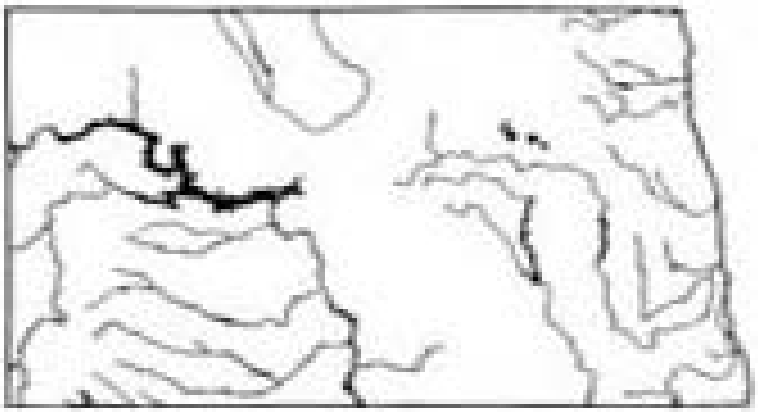

Technical Report No: ND16-02

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by

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ABSTRACT

Due to the increased concern on dissolved organic nitrogen (DON) in surface waters, it is necessary to understand the biodegradability and bioavailability of DON in point and non-point sources. In this study, algae and bacteria were applied under lab condition to undestand the impact of DON to water environment. Biodegradable DON (BDON) was determined using bacteria while bioavailable DON (ABDON) was determined using green algae *Selenastrum capricornutum*, *Chlamydomonas reinhardtii*, and *Chlorella vulgaris* and/or mixed culture bacteria in municipal and animal wastewaters. Results showed that, the ranges of BDON and ABDON in municipal wastewaters were 50-60% and 30-77%, respectively, while the ranges of BDON and ABDON in animal wastewaters were 48-54% and 40-81%, respectively. In both wastewater sources, ABDON (%) for all three algae were not significantly different indicating that *Chlamydomonas reinhardtii* and *Chlorella vulgaris* can be used as a test species for nitrogen determination similar to *Selenastrum capricornutum*.

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BACKGROUND

Nitrogen is an essential nutrient source for living organisms that controls the productivity of aquatic ecosystem. Optimal amount of nitrogen is important to natural surface waters such as lakes, rivers, and estuaries; however, in high concentrations it can be a contaminant in the water ecosystem. Total dissolved nitrogen (TDN) consists of dissolved inorganic nitrogen (DIN) and dissolved organic nitrogen (DON). Biological availability of dissolved organic nitrogen (DON) in aquatic ecosystems accelerates DON transformation into highly soluble inorganic nitrogen forms including ammonia (NH_3), nitrite (NO_2^-), and nitrate (NO_3^-). Excessive DON increases the readily bioavailable and biodegradable nitrogen species in aquatic systems which may resulted in eutrophication in receiving water (Paerl et al., 1997; Seitzinger and Sanders, 1997; Gobler et al., 2005). Therefore, understand wastewater derived DON is crucial to control the cumulative amount of nitrogen in surface waters.

The chemical composition of DON varies which mainly depend on its origination from numerous natural, anthropogenic sources, and autochthonous production. DON consists of complex macromolecules and has been partially characterized; some of the known DON compounds in the environment include urea, dissolved free amino acids (DFAA), dissolved combined amino acids (DCAA), peptides, amino sugars, purines, pyrimidines and other complex macromolecules such as humic and fulvic acid (Bronk, 2007).

Wastewater effluent nitrogen is one of the most important nitrogen sources to receiving waters and its reduction is crucial for especially nutrient sensitive receiving waters. Due to its complex structure, most of the compounds in wastewater derived DON cannot be identified with current technologies. While some portion of DON are readily biodegradable and/or bioavailable to bacterial communities in biological treatment systems, some portion of it are recalcitrant. Bioavailable DON (ABDON) is a portion of DON that is utilized by bacteria and/or algae. ABDON examines the portion of DON that can be minimized by algae-only or algae + bacteria inocula. It evaluates the potential environmental effect of wastewater-derived DON to river and estuaries.

DESCRIPTION OF THE CRITICAL STATE OR REGIONAL WATER PROBLEM TO BE INVESTIGATED

This study would provide valuable information for scientists and engineers on the nature and behavior of water and wastewater derived DON. Results obtained in this study could elucidate the biological removal of nitrogen species including DIN and DON for further understanding of their fate and characterization in municipal wastewater facilities and animal feedlots.

The city of Fargo WWTP has an average flow of fifteen million gallons of wastewater per day. The plant operates a trickling filter process which includes removal of biochemical oxygen demand (BOD), carbonaceous BOD and ammonia nitrogen (NH_3-N). The plant does not have a denitrification process. Therefore, the final effluent contains high amount of nitrate and DON, which has been discharged into the Red River. To improve the quality of surface waters in ND and prevent occurrence of eutrophication, it is necessary to understand nitrate and DON in the effluent.

Runoff from animal feedlots is a major contributor to surface and groundwater impairment

and needed to be treated before reaching the receiving waters. Transport and accumulation of nutrients in downstream surface water can seriously affect the living organisms in the water body.

SCOPE OF STUDY AND OBJECTIVE

The main scope of this study is to collect DON and ABDON data from three different locations in a WWTP. The specific objectives are:

1. To investigate DON and ABDON in three different algae species with/without mixed culture bacteria addition in a two-stage trickling WWTP.

2. To examine mixed culture algae and algae + bacteria interactions for DON and ABDON determination in livestock wastewaters.

CHAPTER 1: Bioavailability of wastewater derived dissolved organic nitrogen to green microalgae *Selenastrum capricornutum*, *Chlamydomonas reinhardtii*, and *Chlorella vulgaris* with/without presence of bacteria

1.1 Introduction

Municipal wastewater effluent DON is one of the most important autochthonous nitrogen sources to receiving waters and its reduction is crucial for especially nutrient sensitive surface waters. Wastewater effluent DON may consist of urea, amino acids, amino sugars, proteins, nucleic acids, fulvic acids, humic acids, and a variety of uncharacterized components. Due to the complex properties of DON, the identifiable effluent DON usually accounts for less than 10% of DON, while major portion (70%) of DON that may consists of mainly polymerized biological compounds, cannot be identified directly with current technologies (Pehlivanoglu-Mantas and Sedlak, 2006).

Although many studies exist on the ABDON from natural and anthropogenic sources (Bushaw et al., 1996; Seitzinger and Sanders, 1997; Vähätalo, et al., 2005; Bronk et al., 2007) limited studies are available on the ABDON in domestic wastewater (Pehlivanoglu and Sedlak 2004; Urgun-Demirtas et al., 2008; Xu et al. 2010; Simsek et al., 2013). Most of the previous studies used a unicellular green microalgae *Selenastrum capricornutum* to investigate bioavailability of nitrogen since *S. capricornutum* has some advantages including easy growing in the laboratory conditions and has high efficiency to utilize the primary nutrients. Moreover, *S. capricornutum* has been used and suggested by United States Environmental Protection Agency as a test species of water quality and fresh water algae toxicity studies (Vanderheever and Grobbelaar, 1998). It has been widely applied in toxicity studies of ionic liquids (Pham et al., 2010), metal oxide nanoparticles (Kahru et al., 2008), and propylene glycol ethers (Staples et al., 2002) to quantify pollutants bioavailability.

Pehlivanoglu and Sedlak (2004) used *S. capricornutum* in wastewater effluent prior to discharge to determine the bioavailability of DON. The bacterial inoculum in the bioassay was isolated from effluent receiving surface waters (Truckee River). Their results showed that DON was not readily available to algae *S. capricornutum*, while around 56% of DON was available to algae and bacteria. Later, Sattayatewa et al. (2009) conducted similar experiments with wastewaters consisting low nitrogen level. About 28 to 57% of DON was biodegradable for bacteria in 40 days of incubation period, while DON in large molecules was likely to be converted to ammonia before final use. The results concluded that 28 to 48% of DON was bioavailable to algae, which utilized the big portion of DON in 3 to 8 days. Similarly, ABDON assay has been performed to examine the bioavailability of specific portion of DON (Liu et al., 2012; Qin et al., 2015). Hydrophilic substances can contribute to 64 to 80% of DON. Liu et al. (2012) evaluated the bioavailability of hydrophilic and hydrophobic DON. They extracted the wastewater effluent to separate hydrophilic and hydrophobic DON. Their results showed that 40 to 85% of hydrophilic DON was bioavailable to algae + bacteria in the bioassay study, while the hydrophobic portion of DON was not readily available to algae in 14 days of incubation period.

