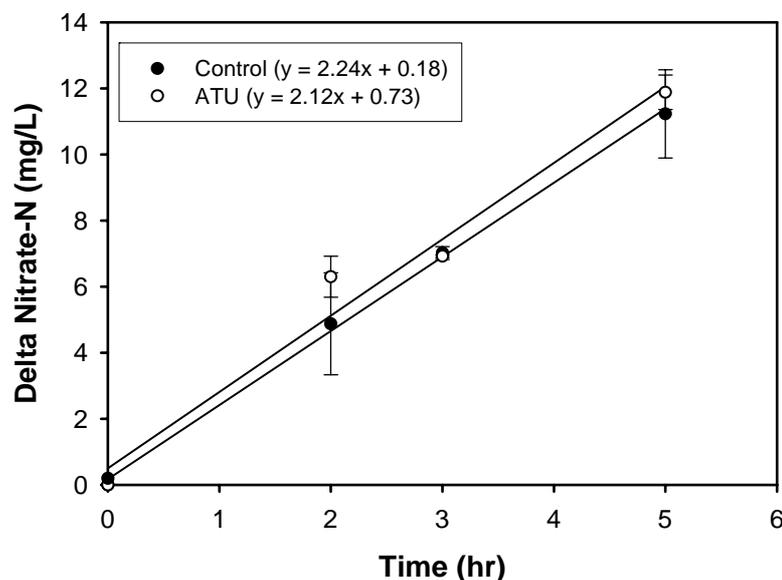
Figure 3. Nitrate formation in the control and ATU experimental samples over time in batch experiments (n = 2).
The slopes represent the average nitrate formation rates.

b. Azide

The impact of azide on nitrite removal and nitrate formation was investigated to study inhibition of nitrite oxidation. Six separate experiments were performed using azide. Figure 4 represents the mean decrease in nitrite removal in both control and azide samples. After 5 hr of incubation, the mean decrease in nitrite concentration was 12.24 ± 2.04 mg-N/L in the control experiments representing 54% nitrite removal. In contrast, only 4.18 ± 1.48 mg-N/L of nitrite was removed in the presence of azide (19% decrease) indicating azide inhibition. The rates of nitrite removal averaged 2.42 mg-N/L-hr and 0.83 mg-N/L-hr in the controls and azide samples, respectively. These results indicate a 66% inhibition of the nitrite removal rate by azide. Statistical analysis of the data confirmed a significant difference in nitrite removal rates between the control and azide tests after five hours of incubation ($\alpha = 0.05$).

Nitrate accumulated in the controls (11.40 ± 1.67 mg-N/L) and formed in the azide tests (4.99 ± 0.75 mg-N/L) also indicated inhibition of nitrate formation (Figure 5). The mean rates of nitrate formation were 2.29 mg-N/L-hr in the absence of azide and 1.00 mg-N/L-hr in the presence of azide. Thus, the nitrate formation rate was reduced 56% percent indicating, as expected, the negative impact of azide on nitrate formation. Statistical analysis confirmed that the difference in rates between the control and tests was significant ($\alpha = 0.05$).

In control samples, the mean nitrite decrease and the mean nitrate increase were comparable (12.23 ± 2.04 mg-N/L and 11.40 ± 1.67 mg-N/L, respectively) and close to the predicted 1:1 stoichiometric ratio. In the presence of azide, the mass of nitrite removed (4.18 ± 1.48 mg-N/L) was similar to the mass of nitrate formed (4.99 ± 0.75 mg-N/L) also indicating a 1:1 ratio. In spite of the inhibition of nitrite removal and nitrate formation, the amount of nitrite consumed in the experiments was nearly equal to the amount of nitrate accumulated indicating nitrite removal and nitrite formation were inhibited to the same degree by azide. Additionally, the rates of nitrite removal and nitrate formation were similar for each of the control and experimental samples. The nitrite removal rate was 2.42 mg-N/L-hr and the nitrate formation rate was 2.29 mg-N/L- hr in the control samples. The rates of nitrite removal and nitrate formation in the azide samples were 0.83 mg-N/L-hr and 1.00 mg-N/L-hr, respectively. Thus, it can be concluded that nitrite was oxidized to nitrate in both control and azide samples, although nitrite removal and nitrate formation were similarly inhibited by azide.

Additionally, total nitrogen calculations were performed to determine if nitrogen was conserved during these experiments. Total nitrogen was calculated by adding the nitrite-nitrogen and nitrate-nitrogen concentrations

measured in the liquid phase for each time point. In the controls, mean total nitrogen remained constant (32.60 ± 0.37 mg-N/L) throughout the experiment, suggesting that all nitrite consumed was converted to nitrate. In the azide experimental samples, mean total nitrogen values also did not change during the 5 hr incubation (33.11 ± 0.47 mg-N/L). Thus, although the rates of nitrite removal and nitrate formation were lower in the azide samples than those in the control, total nitrogen concentration remained constant over time indicating that no nitrogen was lost from this
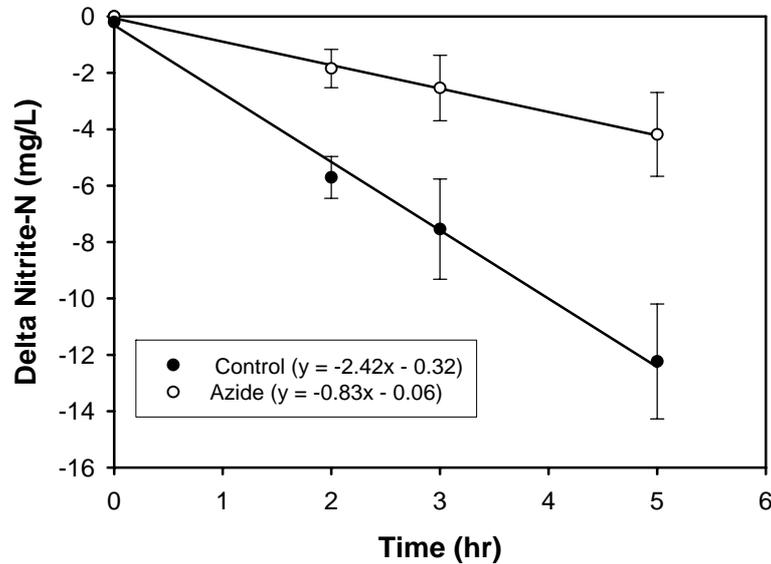
Figure 4. Nitrite removal in the control and azide experimental samples over time in batch experiments (n = 6). The slopes represent the average nitrite removal rates.
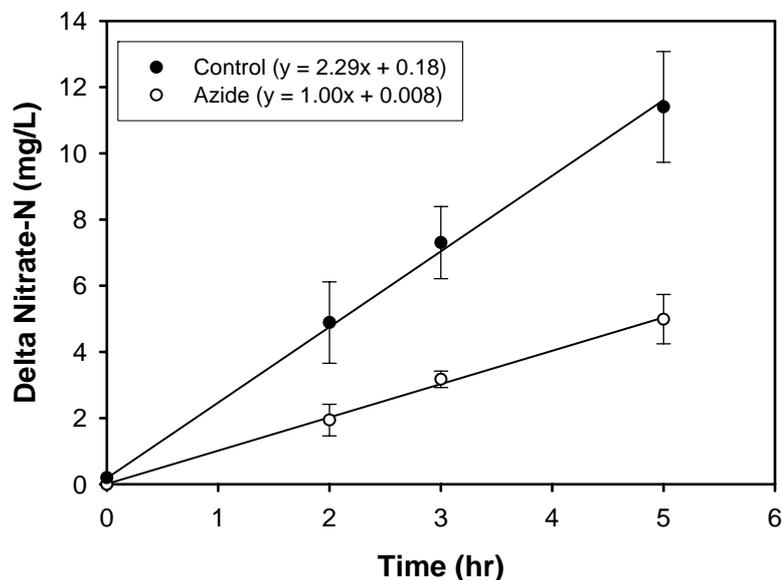
Figure 5. Nitrate formation in the control and azide experimental samples over time in batch experiments (n = 6). The slopes represent the average nitrate formation rates.

system. However, Ginestet et al. [11] and Chandran and Smets [15] illustrated complete inhibition of nitrite oxidation by azide which was not demonstrated in this research.