In this study, three different algal species, *S. capricornutum*, *Chlamydomonas reinhardtii*, and *Chlorella vulgaris* and their combinations with bacteria were used to obtain DON and ABDON

data in the samples collected from three different locations along the two-stage trickling filter (TF) wastewater treatment plant (WWTP). The results were analyzed and compared to investigate if *C. reinhardtii* and *C. vulgaris* were also suitable to use as control species the same as *S. capricornutum* in wastewaters.

1.2 Material and Methods

1.2.1 Sample Collection and Preparation

Grab wastewater samples were collected from the City of Fargo WWTP. The samples were collected from three different locations, which were after primary clarifier, after BOD TF, and after nitrification TF locations along the WWTP. Total six sets of samples were collected from May 2013 to December 2014 and average values were presented in this study. Before performing any analysis, all the samples were filtered twice through 0.2 µm fiber filter (Pull Scientific, USA) in one hour after collection.

1.2.2 Algal and Bacterial Inoculum Preparation

Algal and algal + bacterial inoculum were used to inoculate all the wastewater samples. As algal test species, three different algal species, which were *S. capricornutum, C. reinhardtii,* and *C. vulgaris*, were employed. Algae were obtained from University of Texas Culture Collection of Algae, Austin, TX. The algal strains were grown in Bristol Medium containing: 2.94 mM $NaNO_3$, 0.17 mM $CaCl_2 \cdot 2H_2O$, 0.3 mM $MgSO_4 \cdot 7H_2O$, 0.43mM K_2HPO_4, 1.29 mM KH_2PO_4, and 0.43 mM NaCl. The algae were cultured in 500 ml clear bottles at 20℃ temperature. Bottles were illuminated for 12 hr light/dark cycle by artificial lights (six fluorescent tube lamps, 15 inches long and 15 W each). The stock *S. capricornutum, C. reinhardtii*, and *C. vulgaris* solutions were centrifuged at 3000 rpm for 5 min and rinsed with DDI water twice before inoculated in the samples. The washed algal culture re-suspended in DDI water to form a concentrated algal suspension. The initial algal cell density was measured around 10^5 cells m/L by using a ZEISS LSM microscope.

As bacterial inoculum, approximately 10% diluted MLSS were prepared, which initially obtained from the City of Moorhead WWTP (Moorhead, MN) (initial MLSS was about 2,500 mg suspended solids/L). All the glassware, including double de-ionized water (DDI) were sterilized by autoclaving at 121℃ for 20 - 30 min before conducting any experiment. In this study, *S. capricornutum, C. reinhardtii*, *C. vulgaris*, and bacteria were abbreviated as S, R, V, and B, respectively.

1.2.3 Analytical Methods, DON and ABDON Determination Procedures

About 50 ml filtered samples were used to analyze the initial parameters, which were dissolved ammonia N (DNH_3-N), dissolved nitrite N (DNO_2-N), dissolved nitrate N (DNO_3-N), and TDN. DON was calculated from the mass balance equation (Simsek et al., 2013). All the

measurements were carried out in duplication or triplication for each sample. The diazotization, second derivative ultraviolet spectrophotometric (SDUS) method and salicylate method were used to test nitrite, nitrate, and ammonia, respectively (Table 1). TDN was converted to nitrate after digestion and measured with SDUS method using UV-Visible spectrophotometer (APHA et al., 2005) (Table 1).

Table 1. Analytical method to determine ammonia, nitrite, nitrate, and TDN

Parameter	Testing method	Range (mg/L)	Instrument
Ammonia	Salicylate method	0.02-2.5	Hach DR 6000 spect.
Nitrite	Diazotization method	0.003-0.5	Hach DR 6000 spect.
Nitrate	Second derivative UV spect.	0-3	Varian Cary 50 UV-V spect.
TDN	Second derivative UV spect.	0-3	Varian Cary 50 UV-V spect.

After determining initial parameters, all the samples were placed in 250 ml clear bottles for 14 and 21 days of consecutive incubation using algae and bacteria inoculum. Clear bottles were used to inoculate the samples. The same parameters as in the initial samples were measured and finally ABDON was determined for both 14 and 21 days of incubation periods. The ABDON calculations relied on the changes between initial DON (DON_i, DON before incubation) and final DON (DON_f, DON after incubation) values (Simsek et al., 2012, 2013).

ABDON experiments in this study were divided into 7 portions based on the type of inoculum as; pure cultured algae (S, R, or V), algae + algae (R + V), and algae + bacteria (S + B, R + B, V + B, or R + V + B) inoculum. For the inoculation, 1.5 ml of algae and 1.5 ml of bacteria were used and all the bottles were agitated on an orbital shaker at 100 rpm (VWR standard orbital shaker) with caps were tightly closed. However, all the bottles were aerated daily by opening the caps once or twice a day for 3-4 minutes during the incubations to maintain the oxygen in the samples. After the incubation, wastewater samples were centrifuged with 3000 rpm for 5 min to separate either algae and/or bacteria from the samples before measurements. Control samples were also carried out throughout the experiments for each bioassay (S, R, V, and bacteria) by adding the inoculum to DDW. All the necessary corrections were made using the results obtained from control samples.

1.2.4 Statistical Analyses

Minitab 17 was used in this study for all the statistical analyses. Sample means and standard deviations were calculated. One-way analysis of variance was performed at $P \leq 0.05$ to evaluate the statistical difference in ABDON under different inoculation conditions.

1.3 Results and Discussion

1.3.1 Inorganic nitrogen and TDN

Dissolved inorganic nitrogen (DIN), TDN, and DON concentrations before and after incubation were measured in all the samples in all three locations. DIN, TDN, and DON values before incubation (initial) were presented in Table 2. The figures for DIN and TDN data after incubation values are not presented in this study while the figures for DON after incubation and ABDON data are presented. Initial ammonia after primary clarifier location was the highest and it decreased after BOD and after nitrification locations (Table 2). After the incubation period, about 95-99% of ammonia in all the samples collected from WWTP were nitrified to either nitrite or nitrate by algae or algae + bacteria inoculum.

Initial dissolved nitrite values before incubation were under 0.26 mg-N/L in all the samples in all three locations of WWTP (Table 2). After incubation, nitrite was quite high in all algae and/or bacteria seeded samples after primary location (varied between 15.40 and 26.00 mg-N/L). High nitrite accumulation in the samples in this location possibly occurred due to lack of dissolved oxygen during the incubation. Gonzalez et al. (2008) conducted a study in wastewater using algal-bacterial enclosed system, and found that 65-72% of inorganic N existed as NO_2-N form after the incubation. However, NO_2-N accumulation phenomenon was hardly reported under other field studies and in lagoon or pond systems (Gonzalez et al., 2008). Additional experiments were conducted to monitor DO influence on the partial nitrification by diluting the influent samples for 50% to reduce nitrogen loading in the sample. Diluted samples were incubated for 21 days and the results showed that during the incubation, between 72 and 91% of ammonia was nitrified into nitrate, while the nitrite level was extremely low (NO_2-N < 0.50 mg-N/L). This outcome proved that lower ammonia concentration was required less DO and mitigated the effect of partial nitrification. After BOD TF location, all the nitrite values after incubation reduced and varied between 9.48 and 16.08 mg-N/L. Nitrite after nitrification TF location was under 0.5 mg-N/L in all the samples except the values for the samples seeded with R + B, and V + B were slightly higher (<1.0 mg-N/L).

In after primary clarifier samples, dissolved nitrate after incubation was low in all the samples seeded with algae and algae + bacteria and the highest nitrate value was recorded on the sample seeded with R+V+B, which was less than 6.0 mg-N/L. Nitrate in the samples might be originated from nitrification of nitrite and ammonium or ammonification of DON. Nitrate values after incubation after BOD TF were varied between 5.01 and 9.45 mg-N/L regardless of the type of the inoculum. Previous studies showed that more nitrates were utilized by algae compare to nitrate utilized by bacteria (Sattayatewa et al., 2009; Simsek et al., 2013).

Table 2. Average initial values (mg/L) of the parameters in municipal wastewater samples.

Sampling location	NH_3-N	NO_2-N	NO_3-N	TDN	DON
After primary clarifier	33.74	0.26	0.27	42.53	10.37
After BOD trickling filter	20.50	0.16	8.65	36.27	6.59
After nitrification trickling filter	1.19	0.21	31.90	36.35	3.76

Nitrate after incubation from after nitrification TF location was reduced in algae and algae + bacteria seeded samples. The lowest nitrate in after nitrification TF location was measured in *C. reinhardtii* + *C. vulgaris* + bacteria seeded samples as 19.43 mg/L, which expressed 39.9% reduction from the initial nitrate. This means algae + bacteria can be able to remove 39.9% of nitrate from treated wastewater without advanced treatment (denitrification) application. Overall results showed that when ammonia, nitrite, and nitrate were all existed in the water ecosystem, ammonia was utilized first. Cai et al. (2013) concluded that ammonia was more favorable to algae during algal assimilation process since utilization of ammonia requires less enzyme and energy.

After incubation, TDN was reduced in all the samples compare to the initial TDN value after primary location. TDN reduction and biomass accumulation in the samples showed that algae and algae + bacteria utilized nitrogen for their growth. After incubation, magnitude of TDN reductions in the samples inoculated using each pure cultured algae were as follows: R (%50.79 reduction) > V (%43.14 reduction) > S (%32.74 reduction). The highest TDN reduction was observed in the sample seeded with algae R (TDN reduced to 20.67 mg-N/L). The high nitrogen utilization of algae *C. reinhardtii* and *C. vulgaris* showed that these two species might be used as a test species as well same as algae *S. capricornutum.*

After BOD TF location, average initial TDN (before incubation) was recorded as 36.27 mg-N/L, which was lower than initial TDN after primary clarifier samples expressed that WWTP itself removed about 14.7% of TDN in the BOD TF process. After 21 days of incubation, the trend for TDN after BOD TF samples was similar to the TDNs after primary clarifier samples. TDN reduction in algae inoculated samples increased with the presence of bacteria. The lowest TDN was recorded as 22.4 mg-N/L in *C. vulgaris* + bacteria inoculated sample. Initial TDN after nitrification TF location was measured as 36.35 mg-N/L, which was reduced in all the inoculum conditions. The lowest TDN was determined in *C. reinhardtii* + *C. vulgaris* + bacteria seeded samples as (21.63 mg/L) since the nitrate reduction was higher in this sample.

1.3.2 DON and ABDON

Initial DON after primary clarifier sample was measured as 8.96 mg-N/L, which comprised of 20% of initial TDN (Table 2). After incubation, DON concentration reduced in all the samples (Figure 1a). There was not a significant difference on DON reductions among the algae-only and algae + algae seeded samples and the reductions were not high, varied between 20.5 and 35.3% of initial DON in this location. However, DON reduction in algae + bacteria (S+B, R+B, V+B, and S+R+V+B) seeded samples were high, which varied between 56.6 and 72.3% of initial DON after 21-day of incubation. These results proved that symbiotic relationship between algae and bacteria enhanced DON biodegradability and following bioavailability.

Average initial DON after BOD TF samples was recorded as 6.59 mg-N/L (Table 2) which was lower than DON after primary effluent (36.5% reduction in the treatment plant). These results showed that a portion of DON was removed in BOD TF treatment process. DON/TDN ratio in BOD TF samples (18.23%) was comparable to DON/TDN ratio in primary effluent location

(20.24%) (Figure 2a, 2b). Westgate and Park (2010) determined DON/TDN ratio after secondary treatment locations in five different WWTPs, which employed either activated sludge with diffused or mechanical aeration process or the Ludzack-Ettinger process and found the ratio between 7 to 29%. These results indicated that the organic fraction of the TDN in the effluent were quite high. In some critical areas regulatory agencies may require WWTPs to remove DON in order to reduce TDN discharge concentration. Therefore, knowledge on the structural characterization of DON is becoming increasingly important. After the incubation, DON reduction in all the samples showed that some portion of DON was bioavailable to either algae or algae + bacteria inoculum while some portion of DON was refractory to any combination of inoculum. After 21 days of incubation, the lowest DON was determined in R + V + B seeded sample as average 1.33 mg-N/L, which was about 20% of initial DON.

Average initial DON after nitrification TF was recorded as 3.76 mg-N/L, which comprised of 8.7% of initial TDN in after nitrification location (Table 2). In fact, this DON value was more or less the same as the effluent DON value that was discharged to the river. In some environmentally critical areas, 3.76 mg-N/L of DON is quite high because of stringent TDN effluent discharge limits, which is typically under 5 mg-N/L. Therefore, finding a method to reduce DON in treated effluent is crucial. In this study, DON was reduced significantly in all the samples seeded with algae and/or bacteria (Figure 3a, 3b). Algae + bacteria seeded samples for all three types of algae in this location reduced DON under 1.12 mg-N/L. R + V + B seeded samples showed the highest DON reduction, which comprised of 78.7% of initial DON. Algae-only seeded samples achieved only between 44.7 and 58.8% of DON reduction, which were higher compare to the case in algae + bacteria seeded samples.