c.   ATU and azide

ATU and 24 µM azide were used together in one experiment to investigate the changes in nitrite removal and nitrate formation in the presence of both inhibitors. Figure 6 depicts changes in nitrite concentrations with and without inhibitors. In the control samples, 10.83 mg-N/L of nitrite was consumed over 5 hours and the removal rate was calculated as 2.18 mg-N/L-hr. In the presence of ATU, nitrite decrease over 5 hours was 12.99 mg-N/L and the rate of nitrite removal was 2.56 mg-N/L-hr, similar to results obtained in the control. These results are also similar to findings reported earlier and, again, indicate that ATU did not significantly affect nitrite removal. However, when azide was added, only 1.99 mg-N/L of nitrite was removed over time at a rate of 0.37 mg-N/L-hr, representing 83% inhibition of nitrite removal. As anticipated, the impact of both ATU and azide was similar to the impact of azide alone. The nitrite decrease over 5 hours was 1.99 mg-N/L and the rate of nitrite removal was 0.33 mg-N/L-hr. Results were almost identical to those obtained from the azide alone experiment indicating that AOB did not affect nitrite removal.

With respect to nitrate formation, results were similar in the control and ATU tests, 10.29 mg-N/L and 11.25 mg-N/L, respectively (Figure 7). However, only 4.43 mg-N/L of nitrate was formed when azide only used. Not surprisingly, the same amount of nitrate (4.44 mg-N/L) was formed when ATU and azide were utilized together. The rates of nitrate formation were 2.11 mg-N/L-hr in the control and 2.20 mg-N/L-hr in the ATU experimental samples again indicating no inhibition in the presence of ATU. However, the nitrate formation rate declined in the presence of azide and azide/ATU to 0.92 mg-N/L-hr, representing a 56% reduction. The rate of nitrate formation and the extent of inhibition when both ATU and azide were used indicate that ATU did not affect nitrate formation.

3.2 Effect of Allylthiourea and Azide on CO_2 Fixation

Chemical compounds inhibiting the activity of certain groups of chemoautotrophic bacteria are widely used for assessment of the intensity of microbiological processes. The use of the radioisotope method for determination of CO_2 fixation together with the inhibitor analysis allows one to quantitatively assess the contribution of individual groups of chemoautotrophic bacteria to the total CO_2 assimilation by bacterial groups. The level of nitrite oxidation in the activated sludge treatment was determined from the decrease in $^{14}CO_2$ assimilation in the presence of allylthiourea and azide inhibitors.
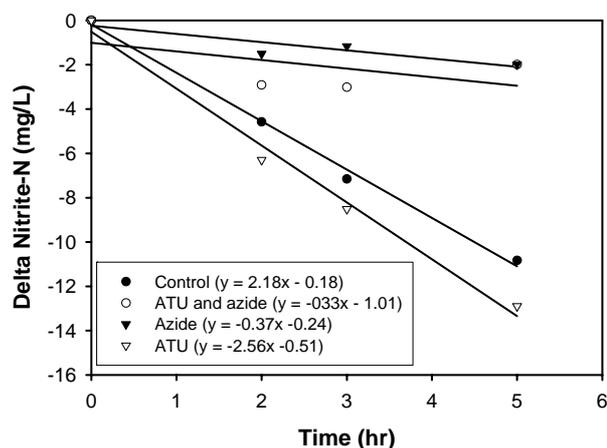
Figure 6. Nitrite removal in the control, ATU, azide, ATU and azide experimental samples over time in batch experiments. The slopes represent the average nitrite removal rates.
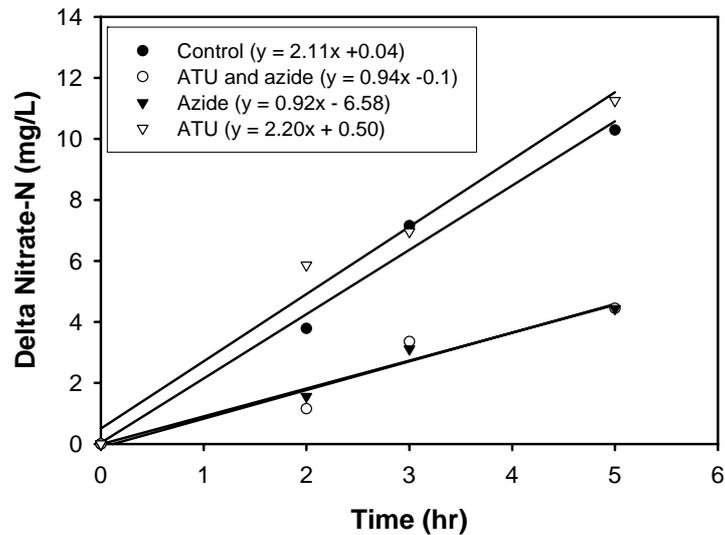
Figure 7. Nitrate formation in the control, ATU, azide, ATU and azide experimental samples over time in batch experiments. The slopes represent the nitrate formation rates.