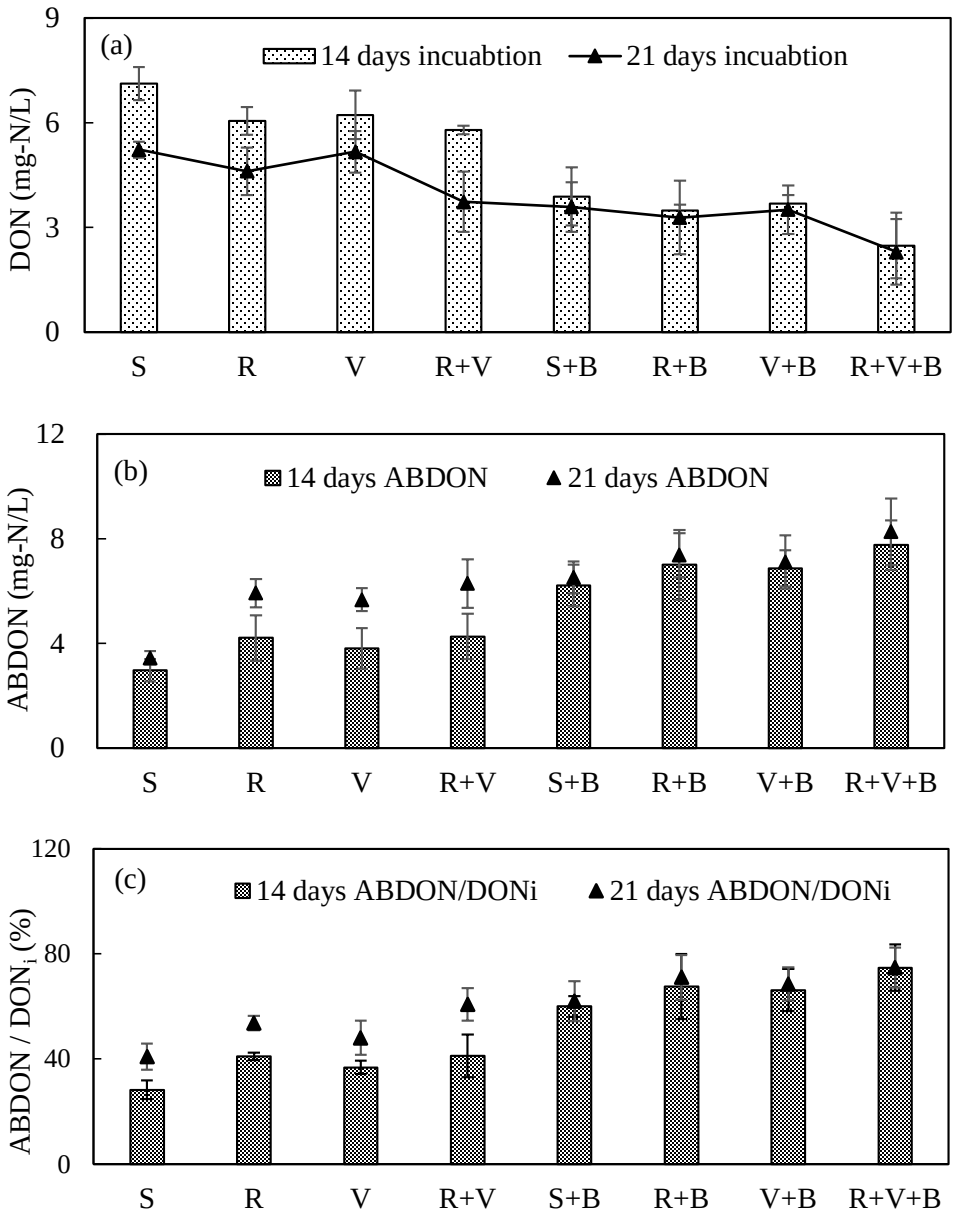

Figure 1. DON, ABDON, and ABDON/DON_i for algae, algae + algae, or algae + bacteria inoculum after primary location for 14 and 21 days of incubation.

Bioavailability of DON after primary location was low in pure cultured algae *S. capricornutum, C. reinhardtii,* and *C. vulgaris,* varied between 3.5 and 5.9 mg-N/L (Figure 1b). However, in algae + bacteria seeded samples (S + B, R + B, and V + B), ABDON was increased significantly ($p \leq 0.05$) because of the symbiotic relationship between algae and bacteria. In algae + bacteria data, *S. capricornutum* + bacteria had the lowest ABDON value (6.5 mg-N/L) compare to other two types of algae; however, this outcome was statistically not different. The highest ABDON value in Figure 1b observed in R + V + B inoculated sample as 8.27 mg-N/L, which was very close to the maximum average initial DON value. These results showed that about 92% of

DON was possible to be bioavailable to algae + bacteria in primary effluent samples when the optimum conditions were met. Previous studies also explained that wastewater derived DON comprised various forms of DON that cannot be bioavailable to algae and/or bacteria because of the complex structure of DON (Pehlivanoglu and Sedlak, 2004; Sattayatewa et al., 2009; Westgate and Park, 2010). Furthermore, Figure 1b proved that the ABDON results for bacteria added samples (algae + bacteria and algae + algae + bacteria) were not very different from 14 to 21 days of incubation results even though 21 days of incubation results were always slightly higher (<2%) than 14 days of incubation results in all the location. These outcomes indicated that 14 days of incubation was actually sufficient to reach more than 90% of attainable ABDON values using the algae and bacteria.

Initial DON fraction of ABDON after primary location was presented in the Figure 1c. For the algae-only seeded samples, the minimum ABDON fraction of DON in 14 and 21 days of incubations were 28.23% (*S. capricornutum*) and 36.80% (*C. vulgaris*), respectively. In general, the bioavailability of DON to pure culture algae (S, R, or V) increased from 12.0% in 14-day of incubation to 16.9% in 21-day of incubation. However, algae + bacteria seeded samples showed very minimal increment (1-2%) of ABDON to DON ratio between 14 and 21 days incubation in this location. These results expressed that 14 days of incubation period for algae + bacteria was appropriate to attain the maximum ABDON level (Urgun-Demirtas et al, 2008; Pehlivanoglu, E. and Sedlak, D.L., 2004), while 21 days of incubation period was more appropriate for algae-only seeded samples. Algae + bacteria results showed that about 20 to 31% more ABDON were achieved comparing to ABDON in algae-only seeded samples because of symbiotic relationship between algae and bacteria (Simsek et al. 2013; Huo et al.; 2013).

ABDON values for S, R, and V inoculum after 21 days of incubation ranged from 2.97 to 3.37 mg-N/L, which were lower than ABDON level in primary effluent (Figure 2b). The results showed that bacteria involvement always increased DON degradability and availability. Initial DON fraction of ABDON was calculated and presented in Figure 2c. DON_i fraction of ABDON for algae-only inoculated samples ranged from 46 – 53% for all three types of algae, while the same fraction in algae + bacteria samples ranged from 72 to 76%. These results showed that bioavailability of DON after BOD TF location in both algae-only and algae + bacteria inoculated samples were high compare to bioavailability of DON after primary location indicating that DON became more bioavailable to algae and bacteria in this (after BOD TF) location. This phenomenon could be explained that both bioavailable and refractory of DON were reduced during the BOD TF treatment process. Studies also suggested that most refractory forms of DON were mainly hydrophobic and easy to remove by adsorption process (Sattayatewa et al., 2009; Liu et al., 2012). Soluble microbial products (SMPs) is a portion of refractory DON which generally is considered resist to degrade (non-bioavailable/non-biodegradable) during bioassay (Sattayatewa et al., 2009). The decreased level of refractory DON indicated that SMPs was not produced during the BOD TF treatment. Released from the dead cells, SMPs were more likely produced under anoxic and anaerobic conditions (Sattayatewa et al., 2009).

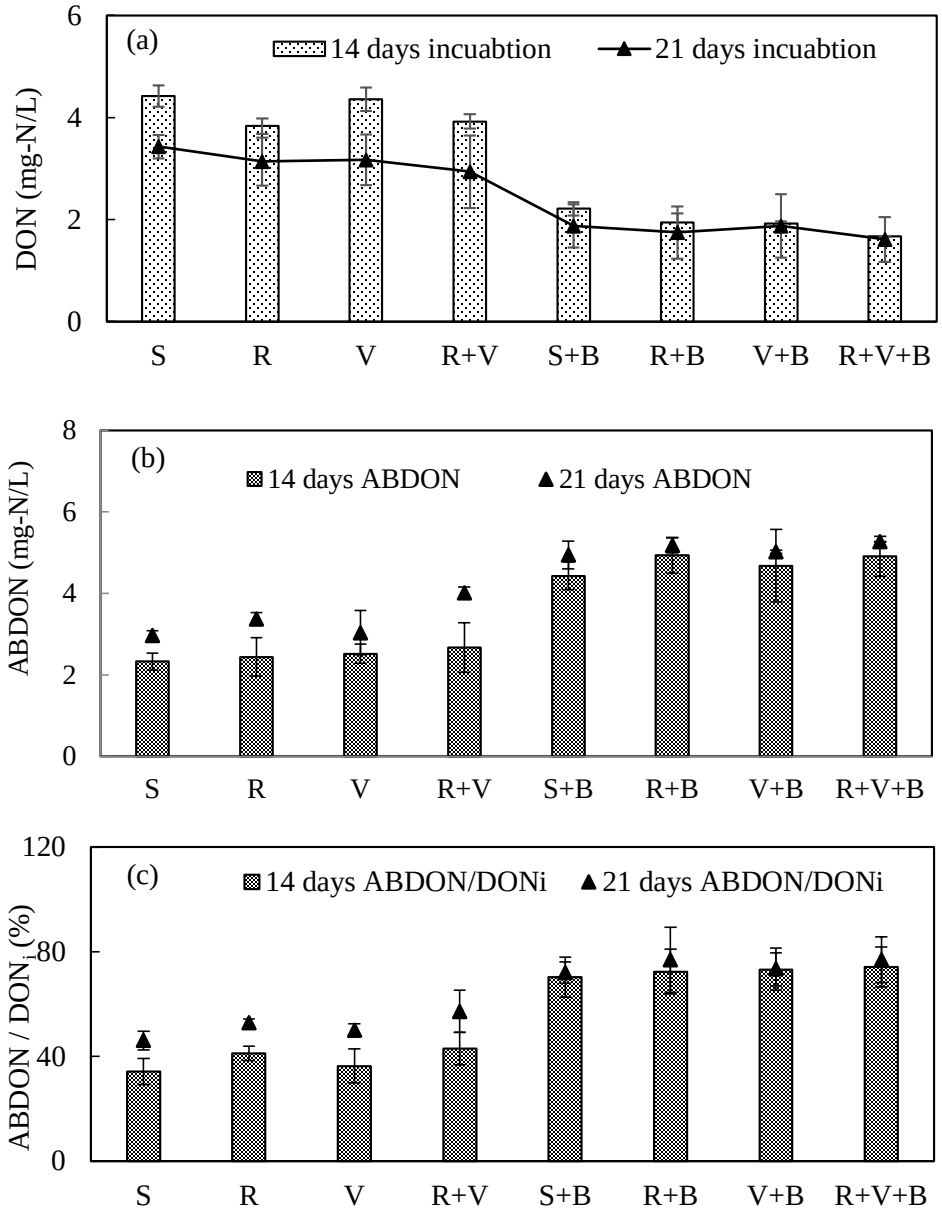

Figure 2. DON, ABDON, and ABDON/DON_i for algae, algae + algae, or algae + bacteria inoculum after BOD TF location for 14 and 21 days of incubation.

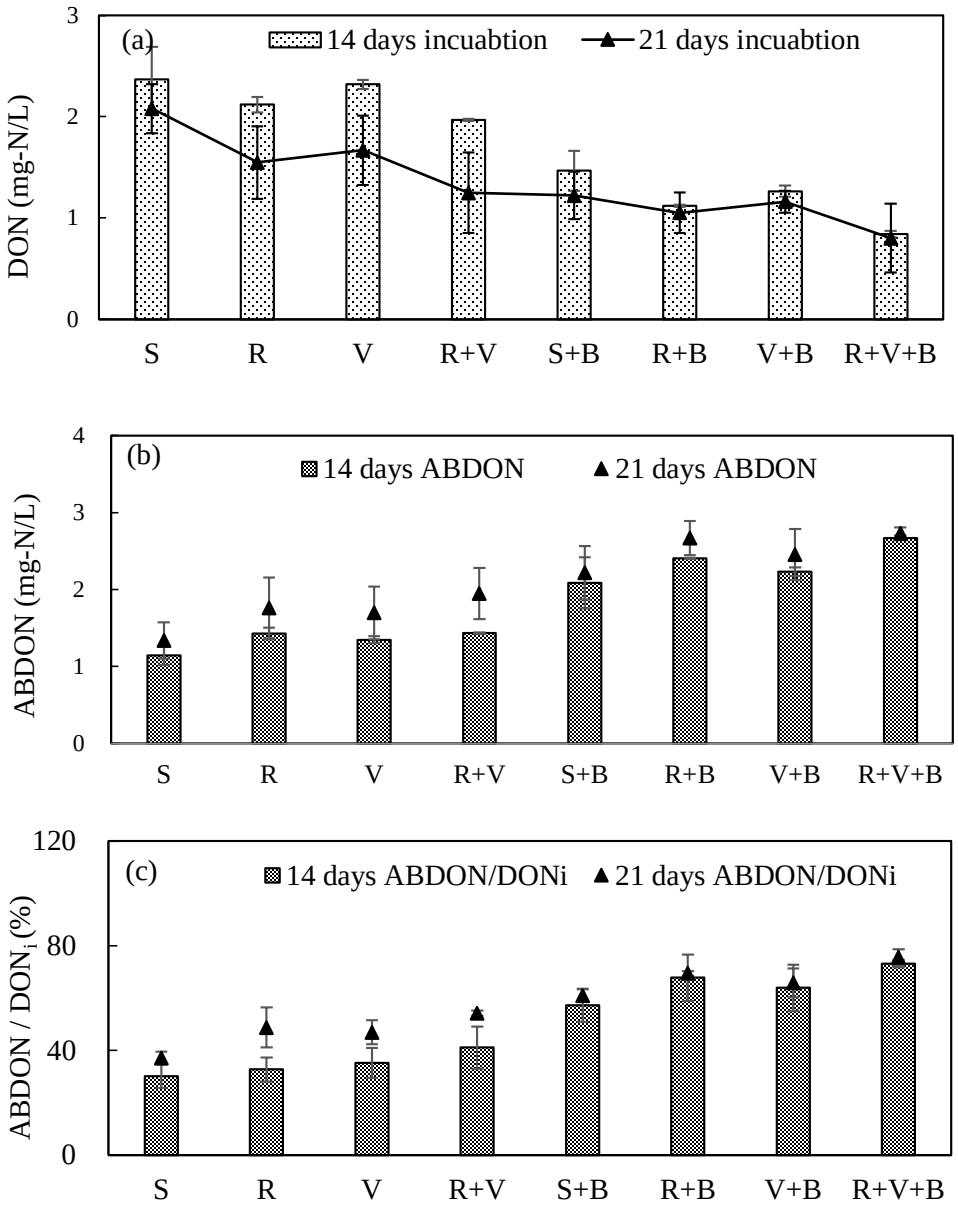

Figure 3. DON, ABDON, and ABDON/DON_i for algae, algae + algae, or algae + bacteria inoculum after nitrification TF location for 14 and 21 days of incubation.

ABDON is presented in Figure 3b and ABDON to DON_i ratio was presented in Figure 3c after nitrification TF location. ABDON was low in algae-only seeded samples compare to ABDON in algae + bacteria seeded samples, which was the similar trend observed in other two locations (after primary clarifier and after BOD TF locations). DIN, DON and TDN levels were different in all three locations; however, ABDON trends were similar. All these results indicated that the differences in DIN levels in three locations did not greatly affect the bioavailability of DON. The average ABDON level for *C. reinhartti* was slightly higher (not statistically significant) than other two algal species in all three locations (after primary, after BOD, and nitrification TFs). Previous

studies explained that on the cell wall of *C. reinhartti*, aminopeptidase (apase) enzyme was found to work functionally to hydrolyze proteins and peptides which can best explain the phenomenon of higher ABDON in the bioassay experiment. Additionally, a strong correlation between organic N and protein molecules such as peptides were found (Langheinrich, 1995; Westgate & Park. 2010)

ABDON/DON_i trend was observed after nitrification TF location (Figure 3c). The highest ABDON after 21 days of incubation was observed in R + V + B seeded samples as 73.1% of initial DON in this location. These results explained that 26.9% of initial DON in after nitrification location was recalcitrant DON, which was not removed in this study using algae *S. capricornutum, C. reinhardtii, C. vulgaris* and mixed culture bacteria. Overall, R + V + B inoculated samples demonstrated the maximum bioavailability of DON in all three locations.

1.4 Conclusions

This study provides important insight on bioavailability of DON using three different algal species (*S. capricornutum, C. reinhartti* and *C. vulgaris*) with bacteria addition in wastewater samples collected from three different locations in a two-stage TF WWTP. In all the locations, about 70 to 80% of DON was bioavailable to mix-cultured algae + bacteria system. ABDON in algae-only seeded samples were quite low compare to algae + bacteria seeded samples proved the symbiotic relationship between algae and bacteria. Among all species, *C. reinhartti* + bacteria achieved the highest ABDON value even though statistically the results were not significant compare to *S. capricornutum* + bacteria and *C. vulgaris* + bacteria inoculum. Similarly, there was no significant difference on ABDON between 14 and 21 days of incubation for all three algae + bacteria seeded samples. These outcomes indicated that 14 days of incubation period was actually sufficient to reach more than 90% of attainable ABDON values using the algae + bacteria inoculum. However, ABDON values in all three single culture algae seeded samples for 14 days of incubation were significantly lower than in the case of 21 days of incubation.

It can be explained that *C. reinhartti* and *C. vulgaris* can be used as a test species likewise *S. capricornutum* to determine nitrogen utilization efficiency by algae. Even though 14 days of incubation period is adequate to achieve the highest ABDON value in algae + bacteria system, 14 days is still a longer process in WWTP. Hence, different techniques might be investigated to reduce the incubation time. Similarly, it is necessary to investigate the impact of bioreactor operation conditions to promote DON degradation rate to enhance ABDON removal in a shorter time. Therefore, it is important to understand the fate and characteristics of DON in wastewater treatment train to evaluate DON and ABDON.