a. Allylthiourea

The mean increases in the amount of inorganic carbon assimilated into biomass by NOB over time in the 2 separate control and ATU experiments were plotted to determine the effect of ATU on CO_2 fixation and to investigate the relationship between nitrite oxidation and CO_2 uptake (Figure 8). After 5 hr of incubation, the mean amount of carbon fixed was 12.97 ± 1.05 µg/L in the control and 7.56 ± 1.38 µg/L in the ATU experiments. Compared to the controls, 41% less CO_2 was incorporated into the biomass in the presence of ATU suggesting that AOB contribute approximately half of the measured CO_2 uptake. The mean rates of CO_2 fixation were found to be 2.62 µg/L-hr and 1.55 µg/L-hr in the controls and in the ATU samples, respectively. A 41% inhibition in the rate was measured indicating, once again, that about half of the CO_2 was fixed by AOB. Statistical analysis of the data also demonstrated that there was a significant difference in the CO_2 fixation rates between the control and ATU tests after five hours of incubation ($\alpha = 0.05$).
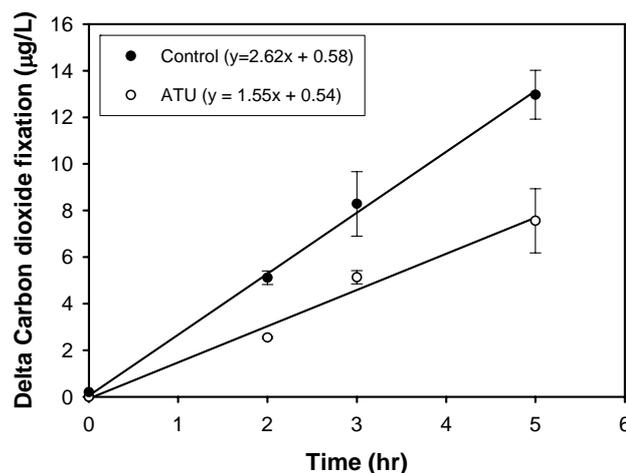
Figure 8. Carbon dioxide fixation in the control and ATU experimental samples over time in batch experiments (n = 2). The slopes represent the carbon dioxide fixation rates.

b. Azide

The effect of azide on CO_2 assimilation by NOB was investigated in 6 separate experiments. Figure 9 represents the mean amount of inorganic carbon assimilated into biomass by NOB during both control and azide experiments over time. After 5 hr of incubation, inorganic carbon incorporated into the biomass was 16.29 ± 4.58 µg/L in the control and 9.12 ± 3.96 µg/L in the azide experiments indicating 44% less assimilation of CO_2 in the presence of azide. The mean rate of CO_2 fixation was 3.25 µg/L-hr in the controls and 1.82 µg/L-hr in the azide samples which represents a 44% reduction in the rate when azide was present. These results indicated that about half of the CO_2 was fixed by NOB. Statistical analysis of the data also demonstrated that there was a significant difference in the CO_2 fixation rates between the control and azide tests ($\alpha = 0.05$).

The reduction in the CO_2 fixation in the presence of azide was not surprising since azide reduced nitrite removal (54%) and nitrate formation (56%). The NOB lacked the energy required to fix inorganic carbon to organic carbon which is typically obtained from the oxidation of nitrite to nitrate. The results showed that azide reduced nitrite removal, nitrate formation and CO_2 fixation as expected.

According to Saralov et al. [17], it is possible to distinguish between CO_2 fixation by chemoautotrophic and by heterotrophic bacteria, using azide. This is due to the fact that generation of ATP in chemoautotrophs in the terminal sections of the respiratory chain, the c-type and α-type cytochrome oxidases, have a high sensitivity to azide and other respiratory inhibitors. Saralov et al. [17] showed that 15 µM azide completely inhibited CO_2 fixation in the *Nitrobacter* sp. Also, Pimenov et al. [18] found that azide exerted a negligible effect on CO_2 fixation by AOB and heterotrophs.

c. ATU and azide

CO_2 fixation was investigated in the presence of both 80 µM ATU and 24 µM azide in one experiment in an attempt to distinguish other bacteria able to assimilate CO_2 from AOB and NOB. Figure 10 depicts changes in CO_2 fixation with and without inhibitors.