CHAPTER 2: Dissolved organic nitrogen in livestock wastewater: biodegradability and bioavailability

2.1 Introduction

Livestock operations contribute to nutrient enrichment in aquatic ecosystems and in turn stimulate overabundance of algal growth and cause a wide range of problems including oxygen depletion (hypoxia and anoxia), fish kills, harm or death to other aquatic organisms, and subsequent habitat loss (Knight et al., 2000; Kadlec and Knight, 1996; Hunt and Poach, 2001; Gilley et al., 2010). Nitrogen is usually the primary growth-limiting nutrient in a water environment where it presents in water as organic and inorganic nitrogen (NO_3^-, NO_2-, NH_4^+, and NH_3). The most dominant N forms in surface waters are nitrate and dissolved organic nitrogen (DON) (Bushaw-Newton and Moran, 1999; Vahatalo and Zepp, 2005; Wiegner et al., 2006).

Livestock wastewaters generated from concentrated animal feeding operations are a crucial agricultural point source containing suspended solids, nutrients, organic matter, pathogens, steroidal hormones, ectoparasiticides, mycotoxins, heavy metals, dioxins and antibiotics (Purdom et al., 1994; Khan et al., 2008; Chadwick et al., 2008; Wei et al., 2011). Some of the chemicals mentioned here are used to improve the reproductive performance of the dairy cattle. Transportation of these chemicals to surface waters causes contamination and may responsible abnormalities (alteration of endocrine function) in aquatic organisms. Proper animal wastewater management should be implemented to protect human and environmental health from exposure to these chemicals (Purdom et al., 1994; Khan et al., 2008).

Animal wastewater has been applied as a growth medium for algal biomass that is used for biogas production (Budiyono et al., 2010; Abou-Shanab et al., 2013). Wastewater treatment systems that integrated with algal biomass production are a cost effective way to produce algal biofuel. Abou-Shanab et al. (2013) examined six different microalgal species treating piggery wastewaters for their biodiesel production capacities. Microalgal-based treatment systems can significantly reduce nutrient concentrations in piggery wastewater at a minimal cost when the optimum conditions are met. *Chlorella vulgaris* was one of the algal species studied. After 20 days of *C. vulgaris* cultivation, TN was reduced from 53 to 27 mg/L. They concluded that TN reduction was tied to the removal of NO_3^-, NO_2^-, NH_4^+, and/or N_2; however, they overlooked organic nitrogen in their study (Abou-Shanab et al., 2013).

Previous studies indicate that some portions of DON in aquatic system are biodegradable and bioavailable to bacteria and/or algae over the time scale and it is an important nutrient source in nitrogen limited surface waters (Liu et al., 2011, Wiegner et al., 2006). Bioavailable DON (ABDON) is a portion of DON that is utilized by algae and algae + bacteria. Removing ABDON in livestock wastewaters before final discharging can reduce eutrophication potential.

In this study, DON and ABDON in livestock wastewaters collected from an animal feedlot and a sheep wastewater storage lagoon were studied. For ABDON bioassays, two different pure culture algal species, *C. reinhardtii* and *C. vulgaris*, mixed culture bacteria, and their combinations were tested. Previous studies showed that green microalgae *C. reinhardtii* and *C. vulgaris* have demonstrated their ability to remove nitrogen species in domestic

wastewater. However, these two species have not been used to test organic nitrogen bioavailability in livestock wastewaters (Kim et al., 2007; Kong et al., 2010; Lee et al., 2006).

2.2 Material and methods

2.2.1 Sample collection and preparation

Grab animal wastewater samples were collected from two different sources: (i) an animal feedlot facility, Animal Nutrition and Physiology Center (ANPC) on North Dakota State University (NDSU) campus and ii) a wastewater storage lagoon of NDSU Sheep Unit. Six sets of samples were collected from each facility from April 2014 to October, 2014. Wastewater samples were filtered first through 1.2 µm pore-size glass fiber filters (GF/C, Whatman Inc. Kent, UK) and subsequently filtered through 0.45 µm pore-size glass fiber filters (GF/F, Whatman Inc. Kent, UK) within one hour after the collection.

In the first part, samples were collected from ANPC, where various types of animals have been raised in this facility depending on the research need. During the sample collection time, there were sheep, pigs, and mostly cattle (about 100-head cattle) in the feedlot. About 60,000 gal/day wastewater is generated in the facility. The wastewater flows through a solid separator unit for hay separation and the liquid portion (wastewater) goes to a liquid storage tank for about three days of storage prior to final discharging into the City of Fargo sewage system. The samples were collected from the storage tank.

In the second part of the study, the wastewater samples were collected from a storage lagoon that receives animal wastewater and runoff from a sheep research feedlot. There were about 200 heads of sheep available during the sample collection period. The lagoon received rainwater as well in some of the sampling time frame. When it was needed, the lagoon wastewater was pumped out to a nearby crop field for disposal and fertilization. The wastewater samples were collected at 0.4 m depth from the lagoon surface.

2.2.2 Experimental design, DON and ABDON determination

The same experimental design and DON and BDON determination procedure were followed as in Section 3.2 of this report. The only difference was that, *S. capricornutum* was not used in this part of the study. Therefore, the abbreviations for the inoculum were assigned as follows: algae-only (R or V), algae + algae (R + V), algae + bacteria [(R or V) + B], or algae + algae + bacteria (R + V + B).

2.3 Results and Discussion

2.3.1 Animal feedlot wastewaters

Inorganic nitrogen and TDN

Average values of dissolved inorganic nitrogen, TDN, and DON concentrations before and after incubation were measured in animal feedlot samples. DIN, TDN, and DON values before incubation (initial) were presented in Table 1 while after incubation values were not presented in this study. After the incubation, about 51 and 57% of ammonia were removed in R-only or V-only seeded samples, respectively. In the R + B and V + B seeded samples, about 90 and 92% of ammonia were removed by algae + bacteria inoculum, respectively. These

results indicated that bacteria addition to the samples increased ammonia reduction since bacteria was mainly responsible to convert ammonia in the wastewater to first nitrite and subsequently to nitrate.

Nitrite after incubation was low in algae-only seeded samples while it was high in algae + bacteria seeded samples (incomplete nitrification) possibly because of lack of oxygen in the samples during the incubation. Similar results were obtained in previous studies (Urgun-Demirtas et al., 2008; Simsek et al., 2013) that observed high nitrite in bacteria inoculated samples and low nitrite in algae-only seeded sample. For algae only samples, ammonia were high after 21 days, while in bacteria involved samples, the major nitrogen form was nitrite. In general, either ammonia or nitrite were high for all inoculum conditions after the incubation. This suggests that partial nitrification occurred in the samples during the incubation. Inadequate respiration during the incubation because of lack of DO might be reduced the nitritation efficiency in the samples as similar results were obtain in a previous study conducted for domestic wastewater (Simsek et al., 2012).

Average initial TDN (before incubation) mostly consists of ammonia and DON since the wastewater sample in animal feedlot was fresh (about 3 days of residence time in storage tank). Average TDN values after incubation for samples seeded with either single cultured (R, V) or mixed cultured (R + V) algae were substantially lower than their TDN values before incubation since algae utilized nitrogen species (mainly ammonia) for their growth. TDN values after incubation reduced for the samples seeded with R (46% reduction) and V (44% reduction), respectively. The reduction of TDN was observed since algae utilized nitrogen during the incubation. Adding bacteria seed into the samples seeded with R and V was not significantly reduced TDN.

Table 3. Average initial values (mg/L) of the parameters in livestock wastewater samples.

Sampling location	NH_3-N	NO_2-N	NO_3-N	TDN	DON
Animal feedlot	33.12	0.12	0.91	42.01	7.71
Storage lagoon	2.33	0.0	0.23	12.08	8.50

DON and ABDON

Average DON concentration before incubation in animal feedlot wastewater sample was recorded as 7.71 ± 0.18 mg-N/L, which comprised of about 18.4% of TDN before incubation (Figure 4a). The magnitude of DON in animal feedlot effluent and typical DON in municipal wastewater (raw wastewater) were quite similar. However, the proportion of DON:TDN was lower than municipal wastewater due to the high TDN level in animal wastewater (Urgun-Demirtas et al., 2008). DON after incubation in algae and algae + bacteria seeded samples was varied between 1.63 and 4.54 mg-N/L, which indicated that 21.1 to 58.9% of initial DON was utilized by either algae and/or bacteria. DON after incubation (DON residue in the sample) to initial TDN ratio was calculated and results showed that between 3.9 and 10.8% of DON in initial TDN was remained in this samples, which consider as refractory (unbiodegradable and unbioavailable) DON (Figure 4a). DON achieved the least reduction in *C. reinhardtii* seeded samples. The highest reduction in DON after incubation to initial TDN was observed in *C. reinhardtii* + *C. vulgaris* + bacteria seeded sample (Figure 1a). DON residue in the sample to TDN after incubation in each bioassay was also calculated and found that

between 6.9 and 19% of TDN after incubation in each samples were DON (data not shown). This indicated that when DON was reducing in each bioassay sample, TDN was also reducing because of algal and bacterial utilization of nitrogen species.

To examine whether the two algae species were in competition or symbiotic relationship on consuming DON, bioavailability of DON in both algae *C. reinhardtii* and *C. vulgaris* and their combination with bacteria was determined and presented in Figure 4b. DON bioavailability was significantly higher ($P \leq 0.05$) in *C. reinhardtii* inoculum compare to in *C. vulgaris* inoculum. The bioavailability of DON increased in both types of algal species with bacteria addition, which was a result of symbiotic relationship between algae and bacteria. Dong et al. (2014) explained that urea in DON is more favorable to nitrate and nitrate-urea mixture media. Further they explained that more encoding proteins were involved in urea assimilation process rather than nitrate transport process. Hence, urea in animal wastewater could enhance the bioavailability of DON in both algal spices.

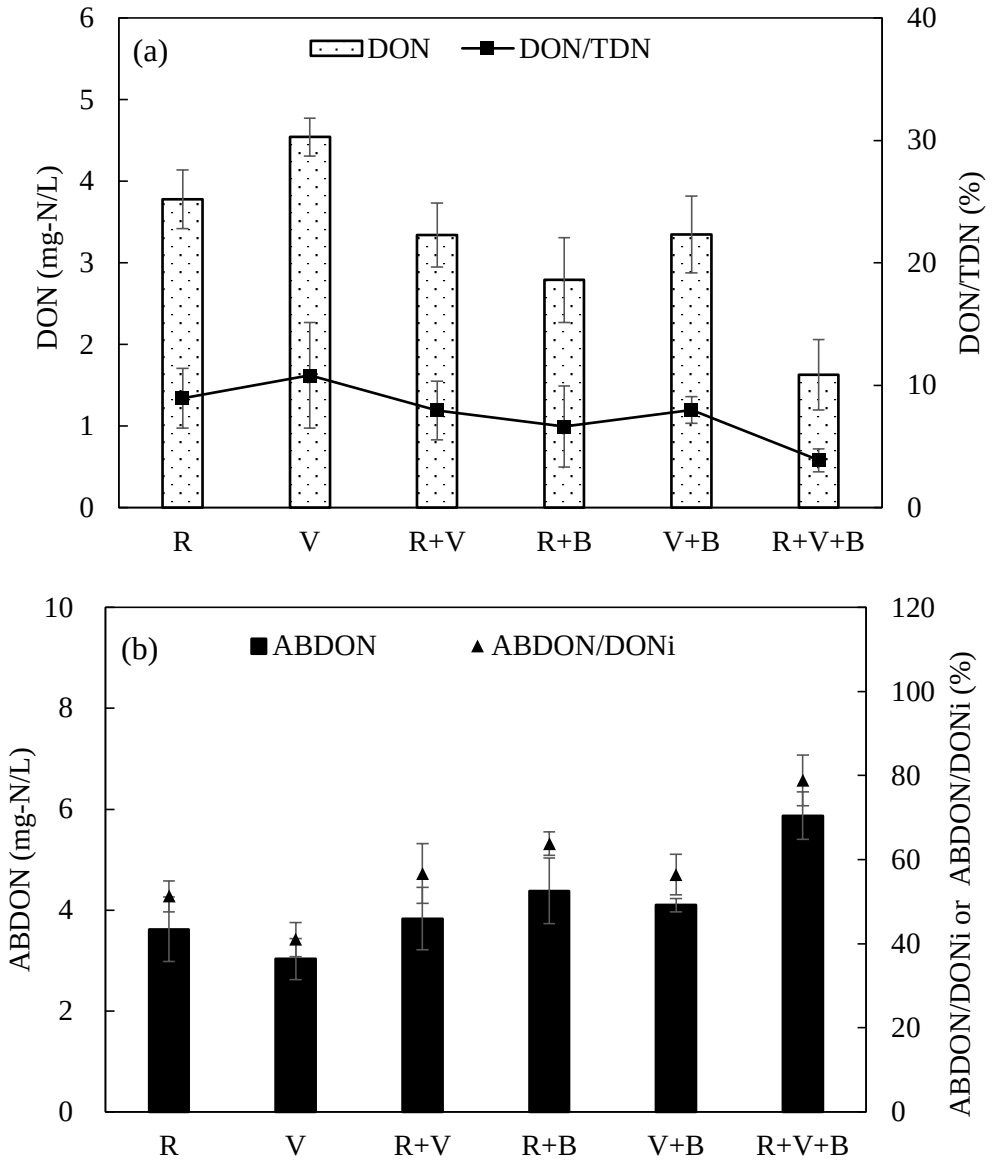

Figure 4. DON, ABDON, and ABDON/DON_i for algae, algae + algae, or algae + bacteria inoculum in animal feedlot samples.

Results showed that initial DON bioavailability that obtained in this study for animal wastewater was higher than in municipal wastewater studies conducted earlier (Urgun-Demirtas et al., 2008; Simsek et al., 2013). ABDON in algae (R or V) and bacteria seeded samples were not statistically different ($P \leq 0.05$). However, the presence of bacteria promoted bioavailability of initial DON in both types of algae. ABDON in mixed cultured algae and bacteria (*C. reinhardtii* + *C. vulgaris* + bacteria) seeded sample was significantly greater ($P \leq 0.05$) than all other combinations since the majority of DON (81%) was bioavailable to algae and bacteria in this sample. Increased DON bioavailability to algae with the presence of bacteria was also concluded in previous studies (Urgun-Demirtas et al., 2008; Simsek et al., 2013). The mutual relationship between algae and bacteria was benefited from nutrient interactions of carbon dioxide, oxygen, vitamin B12, and organic carbon source (Santos and Reis. 2014).

The fractions of ABDON/DONi represented the bioavailability of DON in the samples, respectively. Results showed that certain portion of DON was biodegradable and/or bioavailable to bacteria, algae, and algae + bacteria in wastewaters from animal feedlot sample (Figure 4b). As explained earlier, two sets of samples were diluted to investigate nutrient loading and ensure the availability of DO. ABDON was determined in 1:3 portion of diluted sample to observe oxygen deficiency effect on DON. Results showed that bioavailability of DON was not changed significantly ($P < 0.05$) between non-diluted and 1:3 diluted samples from animal feedlot sample, which proved that the magnitude of inorganic nitrogen (including high NO_2-N accumulation because of partial nitrification) didn't greatly affect DON utilization by algae and bacteria. The bioavailability of influent DON to *C. reinhardtii* in 1:3 diluted samples was slightly higher (about 11%) than in *C. vulgaris*. However, DON bioavailability of influent DON was increased at least 4% with the presence of bacteria.

2.3.4 Storage lagoon wastewaters

Inorganic nitrogen and TDN

Average values of dissolved inorganic nitrogen, TDN, and DON concentrations before and after incubation were measured in storage lagoon samples. DIN, TDN, and DON values before incubation (initial) were presented in Table 3 while after incubation values were not presented in this study. Nitrate, ammonia, and nitrite before incubation were detected in very low concentrations, which were NO_3-N <0.23 mg-N/L, NH_3-N = 2.33 mg-N/L, and NO_2-N <0.30 mg-N/L. These results showed that total dissolved inorganic nitrogen (TDIN) was about 2.86 mg-N/L, which was lower than TDIN in animal feedlot sample. The reason for low inorganic nitrogen in the lagoon was that the residence time of the wastewater was long enough to complete nitrification and following nitrate utilization by bacteria and algae in the lagoon. After incubation, ammonia and nitrite were under detection limit as well in the samples seeded both algae and algae + bacteria. However, nitrate concentration after incubation increased in each location, explained that nitrate was occurred because of bioavailable DON in the samples.