In the controls, 13.72 µg/L CO_2 was assimilated over 5 hrs and the fixation rate was 2.79 µg/L-hr. In the presence of ATU, the amount of CO_2 fixed decreased to 6.58 µg/L and the uptake rate declined to 1.32 µg/L-hr indicating that ATU reduced CO_2 fixation by approximately 52%. When azide was added, similar results to the ATU experiment were obtained; 6.05 µg/L CO_2 was fixed over 5 hrs and the CO_2 uptake rate was 1.21 µg/L-hr, a 56% inhibition. However, when ATU and azide were used together, only 4.23 µg/L of carbon was fixed after 5 hrs. Compared to the control, a 3-fold reduction in carbon incorporated into the biomass was noted. Additionally, the CO_2 fixation rate was reduced to 0.84 µg/L-hr, a 70% reduction in the rate of CO_2 fixation. This data indicate that AOB and NOB fixed 70% of the biomass carbon in the activated sludge.
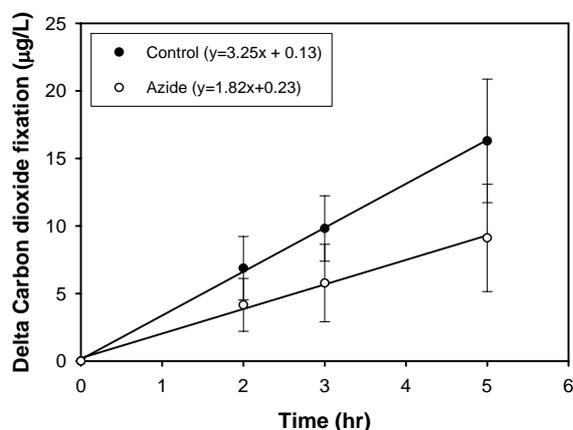
Figure 9. Carbon dioxide fixation in the control and azide experimental samples over time in batch experiments (n = 6). The slopes represent the carbon dioxide fixation rates.
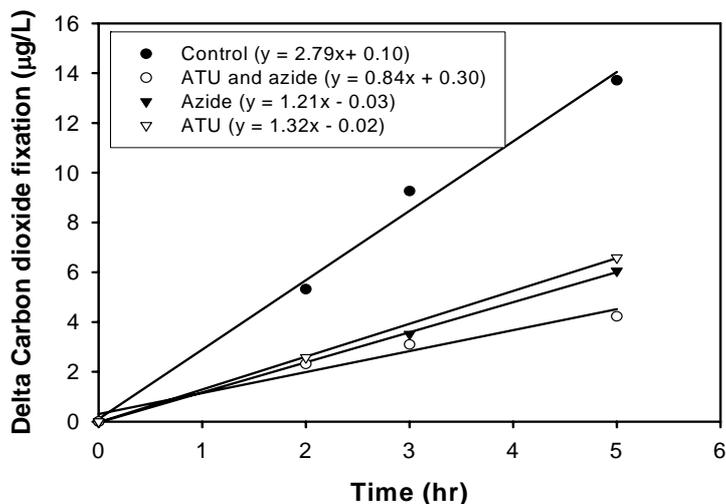
Figure 10. Carbon dioxide fixation in the control, ATU, azide, ATU and azide experimental samples over time in batch experiments. The slopes represent the carbon dioxide fixation rates.

5. CONCLUSION

Nitrite removal rates were equal to nitrate formation rates in the control experiments which indicated that all of the nitrite removed was converted to nitrate. ATU did not inhibit nitrite removal or nitrate formation rates, but did inhibit CO_2 fixation rates by approximately 50%. Since ATU primarily inhibits AOB, it can be concluded that ammonia oxidizers have little effect on nitrite removal and nitrate formation. Azide did inhibit nitrite removal, nitrate formation and CO_2 fixation rates, as expected. Although the rates of nitrite removal and nitrate formation were lower than those in the control, the ratio of nitrite removal to nitrite formation remained at 1:1 indicating a direct link between the two processes. Inhibition of CO_2 fixation by azide also indicated that the activity of NOB was inhibited since the bacteria were not able to fix inorganic carbon. Additionally, it was demonstrated that there was a connection between nitrite removal, nitrate formation and CO_2 fixation. Inhibition during nitrite oxidation results in low CO_2 fixation which is likely due to the reduced energy available. The results also showed that it is possible to distinguish biological activities performed by ammonia oxidizers and nitrite oxidizers.

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