Average TDN before incubation consisted of mostly DON since TDIN was very low in the samples. After incubation, ammonia and nitrite were completely removed in all the samples. However, nitrate concentration after incubation increased in each location, explained

that nitrate was produced after the degradation of ABDON in the samples. These results showed that initially DON degraded to lower weight molecular compounds by bacteria and consequently utilized by algae and/or bacteria (Urgun-Demirtas et al., 2008; Simsek et al., 2013). Overall, the TDN trends observed in lagoon samples were similar with samples from animal feedlot sample. The average TDN concentrations before and after incubation in bacteria seeded samples were similar. About 13% of TDN reduction was observed during the incubation in bacteria-only seeded sample. TDN in bacteria-only seeded sample was very high compare to algae-only inoculated samples explained that bacteria did not utilize TDN as much as algae did. Bacteria were mainly responsible for nitrification in these samples.

DON and ABDON

DON was dominant component of nitrogen in sheep feedlot lagoon samples and comprised of 70.8% of TDN. Previous studies showed that DON in lagoon samples can be derived from N enriched underground water, agricultural ground water, and sediment-water column fluxes across a nutrient gradient (Anderson et al. 2003; Tyler et al. 2001). Additionally, a small portion of DON can be released from soil during runoff. DON release rate in lagoon were influenced by biological processes, hydrometeorlogical factors, rainfall, and surface discharge (Scully et al., 2007). Similar to animal feedlot sample, the minimum DON value after incubation was recorded as average 1.06 mg-N/L, which was a recalcitrant DON in R + V + B seeded sample (Figure 5a).

TDN reduction rates during the incubation in lagoon samples were closely related to the bioavailability of DON. Results showed that more DON reduction was appeared in the samples inoculated with algae + bacteria. Similarly, *C. reinhardtii* + bacteria demonstrated higher DON removal compare to *C. reinhadtii* + bacteria samples. DON to TDN ratios after incubation ranged from 13.2% to 41.2%, which were quiet low compare to the same ratio before incubation (initial sample). The ratios in algae-only seeded samples were higher than algae + bacteria seeded samples indicated that the symbiotic association between algae and bacteria increased DON utilization.

ABDON and its ratio to initial DON data were presented in Figure 5b. Even though the residence time of the lagoon was very high compare to animal feedlot fresh samples, ABDON values in the lagoon samples and in animal feedlot samples were very close each other in all the inoculum conditions. This outcome indicated that, suitable environmental conditions were not occurred in the lagoon to increase biodegradability and bioavailability of DON. However, during the 21 days of incubation, the presence of bacteria enhanced the bioavailability of DON to both *C. reinhardtii* and *C. vulgaris*. The difference of ABDON between sample inoculated with *C. reinhardtii* only and *C. vulgaris* only were not significant ($P<0.05$). The samples inoculated with *C. reinhardtii* + bacteria showed slightly more bioavailability of DON than in the case of *C. vulgaris* + bacteria ($P \leq 0.05$). Similarly, the samples seeded with algae + algae + bacteria demonstrated that approximately 81% of DON was bioavailable to both type o algae + bacteria (mixed culture). The bioavailability of DON to initial DON ratio showed similar trend with ABDON data in all the inoculum condition.

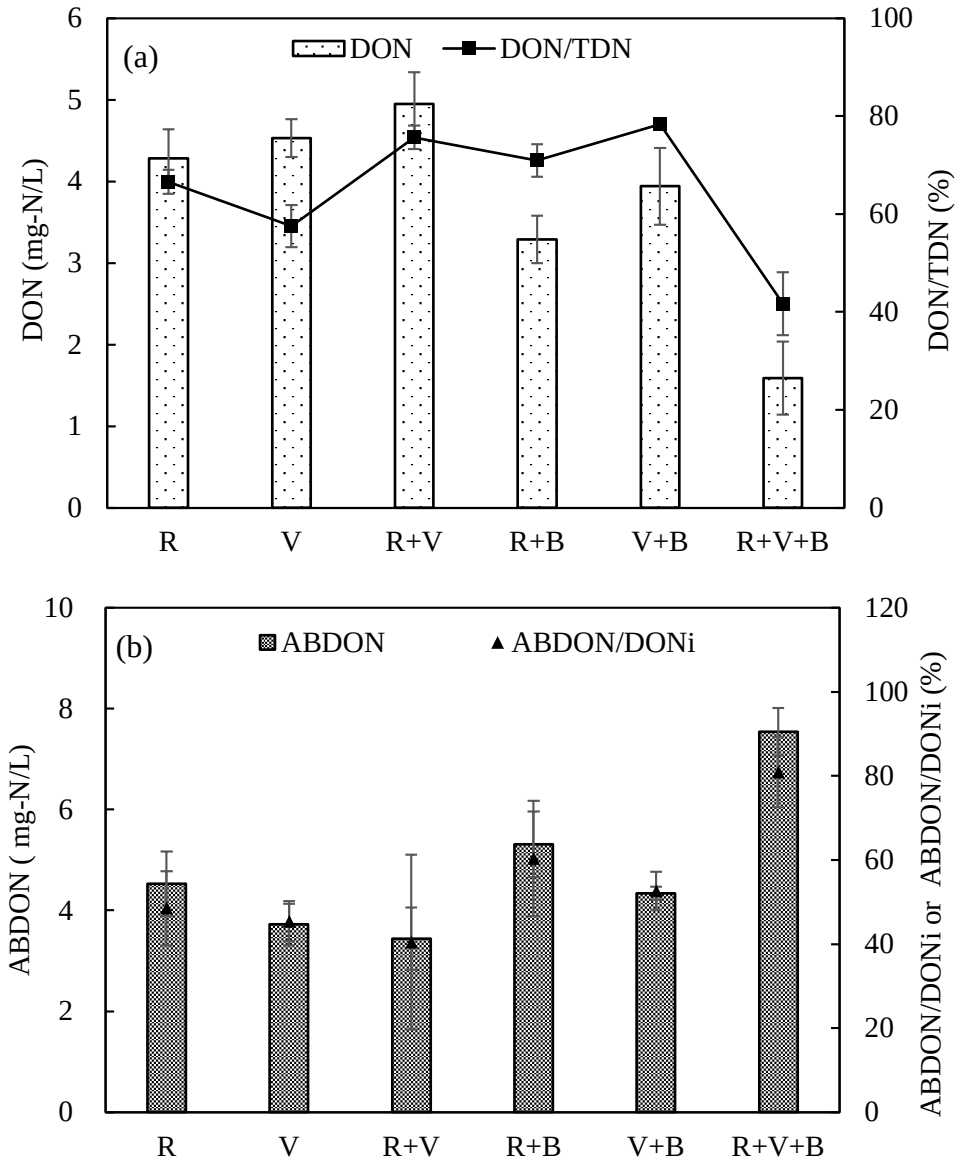

Figure 5 DON, ABDON, and ABDON/DON_i for algae or algae + bacteria inoculum in animal feedlot samples.

2.4 Conclusions

Bioavailability of DON in two different animal wastewater sources were thoroughly investigated. Samples were inoculated using two types of pure culture algal species, *C. reinhardtii* and *C. vulgaris* with/without bacteria addition to determine DON and ABDON. Results showed that the trend in ABDON in both types of wastewater sources were similar. *C. reinhardtii* + *C. vulgaris*+ bacteria seeded samples utilized initial DON more than other combination (algae + algae or algae + bacteria) of inoculum in both sample sources. At least 20% of initial DON was recorded as recalcitrant DON. This portion of DON could be degraded in longer incubation conditions once discharged to the receiving waters. ABDON exertion was slightly higher in *C. reinhardtii* + bacteria inoculated samples comparing to *C. vulgaris* + bacteria inoculated samples indicating that both species can be used to treat DON and inorganic

nitrogen in animal wastewater. This study was conducted under batch conditions with controlled temperature, aeration, and illumination. Further study can be applied in continuous stirred tank reactors or pilot scale reactors to investigate different solid retention times on animal wastewaters.

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